Research Article

Effect of hot-water immersion on eggs and larvae of *Anastrepha grandis* (Macquart, 1846) (Diptera: Tephritidae) "*in vitro*" and on squash (*Cucurbita moschata* Duchesne, 1786)

Efecto de la inmersión en agua caliente sobre los huevos y larvas de *Anastrepha grandis* (Macquart, 1846) (Diptera: Tephritidae) "*in vitro*" y sobre calabaza (*Cucurbita moschata* Duchesne, 1786)

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Abstract. There are risks involved in the production and exportation of fruit fly hosts due to the possible spread of tephritid pests during distribution. *Anastrepha grandis* attacks cucurbit fruits and is considered an A1 quarantine pest in many countries. The objective of this study was to evaluate the effect of hot water treatment on the eggs and larvae of *A. grandis in vitro*, and on 'Atlas' squash (*Cucurbita moschata*). The eggs and third-instar larvae of *A. grandis* were exposed to hot water at temperatures of 42.0, 44.0, 46.0, 46.5, 47.0, 47.5, 48.0, 49.0 and 50.0 (± 0.5) °C for durations of 0 (control), 10, 20, 30 and 60 minutes. Water temperatures of at least 44 °C affected the *in vitro* larval eclosion of *A. grandis* during all exposure times. No adults were obtained when *in vitro A. grandis* larvae were treated at 49 °C and 50 °C at all exposure times and, 48 °C for 30 and 60 minutes. No adults were obtained when squashes infested with *A. grandis* eggs or larvae were treated at temperatures of 49 °C and 50 °C during any exposure time, as well as subjected to 48 °C for 20 minutes. *Anastrepha grandis* larvae were slightly more susceptible to hydrothermal treatment than eggs in squashes. Hot water treatment applies at a temperature of 48 °C for 20 minutes is an effective phytosanitary treatment for squashes cv. Atlas infested with eggs and larvae of *A. grandis*.

Key words: Cucurbitaceae; disinfestation; fruit fly; Insecta; postharvest treatment.

Resumen. Existen riesgos relacionados con la producción y exportación de frutos hospedantes de moscas de la fruta debido a la posible propagación de plagas tefrítidas durante la distribución. *Anastrepha grandis* ataca los frutos de las cucurbitáceas y es considerada una plaga cuarentenaria A1 en muchos países. El objetivo de este estudio fue evaluar el efecto del tratamiento con agua caliente sobre los huevos y larvas de *A. grandis in vitro*, y sobre la calabaza 'Atlas' (*Cucurbita moschata*). Los huevos y las larvas del tercer estadio de *A. grandis* se sumergieron en agua caliente a temperaturas de 42,0, 44,0, 46,0, 46,5, 47,0, 47,5, 48,0, 49,0 y 50,0 (± 0,5) °C durante periodos de 0 (control), a los 10, 20, 30 y 60 minutos. Las temperaturas a partir de 44 °C afectaron la eclosión larvaria *in vitro* de *A. grandis* durante todos los tiempos de exposición. No se obtuvieron adultos cuando se trataron *in vitro* larvas de *A. grandis* a 49 °C y 50 °C en todos los tiempos de exposición, ni a 48 °C durante 30 y 60 minutos. No se obtuvieron adultos cuando se trataron calabazas infestadas con huevos o larvas de *A. grandis* a temperaturas de 49 °C y 50 °C durante cualquier tiempo de exposición, ni a 48 °C durante 20

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minutos. Las larvas de *A. grandis* fueron levemente más susceptibles al tratamiento hidrotérmico que los huevos en las calabazas. El tratamiento de inmersión en agua caliente aplicado a una temperatura de 48 °C durante 20 minutos es un tratamiento fitosanitario eficaz para calabazas infestadas con huevos y larvas de *A. grandis*.

Palabras clave: Cucurbitaceae; desinfestación; Insecta; mosca de la fruta; tratamiento poscosecha.

Introduction

Brazilian fruit growing is considered one of the largest in the world in regard to overall production of fresh fruit and size of cultivated areas. Despite its foreign market production being relatively small, Brazil exported 980 million of tons of fruits and other horticultural crops in 2019 (Abrafrutas 2020).

One of the greatest obstacles to the commercialization of fresh fruit in international trade is the incidence of fruit flies (Diptera: Tephritidae) in vegetable growing areas (Malavasi 2000). Fruit flies are the main pest of the global fruit crop due to the direct damage they cause, and to their ability to adapt to regions they are introduced to (Selivon 2000).

