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Research

Relative Tolerance of Three Morphotypes of the *Anastrepha fraterculus* Complex (Diptera: Tephritidae) to Cold Phytosanitary Treatment

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Abstract

The *Anastrepha fraterculus* (Wiedemann) complex is currently comprised of at least eight morphotypes, including several that are likely to be described as new species. It is critical to evaluate whether the morphotypes differ in tolerance to phytosanitary treatments. Temperatures from 0 to 3°C are used as a phytosanitary treatment for some commodities exported from the region and at risk of infestation by the *A. fraterculus* complex. Description of *A. fraterculus* morphotypes as new species could result in the annulation of phytosanitary treatment schedules for the new species. This study compared the relative cold tolerance of five populations from three morphotypes of the *A. fraterculus* complex: Andean, Peruvian, and Brazilian-1. Both a laboratory and wild strain of the Brazilian-1 morphotype were studied. Differences in mortality of third instars of the five *A. fraterculus* populations reared on nectarines were observed only with short treatment durations at temperatures ranging from 1.38 ± 0.04°C to 1.51 ± 0.08°C (mean ± SEM). Estimated times to achieve the $LT_{99.99682}$ (probit 9) showed that Brazilian-1 wild, Brazilian-1 laboratory, and Cusco population were the most cold tolerant, followed by Andean and Peruvian, the least cold tolerant morphotype (i.e., Brazilian-1 wild = Brazilian-1 laboratory = Cusco population > Andean > Peruvian). These findings suggest that the current cold treatment schedules of 15 d at ≤ 1.11°C and 17 d at ≤ 1.67°C can be applied as cold treatments to any potential new species that may arise from the *A. fraterculus* complex.

Key words: quarantine treatment, postharvest treatment, phytosanitation, South American fruit fly

Cold phytosanitary treatment uses refrigerated air to lower the temperature of the commodity to or below a specific temperature for a specific period to achieve pest mortality at a specified efficacy (IPPC 2018). It is one of the most widely applied phytosanitary measures against tephritid fruit flies and typically consists of temperatures from 0 to 3°C for 15–20 or more days (Heather and Hallman 2008). For instance, cold phytosanitary treatments at 0, 0.56, 1.11, and 1.67°C for 11, 13, 15, and 17 d, respectively, are approved for several fruits against all *Anastrepha* spp. except *Anastrepha ludens* (Loew) (Diptera: Tephritidae) (USDA 2019).

Studies have shown that populations of one of the species covered by these approved cold treatments, *Anastrepha fraterculus* (Wiedemann), can differ substantially at the molecular, genetic, morphological, and behavioral levels (Morgante et al. 1980; Steck 1991; Hernández-Ortiz et al. 2004, 2012, 2015; Yamada and Selivon 2001; Selivon et al. 2004, 2005; Vera et al. 2006; Cáceres et al. 2009; Rull et al. 2013; Devescovi et al. 2014;Dias et al. 2015; Roriz et al. 2019). These studies indicate that the *A. fraterculus* complex is currently comprised of at least eight morphotypes: Andean, Brazilian-1, Brazilian-2, Brazilian-3, Ecuadorian, Mexican, Peruvian, and Venezuelan that are likely to be described as new species (Hernández-Ortiz et al. 2004, 2012, 2015).

Considering the taxonomic instability of the *A. fraterculus* complex, it is essential to test whether the South American morphotypes differ in cold tolerance to anticipate the use of cold phytosanitary treatment against any of them. This is particularly important considering that the data supporting a cold treatment of the nominal species *A. fraterculus* were only gathered from

one morphotype, Brazilian-1 (Willink et al. 2006). One study was done with the Andean morphotype (Valderrama et al. 2005), but it might be insufficient to support a treatment schedule if the Andean morphotype was considered a new species.

Besides the uncertainty associated with a potential genetic variation on cold tolerance among populations of the same species, physical and biological factors, such as cool-down rate, temperature fluctuation, variation in research methodology, and host type, may affect tolerance to cold treatments (Heather and Hallman 2008, Gazit et al. 2014, Hallman et al. 2019a). Thus, it is critical to evaluate the extent to which fruit fly populations of the same or distinct species differ in cold tolerance under the same methodological conditions. This research can appropriately be done using the unique resources of the Insect Pest Control Laboratory (IPCL) of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture at Seibersdorf, Austria, where like studies have previously solved problems confronting phytosanitary decisions (Hallman et al. 2013, 2019a,b). The objective of this study was to determine if morphotypes of the *A. fraterculus* complex differ in tolerance to phytosanitary cold treatment.

