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Quarantine Cold Treatments for *Ceratitis capitata* and *Anastrepha fraterculus* (Diptera: Tephritidae) for Citrus in Argentina: Conclusions After 10 Years of Research

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ABSTRACT: Argentina has quarantine restrictions in some markets due to the presence of two quarantine fruit fly pests: *Ceratitis capitata* and *Anastrepha fraterculus*. One alternative is the use of cold quarantine treatments during transport of the commodities. Since 1996, the Estación Experimental Agroindustrial Obispo Colombres (EEAOC), Tucumán, Argentina, has developed different cold quarantine treatments for citrus. In the present work we present all the data the EEAOC generated in the last ten years in order to facilitate the development of such cold treatments. Fruit flies were obtained from the colonies reared at EEAOC. Four citrus species were analyzed: lemon, grapefruit, orange and tangerines. Different varieties were analyzed for each fruit species. Sensitivity trials aiming at determine the most tolerant stage as well as to asses if there is any influence of varieties on cold tolerance were performed. Finally we compared the tolerance to cold between the two species. Sensitivity trials showed that mature larvae (L3) are the most tolerant stage for both fruit fly species. There was no effect of the varieties and the two fruit fly species were equally sensible to cold. Our results provide strong evidence in favor of concluding that any cold treatment developed for *C. capitata* is effective for *A. fraterculus*.

Key Words: Medfly, South American fruit fly, phytosanitary regulatory measures

INTRODUCTION

Citrus production has grown in the last years in NW Argentina, mainly as a result of an increase in lemon production (Stein, 2007). However, some of the potential markets for Argentinean citrus have been closed due to phytosanitary restrictions. The opening of the Japanese and other East Asian countries markets was limited chiefly by the presence of the medfly, *Ceratitis capitata*, in some regions of Argentina and to a lesser extent by the presence of the South American fruit fly, *Anastrepha fraterculus*. It is worth mentioning that the former is considered to be one of the most damaging pests in agriculture (Christenson and Foote, 1959).

Different National Plant Protection Organizations have a number of policies to determine phytosanitary measures for one same pest. The Animal Plant and Inspection Service (APHIS), from the United States Department of Agriculture (USDA) has standardized cold quarantine treatments for various fruit fly species, regardless of the fruit type and cultivar. Recently modified cold treatments established by APHIS (2006) include T107-a for *C. capitata* at 1.1ºC, 1.67ºC and 2.2ºC for the period of 14, 16 and 18 days respectively and T107-a-1 for *C. capitata* and *Anastrepha spp*. other than *A. ludens*, which is one day longer than the T107-a.

On the other hand, the Ministry of Agriculture, Forestry and Fisheries (MAFF) from Japan requires each country to develop its own treatments for all the varieties proposed for export. Japan has authorized various treatments in citrus for *C. capitata* from different countries: oranges from Spain at 2ºC for 17 days, from Israel at 0.5ºC for 14 days and at 1.5ºC for 16 days, from Australia at 1ºC for 16 days, from South Africa at –0.6ºC for 12 days and for lemons from Spain at 2ºC for 16 days, from Australia at 1ºC for 14 days and from South Africa the same schedule as for oranges (MAFF, 1996).

The origin of cold treatment research can be traced back to the beginning of 20th century when Back and Pemberton (1916) studied the influence of low temperature on different developmental stages of *C. capitata* on peaches and apples, concluding that the third

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instar larvae was the stage most tolerant to cold. More recently, Hill *et al*. (1988) working with Valencia oranges reached similar conclusions; Jessup *et al*. (1993) working with lemons found no statistical differences between the stages for Eureka lemons, but the second instar larvae proved to be the most cold tolerant for the Lisbon variety. Gould (1996), working with carambola fruit infested with eggs and larvae of *A. suspensa* found no differences among stages. No data could be found in cold treatments for *A. fraterculus*.

Since 1996, the Estación Experimental Agroindustrial Obispo Colombres (EEAOC), Tucumán, Argentina, has developed different cold quarantine treatments for citrus for the two species of economic importance found in Argentina, *C. capitata* and *A. fraterculus* in order to open new markets for its citrus production. The present work analyzes all the data the EEAOC generated in the last ten years in order to facilitate the development of cold treatments for citrus.