Anastrepha grandis (Macquart, 1846) is known as the 'South American cucurbit fruit fly (SACF)' and infests cucurbits exclusively, especially pumpkin and squash (*Cucurbita pepo* L., *Cucurbita maxima* Duchesne, and *Cucurbita moschata* Duchesne) (Raga & Baldo 2016; Bolzan *et al.* 2016; Silva *et al.* 2019). SACF is considered a quarantine species (A1) by the United States, Argentina, Chile, Uruguay and the 24 member countries of the Asia and Pacific Plant and Protection Commission. *Anastrepha grandis* has received considerable attention as a result of restrictions on the exportation of melons (*Cucumis melo* L.) in several South American countries (Harper 1987; Silva & Malavasi 1993). This pest has also been recorded in Panama (NAPPO 2009).

Commodities present a significant risk of carrying pests if they have not been managed during pre-harvest production and are therefore subjected to post-harvest phytosanitary treatment (Heather & Hallman 2008; Baldo *et al.* 2021) to ensure the health of vegetables originating from growing areas with low prevalence status (Thomas & Shelly 2000). Originally used for fungal control, there are three methods used to heat commodities for the disinfestation of insects: hot water, vapor heat, and hot air (Lurie 1998). Hot water immersion is commercially viable and adjusted specifically according to each commodity and pest. This technique is the principal phytosanitary treatment for mangoes around the world (Heather & Hallman 2008). The efficacy of the treatment is based on the mortality of immatures to the temperature and time duration of the water immersion of the infested fruits (Sharp and Chew 1987).

The application of high temperatures to commodities provides a quarantine method for disinfesting imported perishables while maintaining product quality (Thomas & Shelly 2000). The objective of this work was to evaluate the effect of hydrothermal treatment on infested squashes with eggs and larvae to guarantee non-emergence of *A. grandis* adults (Hernández *et al*. 2012).

Material and Methods

The study was conducted at the Economic Entomology Laboratory (EEL/BI) located at the Experimental Center of the Biological Institute in Campinas, São Paulo, Brazil.

Fruit origin

In all tests we used Atlas (American butternut) squash (*C. moschata*). The fruits were harvested in the western region of São Paulo state, where growers export to Argentina under pest risk mitigation systems for *A. grandis* (Silva *et al.* 2019). The fruits were immediately placed in plastic boxes for transportation to the laboratory. Before treatment, they were weighed using an analytical balance (model Radwag WTB 2000).

Insect colony

For all tests we used the insects from the *A. grandis* colony established at EEL/BI in 2011 and was maintained at a temperature of 25 ± 2 °C with a relative humidity of 70 ± 20 % and 14:10 light:dark. The duration of the egg-to-adult period was estimated to be 39.9 days at 25 °C (Bolzan *et al*. 2017). Larval development was carried out in squash fruit (*C. pepo, C. maxima, C. moschata*) or cabotiá (*C. moschata* x *C. maxima*).

After three days for egg-laying in cages, the infested fruits were kept in plastic tubs with dimensions of 41 cm x 34 cm x 14 cm, containing a bottom layer of 4 cm vermiculite covered with cotton cloth and secured with an elastic band. Approximately 20 to 30 days after the larval development period, the pupae retained on the vermiculite were sieved and transferred to emergency cages with dimensions of 100 cm (height) x 40 cm (width) x 40 cm (depth). The flies were supplied with water and a diet composed of the following ingredients (Raga *et al*. 2018): crystal sugar (400 g), beer yeast (200 g), wheat germ (100 g), yeast extract (100 g) and Sustagen® (16 g).

Hydrothermal treatment

Initially, the pattern of hot water temperature variation as a function of time was evaluated. A 36-litre capacity Nova Ethics Dubnoff water bath (model 304 TPA) was used with thermostat-controlled heating and constant agitation at approximately 10 rpm. To monitor the internal temperature of the fruits, Pt100 thermocouples were connected to a data logger and personal computer to record the temperatures continuously (Novus Field Logger 512k - RS485 interface, Ethernet and USB, 8 channels).

The eggs and third-instar larva of *A. grandis* were exposed to hot water at temperatures of 42.0, 44.0, 46.0, 46.5, 47.0, 47.5, 48.0, 49.0 and 50.0 (± 0.5) °C for durations of 0 (control), 10, 20, 30, and 60 minutes. The temperature of 49.0 °C was tested only during tests in fruits. An additional temperature near the maximum mortality was necessary to provides more adjusted mortality curves. Temperature-time combinations used during the tests were based in our previous tests.

Hydrothermal testing with *A. grandis* eggs *in vitro*

Anastrepha grandis eggs reaches between 2.0-2.2. mm (Raga & Baldo 2016). Egg collection was performed using a tool for collecting eggs of *Anastrepha fraterculus* (Wiedemann), which also obtained positive results for *A. grandis.* The tool consists of red rigid PVC tubing and exhibits dimensions of 40 mm (height) x 15 cm (diameter), containing approximately 40 holes in the side measuring 8 mm in diameter with a capped base and top. The side of the tube was covered with a layer of Parafilm® and its interior was filled with distilled water. The female perforates the parafilm in the area corresponding to the hole and lays the eggs (Baldo *et al.* 2021).