Materials and Methods

Insects

Five populations from three known morphotypes of the *A. fraterculus* complex were used in our study (Table 1). Experiments were carried out using the same colony without the addition of wild flies during the period of the cold treatments. Colonies were maintained at the IPCL with voucher specimens from all morphotypes periodically collected and deposited at the IPCL. Rearing of laboratory adapted strains (all except Brazilian-1 wild) consisted of routinely collecting and transferring eggs to an artificial diet, followed by pupariation and adult maintenance. Females laid eggs in a silicon sealed oviposition device containing tap water placed on the top of the adult cage. The oviposition device consisted of a Petri dish (13.9 cm) containing an inner hole (11.4 cm) covered with white voile mesh, previously coated with a thin layer of black silicone sealant (Den Braven, The Netherlands). Eggs laid into the oviposition device were collected with a pipette (3 ml) and transferred to an artificial diet based on carrot powder and torula yeast (Tanaka et al. 1970, Rempoulakis et al. 2014). Larvae were held in diet trays (19 × 30 × 2 cm) wrapped with plastic film for 3 d. After incubation, diet trays with larvae were placed into plastic trays (33 × 46 × 12 cm) containing sawdust (GOLDSPANsmoke, Germany) until pupation (12 ± 1 d). The rearing protocol of the wild population from Tucumán (Castelar strain) consisted of mango infestation for 48 h, incubation of infested mangoes into plastic containers (20 × 20 × 14 cm) containing sawdust, and pupae collection after 15–20 d. Puparia from laboratory and wild strains were transferred to screen-mesh cages (45 × 45 × 45 cm), followed by adult emergence. Adults from each *A. fraterculus* morphotype were maintained into different screen-mesh cages with free access to water and dry diet (3 sucrose: 1 hydrolyzed yeast). All insects were reared under laboratory conditions at 25 ± 0.5°C, 65 ± 5% relative humidity, and 14L:10D photoperiod.

Fruit Infestation

Mandarins (*Citrus reticulata* Blanco) from Israel and Spain and nectarines (*Prunus persica* (L.) Batsch var. *nucipersica* Schneid) from Italy and Spain were exposed to sexually mature *A. fraterculus* females for oviposition. To prevent fruit contamination and larval mortality due to fungi infection, apparently by *Penicillium* sp. and *Rhizopus* sp., multiple sanitization measures were applied before and after infestation. Before infestation, fruits were washed, rinsed, soaked for 15 min in antifungal solution (4% sodium benzoate), and rerinsed. Natural infestation consisted of placing 7–10 presanitized fruits in an elevated galvanized steel-mesh platform (~11 cm high) into a screen-mesh cage (45 × 45 × 45 cm) containing 1,000–3,500 sexually mature male and female flies. Females from all *A. fraterculus* morphotypes reached sexual maturity approximately 2–3 wk after adult emergence under the holding conditions described above. Infestation time ranged from 2 to 6 h depending on the fly age and density in the cages. After infestation, a second sanitization round was applied to all infested fruits to prevent fungi growth and development. Infested fruits were soaked for 15 min in antifungal solution (4% sodium benzoate), rinsed, and dried for immediate biometric screening. Following the resanitization procedures after infestation, each fruit was weighted using a digital balance (model IS 32001, VRW, Italy) and its perimeter measured. Subsequently, infested mandarins ($\bar{X}_{weight}$ = 105.6 g ± 1.1, $\bar{X}_{perimeter}$ = 19.9 cm ± 0.05) and nectarines ($\bar{X}_{weight}$ = 148.7 g ± 0.6, $\bar{X}_{perimeter}$ = 20.7 cm ± 0.03) were individually placed into plastic containers (9.5 × 9.5 × 11.5 cm) and incubated at 25°C for up to 10 d until the larvae reached the third instar, considered the most cold tolerant stage of the nominal species *A. fraterculus* (Willink et al. 2006). Natural infestation rates varied among fruits and between replicates across all treatment durations assessed during the cold treatments, an aspect that increases the robustness of the results (Mangan and Hallman 1998, Hallman et al. 2019a).