OBJECTIVES

The present work had three objectives. The first one was to establish which of the developmental stage of *C. capitata* and *A. fraterculus* was most tolerant to cold in different citrus species; the second was to evaluate the influence of the variety within each citrus species on cold sensitivity, and the third was to compare the cold tolerance between *C. capitata* and *A. fraterculus*.

MATERIALS AND METHODS

Fruit fly species and developmental stages. Biological material used in this work consisted of immature stages of *C. capitata* and *A. fraterculus* obtained from a colony maintained at the EEAOC, Tucumán, Argentina. The colony of *C. capitata* originated from the collection of infested fruit, mainly oranges and grapefruit, from NW Argentina and *A. fraterculus* from guavas from Tucumán, Argentina. Each summer, field collected flies were introduced in the colony on four successive occasions in order to maintain wild-like attributes. Quality control was performed for each generation by looking at egg viability, egg/pupae recovery, pupal weight, adult emergence, male/female ratio, flight ability, adult longevity and eggs per female (Orozco et al. 1983, FAO/IAEA/USDA 2003).

The developmental stages used in the trials were: eggs with more than half of their embryonic development complete; immature larvae comprising the first and second instars (L1+L2) and mature larvae comprising the third instar (L3).

Citrus species and varieties. The citrus species and varieties used were: lemon (*Citrus limon*) with Eureka, Lisbon, Lisbon Limoneira 8A, and Genoa varieties; oranges (*C. sinensis*) with Washington Navel and Lanelate in the Navel group, Salustiana, Lue Gim Gong, and Valencia varieties; grapefruit (*C. paradisi*) with Marsh Seedless, Henninger`s Ruby, Rouge La Toma, and Star Ruby varieties; tangerines (*C. reticulata*) and hybrids, with Clemenules, Marisol, and Hernandina in the Clementines group, and Nova, Ellendale, and Murcott varieties. All fruits used in the trials met commercial maturity standards.

Experimental procedures. Fruit was artificially inoculated with 35 individuals per fruit, by cutting an opening at the top of the fruit, placing the eggs or larvae on the fruit pulp, covering the opening with the fruit skin and sealing it with paraffin. The eggs were inoculated on the same day they were introduced in the cold chamber. The immature and mature larvae were inoculated and placed in a chamber at 25 ºC for 24 hours in order to allow them to adapt to the fruit, before introducing them into the cold chamber. The treatment temperature was 2 ± 0.5 ºC. Temperatures were automatically recorded every hour with six fruit pulp sensors per treatment. The treatment began when the readings of at least four sensors showed less than 2 ºC. Between 6 and 8 days of exposure were used for the different developmental stages. After exposure, the fruit was taken to a 25 ºC chamber and checked for mortality. Fruit containing immature and mature larvae were checked after 48 hours, considering that moving larvae were alive. Fruit containing eggs were checked for mortality after 5 days at 25 ºC, considering a live egg as the one from which a larva had hatched. Each treatment included more than 200 viable individuals of the developmental stages mentioned above and was replicated three times.

Sensitivity trials for developmental stages in *C. capitata*. In order to establish the developmental stage most tolerant to cold in *C. capitata*, sensitivity trials were conducted comparing three developmental stages (eggs, immature and mature larvae) on four varieties of the four citrus species (lemon: Eureka, Lisbon, Lisbon Limoneira 8A, and Genoa; oranges: Washington Navel, Salustiana, Lue Gim Gong, and Valencia; grapefruit: Rouge La Toma, Star Ruby, Henninger`s Ruby, and Marsh Seedless; tangerines and hybrids: Clemenules, Nova, Ellendale, and Murcott).

Sensitivity trials for developmental stages in *A. fraterculus*. As regards to *A. fraterculus,* one variety of lemon, orange and grapefruit were evaluated (Eureka, Valencia and Henninger`s Ruby respectively) while two varieties of tangerines and hybrids were evaluated (Hernandina and Murcott).

Influence of varieties on cold sensitivity of mature larvae in *C. capitata*. To determine whether the varieties within each citrus species had an influence on the sensitivity to cold in *C. capitata*, data obtained for third instar larvae was assessed in four varieties of lemon and grapefruit (lemon: Eureka, Lisbon, Lisbon Limoneira 8A, and Genoa; grapefruit: Rouge La Toma, Star Ruby, Henninger`s Ruby, and Marsh Seedless), five varieties of orange (Washington Navel, Salustiana, Lue Gim Gong, Lanelate, and Valencia), and six tangerine and hybrid varieties (Nova, Ellendale, Murcott, Clemenules, Hernandina, and Marisol).