The tool was placed on the cage floor and was exposed to *A. grandis* females 15 to 20 days old. The collector was kept in the cage for approximately 12 hours, on which the

freshly laid eggs were retained to prevent dehydration. The eggs were kept in a glass beaker containing distilled water for a maximum of 2 hours. The collected eggs were then transferred to Petri dishes measuring 15 cm in diameter and were counted under a Nikon Model SMZ 745T stereoscopic microscope (50X magnification).

We evaluated the mortalities of 24 to 48 hour-old *A. grandis* eggs. Each combined treatment (temperature vs. exposure time) consisted of 20 replications in which ten eggs were used during each replication. Using a mercury column thermometer, the time previously set for each treatment was started once the water stabilized at the desired temperature. After the hydrothermal treatments, the eggs were transferred to Petri dishes (2.5 cm in diameter) and stored in a BOD chamber at 25 °C.

Seven days after treatment, the egg mortality of *A. grandis* was evaluated by counting the number of intact (unhatched) eggs under a Nikon stereoscope model SMZ 745T (50X magnification). The number of dead eggs of each treatment was compared with untreated control.

Hydrothermal tests with *A. grandis* larvae *in vitro*

Three weeks after oviposition, squash was placed on a sieve measuring 50 cm in diameter, with a mesh size of 3 mm to 4 mm to facilitate separation of the larvae from the pulp. The sieve was installed over a 50 cm x 40 cm x 9 cm plastic container to retain the *A. grandis* larvae. When we open the squash, the larvae immediately leave the pulp and fall on a vermiculite layer inside the container.

We evaluated the mortality of third-instar larvae of *A. grandis.* The combined treatment (temperature vs. exposure time) had 20 replications of ten larvae each. The methodology for larvae testing with bath equipment was similar to that used for the eggs i*n vitro.* The evaluation of efficacy occurred between 25 and 35 days after exposure by counting the number of emerged adults.

Hydrothermal test with *A. grandis* eggs in squash fruit

Fruits were infested for 48 hours in lab cages (100 cm height x 40 cm width x 40 cm depth), which contained 200 sexually mature *A. grandis* couples (15 to 20 days old). We used three fruits per treatment (exposure time vs. temperature). After infestation, the fruits were treated in a water bath using the same *in vitro* test treatment conditions that resulted in total mortality of *A. grandis* eggs.

Based on the egg period of our colony, the fruits were treated up to 5 days after the beginning of infestation to guarantee there would be no occur premature eclosion during immersion. To monitor the internal temperature of the fruits, a digital temperature Field Logger with four channels (thermocouples) was used in two regions: below the shell (± 0.5 cm depth) and at the centre of the fruit. Two other thermocouples were fixed in two distinct regions within the basket. In addition, a mercury column and digital thermometer were also used to monitor the temperature of the water bath.

We consider each fruit as one replication. After infestation, the fruits were kept individually in a round plastic container with a size of 21 cm (diameter) x 10 cm (height) covered with cotton cloth and fastened with elastic. The containers held approximately 400 ml of vermiculite at the bottom. The infested fruits were kept at 25 ± 2 °C with a relative humidity of 70 ± 10 %. The emerged adults were counted between 45 and 60 days after immersion. We used the same data for the untreated control for all exposure times.

Hydrothermal test with *A. grandis* larvae in squash fruit

The fruits were subjected to infestation for 48 hours in lab cages (same conditions as those above). The cage contained 200 sexually mature *A. grandis* couples (15 to 20 days old). We used three fruits per treatment (exposure time vs. temperature). The fruits were stored for a period of 10 to 15 days to ensure the larval eclosion. Each fruit was considered as one replication.

The methodology adopted for monitoring temperature and conditioning the fruits was the same as described for *A. grandis* eggs in squash. The number of adults was evaluated 25 to 35 days after hydrothermal treatment. We used the same data for the untreated control for all exposure times.

Statistical analysis

The data was analysed with factorial experimental design (temperature vs. exposure time) using the statistical program Assistat 7.7 (Silva & Azevedo 2016). The means were compared using Tukey's test ($p \leq 0.05$).

Probit-9 security levels are normally applied to commodities growing in areas with a lack of information regarding pest infestation (Hansen & Johnson 2007). Mortality data from tests *in vitro* and on fruit was subjected to Probit analysis using the statistical analysis software Statplus 2009 (AnalystSoft) to estimate a mortality percent between 90% and 95%, and Polo-Plus (version 0.03) to estimate 99.9968% mortality (Leora Software 1987).

Results and Discussion

Hydrothermal treatment with *A. grandis* eggs *in vitro*

We used 8,800 *A. grandis* eggs during the *in vitro* tests. A total of 5,201 dead eggs was estimated as a result of hydrothermal treatments (up to 60 minutes). The mean mortality of *A. grandis* eggs observed in the untreated controls was lower than those obtained in the respective treated plots, reaching 1.60%, 1.40%, 1.65%, and 2.05% during exposure times of 10, 20, 30 and 60 minutes, respectively (Tab. 1). Water temperatures starting from 44 °C affected the larval eclosion of *A. grandis* during all times of exposure (Tukey's test). At 50 °C we obtained 100% egg mortality.