Cold Treatment of Infested Fruit

The cold treatment tests of infested nectarines were carried out in a 2 m^3 environmental chamber (model SE-2000–4, Thermotron Industries, Holland, MI). Airflow within the chamber was approximately 28.3 m^3/min. For all treatments, the chamber temperature was set to 0.7°C to achieve target fruit pulp temperatures of 1.7°C or below. The treatment temperature complies with current USDA treatment schedules for the control of *Anastrepha* spp., except *A. ludens*, associated with consignments of nectarines (i.e., T107-a-1, USDA 2019). Fruit, water, and air temperatures inside the environmental chamber were recorded every 15 min using two external four-channel analog data loggers (HOBO UX120-06M, Onset Computer Inc., USA) with four temperature sensors (TMCx-HD, Onset Computer Inc., USA) each. The treatment time was started when two sensors inserted into noninfested nectarines reached ≤ 1.7°C.

After cold treatment, nectarines were held at 25 ± 1°C for at least 24 h before dissection to allow enough time for larval recovery. Untreated controls were also held at 25 ± 1°C for at least 24 h from the time the treated nectarines were placed into the cold chamber. Any moving larvae found during fruit dissection were considered survivors. Nonmoving larvae, regardless of their coloration, were considered dead. A minimum of five replicates with several fruits were performed for each treatment duration. The total number of third instars treated for each treatment duration ranged from 982 to 5,967 due to either uneven infestation rates or unbalanced replicates.

Statistical Analysis

Infestation rates (number of larvae/fruit) were compared between hosts and morphotypes within each host using generalized linear models with Poisson distribution. Survival was analyzed using a generalized linear model with binomial responses. Considering that all insects responded equally (e.g., 100% mortality) to some cold

Table 1. Origin and collection information for the three known morphotypes of the *A. fraterculus* complex used in cold treatment experiments

Morphotype	Collection site	Host	Generation*
Andean	Ibagué, Colombia	*Coffea arabica*	F-56
Brazilian-1	Tucumán, Argentina (laboratory strain)	*Psidium guajava*	F-35
Brazilian-1	Tucumán, Argentina (wild strain)	*Psidium guajava*	F-02
Unknown	Cusco, Peru	Unknown	F-46
Peruvian	La Molina, Peru	*Annona cherimola*	F-65

*Tests were carried out across four consecutive generations.

treatments, the bias reduction correction developed by Firth (1993) was applied to improve the estimates of the model coefficients and avoid underestimation of standard errors (Kosmidis and Firth 2010, Kosmidis 2014, Kosmidis et al. 2020). Duration of the cold treatment (dose), morphotype, and their interaction were modeled as fixed effects. The statistical significance of the fixed effects and their interaction were determined using likelihood ratio tests with type III sums of squares. Post hoc pairwise comparisons of estimated marginal means between the levels of cold treatment and morphotype were performed with Bonferroni adjustment (Holm 1979). A probit model with adjustment for overdispersion was used to estimate the lethal time (LT) of cold exposure to achieve 50, 99.9 and 99.99682% (probit 9) mortality for each *A. fraterculus* morphotype and their fiducial limits at 95% confidence interval (CI). Data from unexposed insects were also included in the probit model. The LT values were then compared between morphotypes using lethal dose ratio tests. Statistical analyses were performed in R (version 3.6.1) using the *brglm2* (Kosmids 2019), *emmeans* (Lenth 2019), *drc* (Ritz et al. 2015), and *multcomp* (Hothorn et al. 2008) packages.

Results

Infestation rates were consistently lower in mandarins than in nectarines, indicating the preference of *A. fraterculus* females for nectarines (χ^2 = 21,699; df = 1; P < 0.0001, Table 2). Due to the low number of mandarins infested, only naturally infested nectarines were exposed to cold treatments.