Influence of varieties on cold sensitivity of mature larvae in *A. fraterculus*. In *A. fraterculus*, third instar larvae susceptibility was compared in three varieties of oranges (Valencia, Salustiana, and Washington Navel), and three tangerine and hybrid varieties (Murcott, Hernandina, and Ellendale).

Tolerance to cold between *C. capitata* and *A. fraterculus*. To compare cold tolerance between *C. capitata* and *A. fraterculus*, the three developmental stages of both fruit fly species on one variety of each of the four citrus species were examined (lemon: Eureka; orange: Valencia; grapefruit: Henninger's Ruby and tangerine: Murcott).

Statistical analysis. In order to achieve a minimum number of 200 viable individuals per treatment, control fruit was set apart just before introduction in the cold chamber. The total number of insects treated resulted from deducting the proportion of dead insects in the control from the total of insects inoculated. Mortality was corrected as described by Abbott (1925). Mortality data was analysed using the Probit method (Finney, 1971), comparing 95% confidence intervals (CI 95%) of the lethal time 50 (LT 50) of the different treatments. If the CI did not overlap, they were considered different.

RESULTS

Probit analysis results for *C. capitata* developmental stages are shown in Tables 1 to 4 for lemon, orange, grapefruit, and tangerine and hybrids respectively. Probit analysis results for *A. fraterculus* developmental stages are shown in Tables 5 to 8 for lemon, orange, grapefruit, and tangerine and hybrids respectively. Probit analysis to compare third instar larvae tolerance to cold between *C. capitata* and *A. fraterculus* are shown in Table 9.

Table 1. Cold sensitivity for different developmental stages of *C. capitata* in lemon varieties.

STAGE		Eggs		Immature larvae		Mature larvae	
VARIETY	**Rep.**	**LT 50**	**CI 95%**	**LT 50**	**CI 95%**	**LT 50**	**CI 95%**
Eureka	I	1.881	1.617 - 2.127	3.080	2.912 - 3.227	6.624	6.095 - 7.114
	II	1.929	1.631 - 2.203	1.887	0.631 - 2.733	7.056	6.827 - 7.252
	III	1.855	1.616 - 2.078	2.563	1.419 - 3.266	7.178	7.062 - 7.289
Lisbon	I	1.471	1.265 - 1.661	3.370	2.579 - 3.948	6.344	5.508 - 7.259
	II	1.429	0.842 - 1.892	3.352	2.485 - 3.957	6.455	5.810 - 7.143
	III	1.511	1.299 - 1.708	3.201	2.676 - 3.592	6.978	6.218 - 7.615
Lisbon Limoneira 8 A	I	2.727	2.022 - 3.262	4.551	3.542 - 5.318	6.763	6.024 - 7.345
	II	3.416	2.739 - 4.011	4.860	4.135 - 5.455	6.891	6.409 - 7.310
	III	3.622	2.265 - 4.617	4.320	3.593 - 4.887	6.496	5.463 - 7.488
Genoa	I	2.608	2.145 - 2.942	3.467	2.979 - 3.839	6.075	4.791 - 7.349
	II	2.174	1.778 - 2.486	3.455	2.881 - 3.894	5.782	5.293 - 6.260
	III	2.476	1.955 - 2.861	3.309	2.169 - 4.075	5.958	4.391 - 7.366

Sensitivity trials for developmental stages in *C. capitata*. The results of the sensitivity trials for developmental stages for *C. capitata* on lemons showed no differences in LT 50 confidence intervals between eggs and immature larvae in all varieties, except in Lisbon. In all cases, mature larvae had the highest LT values, and were statistically different from eggs and immature larvae.

In oranges, the comparison of LT 50 between eggs and immature larvae showed differences in the Valencia and Salustiana varieties and no differences in the other two varieties. Immature and mature larvae showed differences in the LT 50 for all varieties with the exception of Salustiana. In grapefruits, no differences in LT 50 were found between eggs and immature larvae whereas mature larvae displayed higher LT 50 than earlier stages. In tangerines and hybrids, there were no differences between LT 50 for eggs and immature stages in Murcott and Clemenule, however, there were differences LT 50 in Ellendale and Nova. For all varieties except Ellendale immature and mature larvae had different LT 50's.