Our results indicated that *A. grandis* eggs showed a higher tolerance than medfly eggs, which is likely due constant exposure to warm soil conditions during cucurbit cultivation. Mortality caused by the hydrothermal treatment of mangoes with *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) consists of raising the water temperature to 46.1 °C for 75 minutes (Assis 2001). Starting from 42.0 °C, the increase of exposure times in hot water led to an increase in the percentage of dead *C. capitata* (medfly) eggs, and 100% egg mortality was reached at 46.0, 48.0 and 50.0 °C (Vieira *et al*. 2010). Brown *et al*. (2020) demonstrated that the thermal history of *Bactrocera tryoni* (Froggatt) eggs prior to quarantine treatment could affect the efficacy of temperature quarantine treatments

No differences in egg mortality were detected between 30 and 60 minutes at all temperatures, while from 47.5 °C there were no differences observed across all temperatures (Tab. 1). It is therefore likely that temperatures above 47.5 °C caused irreversible damage to the embryonic development of *A. grandis*.

Within *in vitro* conditions, *A. grandis* eggs are susceptible to hot water treatment (Fig. 1). *A. grandis* eggs treated at 47.5 °C exhibited 92.0% mortality. In general, there was a decrease in the estimated temperature for obtaining different levels of mortality [Lethal dose (LD) 50 - LD 99.9968] according to the increase of exposure time of *A. grandis* eggs to hot water

treatment (Tab. 2). Based on our data, *A. grandis* eggs are much more heat-resistant than medfly eggs (Gazit *et al.* 2004).

Regardless of temperature, 10 minutes of exposure showed the highest Probit-9 value (52.29 °C) and a minor value occurred at 30 minutes (Tab. 2). However, the degree of temperature is the primary factor causing lethal or sublethal effects on fruit fly immatures.

The embryonic period may be a determining factor of greater or lesser heat tolerance. Eggs of Tephritidae at the beginning of the embryonic period are more susceptible than at near-larval hatching (Corcoran 1993; Wedell *et al.* 1997).

The curves indicate a shift to higher mortality as the temperature increases (Sharp & Chew 1987; Corcoran 1993); however, the nonlinear (rather than logarithmic) characteristics (Jang & Chan 1993) may be more appropriate for describing thermal mortality (Fig. 1).

Table 1. Average number dead eggs of *A. grandis* (n = 10) after hydrothermal treatment *in vitro*. Control plots were kept at 25 °C. / Número promedio de huevos muertos de *A. grandis* (n = 10) después del tratamiento hidrotermal *in vitro*. Las parcelas de control se mantuvieron a 25 °C.

Temperature (°C)	Time (min)			
	10	20	30	60
Control	1.60 gA	1.40 hA	1.65 gA	2.05 fA
42	2.00 fgBC	1.90 ghC	2.60 fAB	2.85 fA
44	2.80 fB	2.60 gB	3.55 eA	3.75 eA
45	4.85 eA	3.75 fB	5.30 dA	5.25 dA
46	6.75 dA	5.75 eB	7.30 cA	7.25 cA
46,5	7.80 cB	7.30 dB	8.70 bA	8.60 bA
47	8.45 bcB	8.40 cB	9.40 abA	9.30 abA
47,5	9.20 abA	9.10 bcA	9.70 aA	9.75 aA
48	9.90 aA	9.90 abA	9.85 aA	9.95 aA
50	10.00 aA	10.00 aA	10.00 aA	10.00 aA

dms for columns = 0.8384; dms for lines = 0.6808. The means followed by the same uppercase letter in the row and the same lowercase letter in the column do not differ from each other by the Tukey test ($p < 0.05$).

Table 2. Estimated lethal temperature (LD50, LD90, LD95, LD99.9968) for non-emergence of adults from eggs of *A. grandis* subjected to hydrothermal treatment *in vitro* at different immersion times. / Temperatura letal estimada (TL50, TL90, TL95, TL99,9968) para la no emergencia de adultos de huevos de *A. grandis* sometidos al tratamiento hidrotermal *in vitro* a diferentes tiempos de inmersión.