Mortality of third instars from three morphotypes of the *A. fraterculus* complex infesting nectarines exposed or unexposed (controls) to cold treatments below 1.7°C, precisely 1.38 ± 0.04°C (mean ± SEM, treatments with Andean, Brazilian-1 laboratory, Cusco population, and Peruvian) and 1.51 ± 0.08°C (mean ± SEM, treatments with Brazilian-1 wild), from 3 to 17 d is shown in Table 3. As expected, larval mortality increased significantly with duration of cold treatment (dose: χ^2 = 17,198; df = 4; P < 0.0001). Morphotypes of the *A. fraterculus* complex responded differently to cold treatment. Third instars from Cusco population, Andean, and Peruvian morphotypes were more susceptible to cold treatments than the Brazilian-1 (laboratory and wild) morphotype (morphotype: χ^2 = 83; df = 4; P < 0.0001), particularly at sublethal doses. Brazilian-1 wild was the most cold-tolerant of all morphotypes in treatments of 8 and 9 d, and Brazilian-1 laboratory was more tolerant than Andean, Cusco population, and Peruvian after 8 d of treatment (dose × morphotype: χ^2 = 411; df = 16; P < 0.0001). However, no difference in mortality was found among *A. fraterculus* morphotypes at 10 and 15 d of cold treatment (Table 3). Interestingly, infested nectarines exposed to cold treatments for 15 d yielded no survivors for all *A. fraterculus* morphotypes, except for Brazilian-1 wild, in which one survivor was found. While this survivor was moving, and, thus, was counted as alive, it died as a coarctate larva and did not survive to the adult stage. No moving larva was found among the 1,758 third instars from Brazilian-1 wild exposed to 10 d of cold treatment. Increasing the duration of cold treatment to 17 d for nectarines infested by Brazilian-1 wild yielded no survivors among the 3,416 larvae treated (Table 3).

Comparisons of LT estimates for 50, 99.9, and 99.99682% (probit 9) mortality at 95% CI show significant differences between *A. fraterculus* morphotypes (Table 4). The estimated LT_{50} values for Brazilian-1 wild and Brazilian-1 laboratory were the highest among all morphotypes, followed by Peruvian, Andean, and Cusco population (i.e., Brazilian-1 wild = Brazilian-1 laboratory > Peruvian = Andean > Cusco population). At the 99.9% level of control, Brazilian-1 (wild and laboratory) was the most cold-tolerant morphotype followed by Cusco population, Andean, and Peruvian, the least cold-tolerant morphotype (i.e., Brazilian-1 wild = Brazilian-1 lab > Cusco population = Andean > Peruvian). At the 99.99682 % level of control, Brazilian-1 wild, Brazilian-1 laboratory, and Cusco population were the most cold-tolerant, followed by Andean and Peruvian, the least cold-tolerant morphotype (i.e., Brazilian-1 wild = Brazilian-1 laboratory = Cusco population > Andean > Peruvian).

Cool down time was 240 ± 39 min (mean ± SE) for noninfested fruit. Infested nectarines were treated at temperatures of 1.38 ± 0.04°C to 1.51 ± 0.08°C (mean ± SEM) for 3, 8, 9, 10, 15 (all morphotypes), and 17 d (Brazilian-1/wild). Temperatures (mean ± SD) recorded in thermocouples across blocks are summarized for noninfested nectarines, water, and air in Supplementary Tables S1–S4.

Discussion

The results of our study provide evidence that phytosanitary cold treatment against third instars of the Brazilian-1 morphotype (aka *Anastrepha* sp. 1 *aff. fraterculus*) can also be applied against the Cusco population, Andean, and Peruvian (aka *Anastrepha* sp. 4 *aff. fraterculus*) morphotypes of the *A. fraterculus* complex. Third instars of the three *A. fraterculus* morphotypes evaluated in our study differed in their mortality only with short treatment durations at 1.38 ± 0.04°C and 1.51 ± 0.08°C (mean ± SEM). Brazilian-1 wild was more tolerant than all *A. fraterculus* morphotypes in cold treatments of 8 and 9 d. Cusco population, Andean, and Peruvian morphotypes were more susceptible to cold treatment of 8 d than Brazilian-1 laboratory. In contrast, no significant differences in acute mortality of third instars were found among the *A. fraterculus* morphotypes after cold treatment durations of 10 and 15 d. Considering the estimated lethal times (LTs) to achieve 99.99682 % (probit 9) efficacy, no differences were found in Brazilian-1 wild (LT = 13.67 ± 0.72 d), Brazilian-1 laboratory (LT = 13.30 ± 0.43 d), and Cusco population (LT = 12.84 ± 0.62 d), but they were significantly different from