Sensitivity trials for developmental stages in *A. fraterculus*. Results of the sensitivity trials for three developmental stages of *A. fraterculus* on lemons showed differences in LT 50 between eggs and immature larvae, and between the latter and mature larvae. In oranges, comparison of LT 50 among eggs, immature larvae and mature larvae revealed differences in cold tolerance among different developmental stages. In grapefruit, there were no differences in LT 50 between eggs and immature larvae, but there were differences in LT 50 between those stages and the mature larvae. In contrast, no differences in LT 50 were detected among different stages in tangerines and hybrids.

Influence of varieties on the sensitivity of mature larvae of *C. capitata*. No differences in LT 50 were found in cold sensitivity of *C. capitata* mature larvae among the four varieties of lemon and grapefruit, the six varieties of tangerines and hybrids, nor the five orange varieties.

Influence of varieties on the sensitivity of mature larvae of *A. fraterculus*. No differences in cold sensitivity of *A. fraterculus* mature larvae were found among three tangerines and hybrid varieties, nor among three orange varieties.

Tolerance to cold between *C. capitata* and *A. fraterculus*. We failed to find significant differences when comparing cold sensitivity between *C. capitata* and *A. fraterculus* on one variety of each citrus species (Eureka lemons, Valencia oranges, Henninger´s Ruby grapefruit, and Murcott tangerines).

Table 2. Cold sensitivity for different developmental stages of *C. capitata* in orange varieties.

STAGE		Eggs		Immature larvae		Mature larvae	
VARIETY	**Rep.**	**LT 50**	**CI 95%**	**LT 50**	**CI 95%**	**LT 50**	**CI 95%**
Valencia	I	1.205	0.715 - 1.621	3.966	3.659 - 4.229	7.400	6.181 - 8.364
	II	2.268	1.180 - 2.693	3.936	3.263 - 4.424	6.400	4.939 - 7.673
	III	1.036	0.747 - 1.290	4.162	3.792 - 4.485	6.283	5.371 - 7.122
Lue Gim Gong	I	1.261	1.036 - 1.463	4.665	4.336 - 4.967	5.829	5.415 - 6.241
	II	3.374	2.651 - 4.037	4.765	4.032 - 5.422	5.937	5.745 - 6.126
	III	3.839	3.266 - 4.321	4.524	3.892 - 5.067	5.541	5.070 - 5.982
Salustiana	I	1.425	0.856 - 1.885	3.712	3.373 - 4.054	5.667	4.919 - 6.295
	II	1.523	0.999 - 1.961	3.606	3.161 - 4.059	5.735	5.253 - 6.168
	III	2.104	1.717 - 2.429	5.348	4.647 - 5.925	5.775	5.333 - 6.172
Washing-ton Navel	I	2.730	2.278 - 3.139	2.945	2.153 - 3.590	5.303	4.853 - 5.712
	II	2.647	1.893 - 3.250	2.768	2.007 - 3.377	5.203	4.960 - 5.432
	III	2.766	2.247 - 3.189	2.399	1.558 - 3.038	5.641	5.200 - 6.044
Lanelate	I					4.817	3.834 - 5.584
	II		N / D		N / D	4.594	3.876 - 5.166
	III					4.468	4.195 - 4.719

Table 3. Cold sensitivity for different developmental stages of *C. capitata* in grapefruit varieties.