Level Probit	Time of Exposure				
	p- level	10 min	20 min	30 min	60 min
	Estimated Temperature (˚C)				
LD50	0.942	44.956	45.386	44.370	44.271
LD90	0.177	49.074	49.559	47.940	48.011
LD95	0.620	50.309	50.810	49.003	49.128
LD 99.9968 (Probit 9)	0.531	52.294	52.260	51.357	51.372

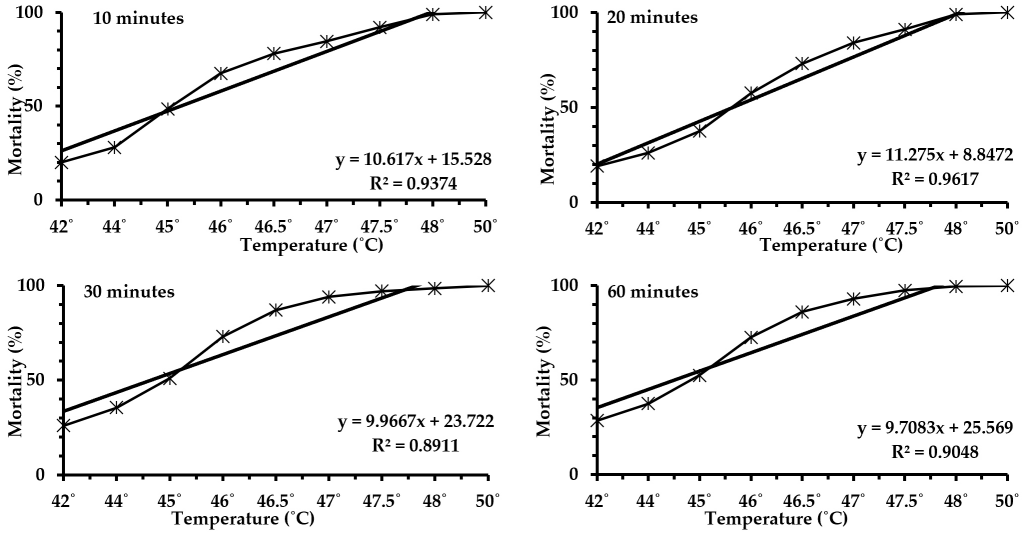

Figure 1. Mortality (%) of *A. grandis* eggs *in vitro* subjected to hydrothermal treatment at different temperatures and times of exposure. / Mortalidad (%) de huevos de *A. grandis in vitro* sometidos al tratamiento hidrotermal a diferentes temperaturas y tiempos de exposición.

Hydrothermal treatment with *A. grandis* larvae *in vitro*

The number of *A. grandis* adults from treated larvae *in vitro* was reduced significantly (Tukey's test) at all temperatures and respective hot water exposure times when compared to control plots, with the exception of 10 and 20 minutes below 42 °C (Tab. 3). No adults were obtained when *A. grandis* larvae were treated at 49 °C and 50 °C at all exposure times and, 48 °C for 30 and 60 minutes. Similarly, no adults were obtained when *A. grandis* larvae were subjected to temperatures of 47 °C and 47.5 °C for 60 minutes. There was no statistical difference between the average number of adults when *A. grandis* larvae were treated from 47.5 °C to 50 °C (Tab. 3).

The reduction in the number of adults from larvae treated *in vitro* for 10 and 20 minutes was more pronounced from 45 °C to 47 °C, while for 30 and 60 minutes this phenomenon occurred between 42 °C and 45 °C (Fig. 2). The temperatures of 49 °C and 50 °C for the duration of 10 minutes resulted in complete emergency inhibition. Hallman (1994) obtained 100% mortality of *Anastrepha suspensa* (Loew) larvae reared at 20 °C and subjected *in vitro* to hot water at 43 °C for 43 minutes.

The immersion time of 20 minutes from 47 °C showed a significant reduction in the number of adults of *A. grandis*. At 60 minutes, the results between 46 °C and 50 °C were statistically similar (Tab. 3; Fig. 2). Third-instar larvae of *A. grandis* immersed for 30 minutes between 42 °C and 45 °C were found to have a marked decrease in the number of adults; meanwhile, at 47.5 °C only one adult was found among the 200 treated larvae. Complete non-emergence of adults was reached at 48 °C for 30 and 60 minutes of exposure.

The number of adults emerged from third-instar larvae and treated at 42 °C for 10 and 20 minutes was similar to the untreated control (Tab. 3). A 60-minute immersion period required only 47 °C to supress adult emergence (Fig. 2). The curves obtained for 30 and 60-minute periods are similar, indicating that the treatment of *A. grandis* larvae for 30 minutes would be adequate for meeting the adult non-emergence quarantine criteria. Therefore, an exposure time of 30 minutes in hot water may be most favourable for ensuring the quality of commodities.

When treating 1 to 2-day-old *A. suspensa* larvae (contained in glass tubes) in hot water at a temperature of 46.1 °C, Sharp & Chew (1987) estimated that immersion times of

4.4; 5.6; 5.9 and 6.6 minutes would be required to achieve 50%, 90%, 95% and 99% larval mortality.