Table 2. Number of infested and noninfested fruits after being exposed to sexually mature females of morphotypes of the *A. fraterculus* complex and their infestation rates (larvae/fruit)

Host	Morphotype or population	Total no. of infested fruit	Total no. of noninfested fruit	No. larvae/fruit (mean ± SE)
Mandarin	Andean	27	330	6 ± 1
	Brazilian-1 lab.	56	363	5 ± 1
	Cusco	26	310	10 ± 1
	Peruvian	43	270	18 ± 1
Nectarine	Andean	276	11	125 ± 6
	Brazilian-1 lab.	279	9	127 ± 7
	Brazilian-1 wild	322	30	54 ± 3
	Cusco	248	14	117 ± 7
	Peruvian	265	16	131 ± 7

Andean (LT = 11.63 ± 0.37 d) and Peruvian (LT = 9.83 ± 0.33 d) that also differed from each other. The LT estimates of such extreme level of control against one of the most cold tolerant *A. fraterculus* morphotype, apparently Brazulian-1 wild, indicate that 99.99682% efficacy could be achieved with a treatment schedule of less than 15 d at temperatures below 1.7°C.

Although the results from previous studies evaluating the cold tolerance of *A. fraterculus* populations cannot be directly compared because of critical methodological differences (e.g., LT estimates and target temperature), they share a few similarities with our findings. Depending on the citrus species and variety, the LT_{50} estimates for fruits artificially infested with third instars of an Argentinean population (Tucumán) treated at temperatures below 2°C ranged from 1.13 to 7.94 d (Willink et al. 2006). Even though our results are based on naturally infested nectarines treated at temperatures below 1.67°C, the LT_{50} estimates from Brazilian-1 wild (LT = 4.74 ± 0.04 d) and Brazilian-1 laboratory (LT = 4.62 ± 0.05 d) from Tucumán are within the range reported by Willink et al. (2006). For *A. fraterculus* populations from Colombia, no survivors were found in feijoa, *Acca sellowiana* (O.Berg) Burret, artificially infested with third instars after 8 d treatment at 1.1°C (Valderrama et al. 2005). Similarly, we also found no survivors on treatments of third instars (5,418 larvae) of the Andean morphotype from Ibagué for 8 d at 1.38 ± 0.04°C (mean ± SEM).

Unlike Myers et al. (2016) and Hallman et al. (2019a) that found no significant differences in cold tolerance among *B. dorsalis* (Hendel) (Diptera: Tephritidae) and Ceratitis *capitata* (Wiedemann) (Diptera: Tephritidae) populations, respectively, the *A. fraterculus* morphotypes evaluated in our study differed significantly in their cold tolerance at sublethal doses and LT estimates to achieve either 99.9 or 99.99682% levels of control. Curiously, the findings from these comparative studies correlate well with the taxonomic status of the group evaluated. For instance, the four *B. dorsalis* populations evaluated by Myers et al. (2016) were considered different species before the formal taxonomic revision that led to the synonymization of *Bactrocera invadens*, *Bactrocera papayae*, and *Bactrocera philippinensis* with *Bactrocera dorsalis* (Schutze et al. 2015a,b). For the *A. fraterculus* complex, however, the differences on cold tolerance among morphotypes reported in our study correlate with the taxonomic uncertainty within the group (Hendrichs et al. 2015, Schutze et al. 2017). That is, contrary to the similar response to cold treatments found in studies with populations from the same species (Myers et al. 2016, Hallman et al. 2019a), the differences in cold tolerance and infestation rates among morphotypes of the *A. fraterculus* complex reported here further suggest that some of these populations may belong to different species.