STAGE		Eggs		Immature larvae		Mature larvae	
VARIETY	**Rep.**	**LT 50**	**CI 95%**	**LT 50**	**CI 95%**	**LT 50**	**CI 95%**
Marsh seedless	I	3.751	2.349 - 4.859	4.272	2.715 - 5.056	6.910	5.562 - 7.891
	II	4.032	3.480 - 4.796	3.618	3.152 - 3.965	6.765	6.391 - 7.111
	III	1.686	1.492 - 1865	3.788	2.710 - 4.441	6.961	6.674 - 7.225
Henninger's Ruby	I	1.117	0.688 - 1.488	4.194	3.838 - 4.491	5.650	4.700 - 6.457
	II	1.230	0.666 - 1.704	4.113	3.932 - 4.279	6.133	5.630 - 6.622
	III	3.480	2.891 - 4.042	4.204	3.925 - 4.446	5.602	5.384 - 5.819
Star Ruby	I	3.701	3.275 - 4.109	3.829	3.043 - 4.187	7.169	6.514 - 7.731
	II	3.376	2.622 - 4.056	3.473	2.827 - 3.911	6.724	5.746 - 7.485
	III	2.461	1.954 - 2.913	3.494	2.967 - 3.831	6.387	5.288 - 7.340
Rouge La Toma	I	2.036	1.315 - 2.703	3.534	2.881 - 4.004	5.646	5.035 - 6.233
	II	2.145	1.781 - 2.451	3.662	2.951 - 4.123	5.231	4.129 - 6.158
	III	1.877	1.145 - 2.540	2.888	2.386 - 3.251	5.387	4.379 - 6.267

Table 4. Cold sensitivity for different developmental stages of *C. capitata* in tangerines and hybrid varieties.

STAGE		Eggs		Immature larvae		Mature larvae	
VARIETY	Rep.	LT 50	CI 95%	LT 50	CI 95%	LT 50	CI 95%
Nova	I	1.690	1.432 - 1.927	3.254	2.381 - 4.022	5.888	5.016 - 6.648
	II	1.755	1.651 - 1.857	3.888	3.294 - 4.458	6.495	6.271 - 6.711
	III	1.818	1.600 - 2.023	4.702	4.313 - 5.053	5.965	5.115 - 6.695
Ellendale	I	0.915	0.326 - 1.405	3.409	3.040 - 3.757	4.782	4.174 - 5.305
	II	1.048	0.384 - 1.581	3.591	3.200 - 3.962	4.614	3.961 - 5.159
	III	1.188	0.522 - 1.713	3.234	2.493 - 3.889	5.443	5.021 - 5.823
Murcott	I	1.969	1.740 - 2.173	5.102	4.607 - 5.545	6.340	5.821 - 6.816
	II	1.974	1.847 - 2.093	4.965	4.143 - 5.662	6.530	5.732 - 7.234
	III	1.974	0.619 - 2.991	3.666	2.781 - 4.422	6.955	5.711 - 7.936
Clemenule	I	2.724	1.488 - 3.737	2.033	1.847 - 2.201	5.635	5.325 - 5.923
	II	2.323	1.536 - 2.931	2.217	1.588 - 2.724	4.454	3.965 - 4.860
	III	2.066	0.780 - 3.167	2.491	2.239 - 2.710	5.330	4.913 - 5.709
Hernandina	I					5.607	5.386 - 5.818
	II	N / D		N / D		5.513	5.140 - 5.856
	III					5.097	4.613 - 5.528
Marisol	I					5.282	4.833 - 5.690
	II	N / D		N / D		4.871	4.290 - 5.356
	III					5.334	4.903 - 5.716

Table 5. Cold sensitivity for different developmental stages of *A. fraterculus* in lemon.

STAGE		Eggs		Immature larvae		Mature larvae	
VARIETY	Rep.	LT 50	CI 95%	LT 50	CI 95%	LT 50	CI 95%
Eureka	I	1.223	1.017 - 1.411	3.727	3.504 - 3.901	5.658	5.272 - 6.007
	II	1.874	1.265 - 2.349	3.366	2.976 - 3.599	5.929	4.943 - 6.727
	III	1.910	1.563 - 2.209	3.099	2.711 - 3.355	7.083	6.337 - 7.624

Table 6. Cold sensitivity for different developmental stages of *A. fraterculus* in orange varieties.

STAGE		Eggs		Immature larvae		Mature larvae	
VARIETY	**Rep.**	**LT 50**	**CI 95%**	**LT 50**	**CI 95%**	**LT 50**	**CI 95%**
Valencia	I	1.634	0.871 - 2.212	3.859	3.527 - 4.133	5.866	5.488 - 6.210
	II	1.860	1.141 - 2.434	3.671	2.990 - 4.145	6.097	5.250 - 6.827
	III	1.775	1.054 - 2.339	3.892	3.513 - 4.201	5.368	4.663 - 5.964
Salustiana	I					4.382	3.204 - 5.265
	II	N / D		N / D		4.340	3.153 - 5.218
	III					4.535	3.369 - 5.410
Washington	I					5.821	5.317 - 6.267
	II	N / D		N / D		5.762	5.047 - 6.382
	III					5.425	4.982 - 5.823

Table 7. Cold sensitivity for different developmental stages of *A. fraterculus* in grapefruit.