The average viability of the *A. grandis* pupae in untreated plots was 80.37% (Tab. 3). The lack of emergence of adults from third-instar treated larvae provided a reduction in probit levels (Tab. 4). In general, a decrease in estimated temperature (LD 50 - LD 99.9968) was obtained after an increasing of exposure time of *A. grandis* larvae in hot water (Tab. 4). Larvae treated at 50.17 °C for 10 minutes was estimated for Probit-9. Hernández *et al*. (2012) estimated that a duration of 5.27 minutes per 48 °C would result in Probit-9 for *C. capitata* third-instar larvae *in vitro*.

Table 3. Average number of adults of *A. grandis* emerged when larvae (n = 10) were submitted to hydrothermal treatment *in vitro.* Control plots were kept at 25 °C. / Número promedio de adultos de *A. grandis* que emergieron cuando las larvas (n = 10) se sometieron al tratamiento hidrotermal *in vitro*. Las parcelas de control se mantuvieron a 25 °C.

Temperature (°C)	Time (min)			
	10	20	30	60
Control	8.15 aA	7.95 aA	8.10 aA	7.95 aA
42	7.90 aA	7.55 abAB	7.00 bBC	6.90 bC
44	6.50 bA	6.80 bA	4.30 cB	4.70 cB
45	5.80 bA	5.65 cA	1.65 dB	2.15 dB
46	3.15 cA	2.10 dB	0.80 eC	0.70 eC
46.5	2.05 da	1.00 eB	0.25efC	0.25 eC
47	0.85 eA	0.60 efAB	0.10 efB	0.00 eB
47.5	0.25 efA	0.25 efA	0.05efA	0.00 eA
48	0.05 fA	0.05 fA	0.00 fA	0.00 eA
49	0.00 fA	0.00 fA	0.00 fA	0.00 eA
50	0.00 fA	0.00 fA	0.00 fA	0.00 eA

Dms for columns = 0.7903 dms for rows = 0.6305 CV% = 10.40 - Averages followed by the same uppercase letter in the row and same lowercase letter in the column do not differ from each other by the Tukey test ($p < 0.05$).

Table 4. Estimated lethal temperature (LD50, LD90, LD95, LD99.9968) for providing non-emergence of adults when *A. grandis* larvae were subjected to hydrothermal treatment *in vitro* at different immersion times. / Temperatura letal estimada (TL50, TL90, TL95, TL99,9968) para la no emergencia de adultos cuando las larvas de *A. grandis* se sometieron al tratamiento hidrotermal *in vitro* a diferentes tiempos de inmersión.

Level Probit	Time of Expeosure				
	p- level	10 min	20 min	30 mins	60 min
	Estimated Temperature (°C)				
LD50	0.008	44.74	44.41	43.22	43.32
LD90	0.792	48.03	47.58	45.83	45.85
LD95	0.258	49.01	48.57	46.60	46.59
LD99.9968 (Probit 9)	0.618	50.17	49.70	49.80	49.01

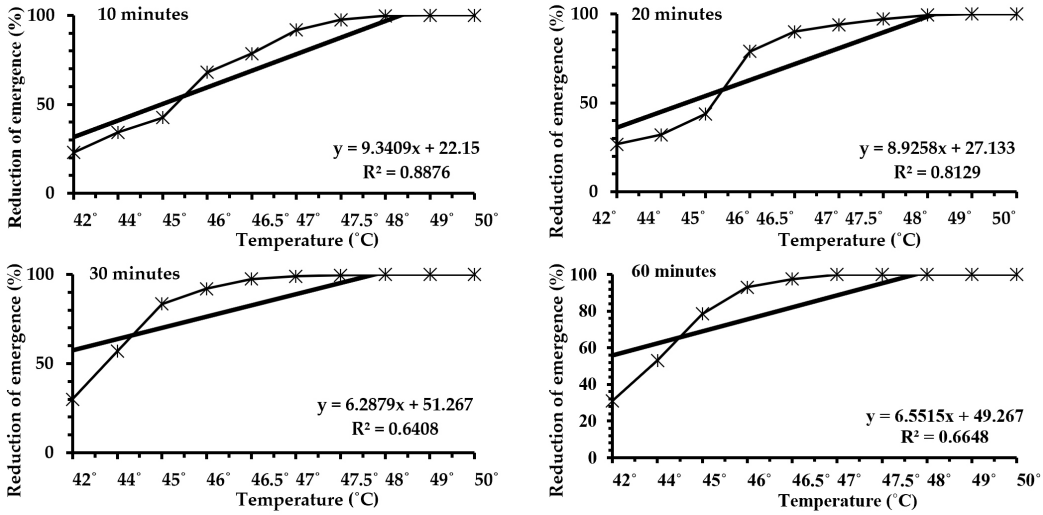

Figure 2. Percent reduction of adult emergence of *A. grandis* when larvae were subjected to hydrothermal treatment *in vitro* at different temperatures and exposure times. / Reducción porcentual de la emergencia de adultos de *A. grandis* cuando las larvas se sometieron al tratamiento hidrotermal *in vitro* a diferentes temperaturas y tiempos de exposición.