The greater susceptibility of the laboratory domesticated populations of *A. fraterculus* reared on artificial diet to cold treatments of 8 and 9 d relative to Tucumán/wild, a wild collected strain reared on fruit, should not be ignored. Furthermore, Tucumán/wild was the only population in our study in which a single larva (out of 3,865) survived to the treatment duration of 15 d but did not pupariate (temperature details for Block 2 in Supplementary Table S2). The influence of laboratory domestication and artificial diet on cold tolerance in tephritid fruit flies is unknown, particularly in the context of phytosanitary treatments (Mangan and Hallman 1998). Nevertheless, basic research with non-pest insects suggests that both laboratory domestication and diet composition have the potential to reduce cold-stress tolerance. For instance, inbreeding decreased the evolutionary potential of the tropical butterfly, *Bicyclus anynana* (Butler) (Lepidoptera: Nymphalidae), and, consequently, reduced its ability to respond to selection for increased cold stress resistance in a tolerance assay of 1°C for 19 h (Dierks et al. 2012). In *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae), a high dietary sugar intake in larvae and adults increased mortality 24 h after a treatment of 0°C for 16 h (Colinet et al. 2013). It remains to be determined, however, whether similar responses can be observed in tephritid fruit flies after the long periods of cold exposure required by phytosanitary cold treatments.

Besides laboratory domestication and larval diet, another important research aspect to consider while proposing generic cold treatments is host suitability. Usually, third instars reared in poor hosts are more susceptible to phytosanitary cold treatments than larvae reared in suitable hosts (De Lima et al. 2007, Gazit et al. 2014). For example, the durations of cold treatment schedules of 3°C or below against *C. capitata* are 23 continuous days for *Citrus paradisi* Macfad (IPPC 2017a), 20 continuous days for *Citrus sinensis* (IPPC 2017b) and *Citrus reticulata* × *Citrus sinensis* (L.) Osbeck (IPPC 2017c), and just 18 continuous days for the conditional host *Citrus limon* (L.) Osbeck (IPPC 2017d). We have accounted for host suitability by using nectarine, a suitable host for all morphotypes of the *A. fraterculus* complex evaluated in our study.

Our findings suggest that the schedules T107-a-1 and T107-c (i.e., 15 d at ≤ 1.11°C or 17 d at ≤ 1.67°C, USDA 2019) can be applied as cold treatments to any new species that may arise from the *A. fraterculus* complex. The development of broadly applicable (generic) phytosanitary treatments does not require systematic testing against all pest species of a group (Hallman et al. 2010). The schedule T107-a-1 used for *C. capitata* and *Anastrepha* spp. (excluding *A. ludens*) to treat 18 fruits, for example, was established without evaluating all quarantine species of the genus *Anastrepha* nor efficacy on all the 18 fruits. We reinforce that the use of broadly applicable phytosanitary treatments against members of cryptic species complexes constitutes a proactive strategy to prevent agricultural trade barriers and ensure plant health protection in case of new species arise from these complexes.

Table 3. Numbers of replicates, fruit, treated larvae, live larvae, larvae per fruit, and mortality (%) of three morphotypes of the *A. fraterculus* complex third instars reared in nectarines exposed to 1.38 ± 0.04°C and 1.51 ± 0.08°C (mean ± SEM) for 0–17 d