STAGE		Eggs		Immature larvae		Mature larvae	
VARIETY	**Rep**	**LT 50**	**CI 95%**	**LT 50**	**CI 95%**	**LT 50**	**CI 95%**
Heninnger's Ruby	I	1.803	0.975 - 2.455	4.174	3.243 - 4.761	6.171	5.429 - 6.853
	II	2.510	1.531 - 3.250	3.591	2.488 - 4.120	6.279	5.859 - 6.863
	III	2.217	0.790 - 3.242	4.109	3.306 - 4.602	6.972	5.707 - 7.949

Table 8. Cold sensitivity for different developmental stages of *A. fraterculus* in tangerines and hybrid varieties.

STAGE		Eggs		Immature larvae		Mature larvae	
VARIETY	**Rep.**	**LT 50**	**CI 95%**	**LT 50**	**CI 95%**	**LT 50**	**CI 95%**
Murcott	I	4.902	1.011 - 6.986	3.724	2.787 - 4.598	6.031	4.252 - 7.439
	II	2.643	1.634 - 3.724	4.016	3.314 - 4.598	6.245	4.880 - 7.319
	III	2.366	1.636 - 2.971	2.993	2.361 - 3.529	5.788	5.220 - 6.290
Hernadine	I	4.957	4.191 - 5.628	4.249	2.008 - 5.721	6.106	3.967 - 7.584
	II	5.115	2.836 - 7.358	2.452	1.074 - 3.560	5.341	4.070 - 6.332
	III	4.840	3.273 - 6.212	3.120	0.934 - 4.383	5.888	4.890 - 6.722
Ellendale	I					5.960	4.874 - 6.690
	II	N / D		N / D		5.451	1.135 - 7.704
	III					5.166	4.837 - 5.464

Table 9. Cold sensitivity for third instar larvae of *C. capitata* and *A. fraterculus* in four citrus species.

			C. capitata		*A. fraterculus*	
Species	**Variety**	**Rep**	**LT 50**	**CI 95%**	**LT 50**	**CI 95%**
Lemon	**Eureka**	I	6.624	6.095 – 7.114	5.658	5.272 – 6.007
		II	7.056	6.827 – 7.252	5.929	4.943 – 6.727
		III	7.178	7.062 – 7.289	7.083	6.337 – 7.624
Orange	**Valencia**	I	7.400	6.181 – 8.364	5.866	5.488 – 6.210
		II	6.400	4.939 – 7.673	6.097	5.250 – 6.827
		III	6.283	5.371 – 7.122	5.368	4.663 – 5.964
Grapefruit	**Henninger´s Ruby**	I	5.650	4.700 – 6.475	6.171	5.429 – 6.853
		II	6.133	5.630 – 6.622	6.279	5.859 – 6.863
		III	5.602	5.384 – 5.819	6.972	5.707 – 7.949
Tangerine	**Murcott**	I	6.340	5.821 - 6.816	6.031	4.252 – 7.439
		II	6.530	5.732 - 7.234	6.245	4.880 – 7.319
		III	6.955	5.711 - 7.936	5.788	5.220 – 6.290

DISCUSSION

The present work evaluated the cold sensitivity of two fruit fly species in four citrus species covering several varieties. Unlike Jessup *et al*. (1993) who considered eggs to be the most cold tolerant stage, the results of this work are consistent with the conclusions of previous reports (Back and Pemberton, 1916 and Hill *et al*., 1988) which consider third instar larvae to be the most cold tolerant stage for *C. capitata*.

Results for *A. fraterculus* showed a similar pattern for cold tolerance as the one observed for *C. capitata*. Our results provide evidence in favour of concluding that APHIS requirement of m ore time under cold treatment for Anastrepha is unnecessary.

CONCLUSIONS

Our results allow us to conclude that: a) third instar larvae should be used to develop cold quarantine treatments for citrus; b) variety shows no influence on the effect of cold treatment on immature fly stages and c) treatments developed for *C. capitata* are effective for *A. fraterculus*.

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