Hydrothermal treatment with *A. grandis* eggs in squash fruit

The mean weight of the squash was 399.62 g (SD = 61.34; n = 328 fruit). The number of adults obtained in the control plots were similar to those plots whose eggs were treated at 42 °C during all exposure times (Tab. 5). For all times of exposure at 46 °C and above, there was a significant reduction (Tukey's test) in the number of adults when compared to the untreated control. No adults were obtained when squashes infested with *A. grandis* eggs were treated at 49 °C and 50 °C, regardless of exposure time, as well as when subjected to 48 °C for 20, 30 and 60 minutes in a water bath (Tab. 5). The *A. grandis* eggs heat up quickly to the desired temperature due to the thin layer of the epicarp, as well as the lower firmness of the mesocarp in relation to the epicarp (Martínez-Valdivieso *et al*. 2015).

When fruits are subjected to hydrothermal treatment, embryonic development is not linear to exposure time; this is likely a result of an internal temperature gradient that is not initially uniform due to the irregular shape of the fruit (Tab. 5). Jang & Chan (1993), in kinetics studies on the thermal death of fruit flies, determined that insects did not follow the logarithmic function of death, with death occurring at a constant rate. These discussions indicate that mortality curves with nonlinear characteristics may be more appropriate for describing thermal mortality in insects.

A decrease in the number of adults was observed as temperature increased during the respective immersion times of the fruit infested by *A. grandis* eggs (Tab. 5). The reduction of *A. grandis* emergence was more pronounced from 45 °C to 47 °C during all exposure times (Fig. 3). This information may be decisive when choosing the most appropriate conditions for higher efficacy of disinfestation since hot treatment may affect the final quality of the commodity (Chitarra & Chitarra 2005).

No significant reduction of emergence was observed when squash was treated from 42 °C to 45 °C for 30 minutes; however, infested squash subjected to 45 °C for 60 minutes of exposure significantly reduced emergence (Tab. 5, Fig. 3). Hot water at 48 °C for 10 minutes was efficient for non-tephritid disinfestation, and for retaining the fruit quality of persimmon cv. 'Fuyu' (Lee *et al*. 2010).

A minimum temperature of 49.49 °C for 60 minutes was required to provide Probit-9 (99.9968% efficacy at 95% confidence level) to prevent the emergence of adults from squash cv. Atlas infested by *A. grandis* eggs (Tab. 6). In the case of fruit subjected to a hot water bath for 10 minutes, a minimum of 54.55 °C was required to achieve the same efficacy. The non-obligatory mortality criteria of the immature *in situ* phase provides a decrease in estimated temperature at Probit-9 level (Tab. 6). As result, lethal disturbances in treated insects can be observed in immature stages subsequent to those exposed to hot water bath, *e.g.*, the heat shock protein phenomenon is characterised by dramatic and rapid changes in both the transcription and translation of proteins in response to sublethal heat stress (Lurie & Jang 2007).

Both young and mature eggs of *C. capitata* exhibited 100% mortality after submersion mangoes cv. Ataulfo in water at a temperature of 46.1 °C for 25 minutes (Hernández *et al*. 2012). Albergaria *et al*. (2007) obtained mortalities of 60.0% and 83.2% of *C. capitata* eggs in Valencia oranges treated in hot water at both 44 °C and 46 °C for 30 minutes, respectively. For both temperatures, the authors estimated that times of 96.10 and 81.79 minutes, respectively, would be lethal (LT99) to medfly eggs. Nascimento *et al*. (1992) treated mangoes in hot water between 45.9 °C and 46.3 °C and estimated a lethal time (LT99.9968) of 39.7 minutes for *A. fraterculus* eggs, 65.7 minutes for *Anastrepha obliqua* (Macquart), and 59.4 minutes for *C. capitata* eggs.

Table 5. Average number of adults of *A. grandis* obtained per squash fruit cv. Atlas infested by *A. grandis* eggs was subjected to hydrothermal treatment. Control plots were kept at 25 °C. / Número promedio de adultos de *A. grandis* obtenidos por fruto de calabaza cv. Atlas infestado por huevos de *A. grandis* que se sometió al tratamiento hidrotermal. Las parcelas de control se mantuvieron a 25 °C.

Temperature (°C)	Time (min)			
	10	20	30	60
Control	62.62 aA	62.62 aA	62.62 aA	62.62 aA
42	46.37 tab	45.62 tab	51.62 aA	58.50 aA
44	36.75 bcB	42.25 bcAB	57.50 aA	50.62 aAB
45	32.62 bcdA	37.25 bcdA	43.12 aA	15.75 bB
46	18.50 cdeAB	23.62 cdeA	20.37 bAB	6.50 bB
46.5	15.25 ofAB	20.00 defA	5.25 bcAB	3.00 bB
47	5.37 eA	8.50 efA	2.12 bcA	0.37 bA
47.5	4.87 eA	5.87 efA	0.50 bcA	0.12 bA
48	1.37 eA	0.00 eA	0.00 eA	0.00 eA
49	0.00 eA	0.00 eA	0.00 eA	0.00 eA
50	0.00 eA	0.00 eA	0.00 eA	0.00 eA

DMS for columns = 20.1499 / DMS for rows = 16.0597. Means followed by the same uppercase letter in the row and the same lowercase letter in the column do not differ from each other by the Tukey test ($p < 0.05$).