Treatment duration (days)	Morphotype or population	No. of replicates	Total no. of treated fruit	Total no. of treated larvae	Total no. of live larvae	No. larvae per fruit (mean ± SE)	Mortality (mean ± SE)[a]
0	Andean	15	58	8,325	8,081	144 ± 14	5.61 ± 2.42 AB
	Brazilian-1 lab.	13	58	8,136	8,013	140 ± 18	4.28 ± 1.36 C
	Brazilian-1 wild	9	57	2,448	2,329	43 ± 5	4.03 ± 1.55 C
	Cusco	14	53	7,327	7,052	138 ± 14	7.62 ± 2.60 A
	Peruvian	16	57	7,372	7,209	129 ± 15	5.39 ± 2.36 B
3[b]	Andean	4	23	3,339	2,474	145 ± 21	44.01 ± 7.10
	Brazilian-1 lab.	4	14	1,851	1,141	132 ± 27	40.47 ± 7.57
	Brazilian-1 wild	5	26	982	680	38 ± 5	36.20 ± 5.25
	Cusco	4	16	1,960	1,129	122 ± 24	47.44 ± 7.14
	Peruvian	4	20	2,711	2,185	136 ± 22	29.39 ± 6.01
5[b]	Andean	6	41	5,572	859	136 ± 16	88.79 ± 3.08
	Brazilian-1 lab.	7	45	5,551	2,113	116 ± 13	64.33 ± 3.63
	Brazilian-1 wild	5	26	1,577	663	61 ± 12	53.00 ± 5.65
	Cusco	5	36	3,180	406	88 ± 15	87.10 ± 4.10
	Peruvian	6	38	3,185	372	84 ± 11	85.31 ± 3.94
8	Andean	8	41	5,418	0	132 ± 15	100.00 ± 0.00 A
	Brazilian-1 lab.	8	41	4,951	134	121 ± 17	96.16 ± 2.57 B
	Brazilian-1 wild	5	29	2,010	44	69 ± 10	94.25 ± 2.80 C
	Cusco	7	37	2,842	1	77 ± 15	99.98 ± 0.02 A
	Peruvian	7	38	5,415	0	143 ± 22	100.00 ± 0.00 A
9	Andean	8	36	4,305	0	120 ± 17	100.00 ± 0.00 A
	Brazilian-1 lab.	8	41	5,089	16	124 ± 18	99.77 ± 0.15 A
	Brazilian-1 wild	4	22	1,480	17	67 ± 10	99.15 ± 0.00 B
	Cusco	8	36	3,918	0	109 ± 17	100.00 ± 0.00 A
	Peruvian	8	37	5,618	0	152 ± 21	100.00 ± 0.00 A
10	Andean	8	36	3,880	0	108 ± 15	100.00 ± 0.00 A
	Brazilian-1 lab.	8	42	5,967	0	142 ± 25	100.00 ± 0.00 A
	Brazilian-1 wild	6	42	1,758	0	42 ± 6	100.00 ± 0.00 A
	Cusco	8	33	4,645	0	141 ± 21	100.00 ± 0.00 A
	Peruvian	8	35	4,975	0	142 ± 21	100.00 ± 0.00 A
15	Andean	8	41	3,641	0	89 ± 12	100.00 ± 0.00 A
	Brazilian-1 lab.	8	38	4,238	0	112 ± 15	100.00 ± 0.00 A
	Brazilian-1 wild	6	62	3,865	1	62 ± 8	99.98 ± 0.02 A
	Cusco	8	37	5,179	0	140 ± 19	100.00 ± 0.00 A
	Peruvian	8	40	5,366	0	134 ± 19	100.00 ± 0.00 A
17[b]	Brazilian-1 wild	4	58	3,416	0	59 ± 7	100.00 ± 0.00

[a]Different letters indicate statistically significant differences between groups (estimated marginal means contrasts from the bias reduction GLM binomial model, $P < 0.05$)

[b]Doses were included only in the probit model

Table 4. Probit model estimates and 95% fiducial limits of days cold treatment at 1.38 ± 0.04°C and 1.51 ± 0.08°C (mean ± SEM) required to produce 50%, 99.9%, and 99.9968% mortality of third instars in nectarines

Morphotype or Population	LT* (95% fiducial limits) in days		
	LT_{50}	$LT_{99.9}$	$LT_{99.99682}$ (probit 9)
Andean	3.66 (3.59, 3.73) B	8.94 (8.73, 9.16) B	11.63 (11.26, 12.00) B
Brazilian-1 lab.	4.62 (4.58, 4.67) C	10.46 (10.21, 10.71) D	13.30 (12.87, 13.73) A
Brazilian-1 wild	4.74 (4.70, 4.77) C	10.74 (10.33, 11.15) D	13.67 (12.95, 14.36) A
Cusco	3.26 (3.15, 3.38) A	9.41 (9.07, 9.76) AB	12.84 (12.22, 13.47) A
Peruvian	3.71 (3.69, 3.74) B	7.88 (7.68, 8.08) C	9.83 (9.50, 10.15) C

*Lethal time (LTs followed by different letters indicate statistical significance, lethal dose ratio tests, $P < 0.05$).

Supplementary Material

Supplementary data are available at *Journal of Economic Entomology* online.

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