Table 6. Lethal estimated temperature (LD50, LD90, LD95, LD99.9968) for providing non-emergence of adults when squash cv. Atlas infested by *A. grandis* eggs and larvae was subjected to hydrothermal treatment at different immersion times. / Temperatura letal estimada (TL50, TL90, TL95, TL99,9968) para la no emergencia de adultos cuando la calabaza cv. Atlas infestada por huevos y larvas de *A. grandis* se sometió al tratamiento hidrotermal a diferentes tiempos de inmersión.

Level Probit		Time of Exposure				
		p- level	10 min	20 min	30 min	60 min
		Estimated Temperature (˚C)				
Eggs	DL50	0.295	44.32	44.93	45.14	44.43
	DL90	0.363	47.37	49.01	47.55	46.25
	DL95	0.536	48.28	50.23	48.26	46.77
	DL99.9968 (Probit 9)	0.271	54.55	55.00	51.55	49.49
Larvae	DL50	0.942	45.51	44.23	42.69	43.26
	DL90	0.177	47.12	46.74	44.71	45.12
	DL95	0.620	47.71	47.47	45.30	45.67
	DL 99.9968 (Probit 9)	0.327	51.64	52.53	49.32	49.34

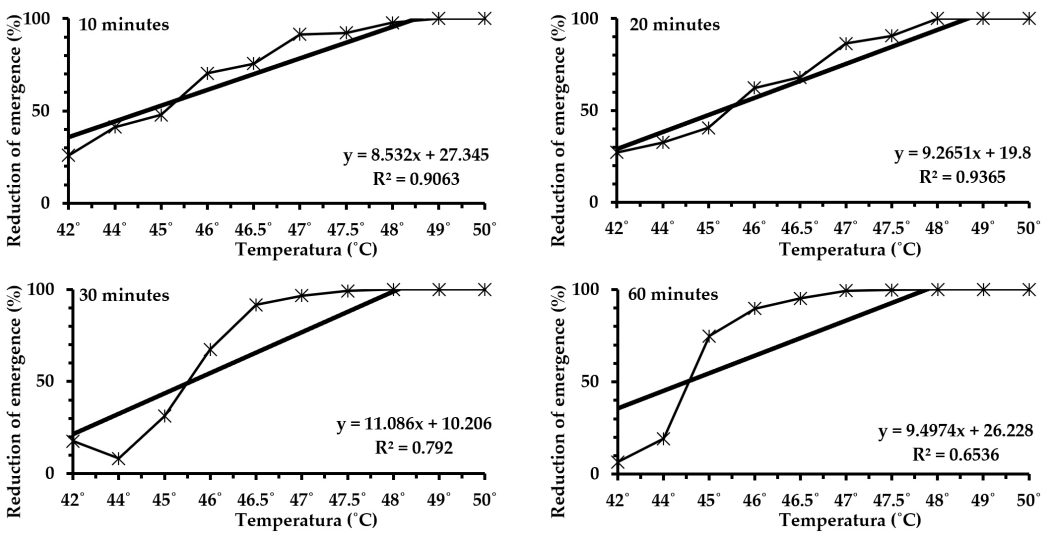

Figure 3. Percent reduction of adult emergence of *A. grandis* when Atlas squashes infested by eggs was subjected to hydrothermal treatment at different temperatures and times of exposure. / Reducción porcentual de la emergencia de adultos de *A. grandis* cuando las calabazas Atlas infestadas por huevos fueron se sometieron al tratamiento hidrotermal a diferentes temperaturas y tiempos de exposición.

Hydrothermal treatment with *Anastrepha grandis* larvae in squash fruit

The mean weight of squash was 334.05 g (SD = 63,22; n = 352 fruit). The number of adults obtained in the control plots was no significant to those obtained at 42 °C and 44 °C (Tukey's test), when squashes infested with third-instar larvae of *A. grandis* were immersed in hot water for 10 minutes (Tab. 7). The number of *A. grandis* adults in all temperatures differed from untreated control plots at 30 and 60 minutes. The thermal tolerance was drastically reduced between 44.0 °C and 47.0 °C (Fig. 4). No adults were obtained when squashes

infested with *A. grandis* larvae were treated at ≥49 °C in a bath at any exposure time, as well as when subjected to 48 °C for 20 minutes, and 47.5 °C for 30 and 60 minutes (Tab. 7).

For Denlinger & Yocum (1998), higher temperatures require shorter exposure times necessary for killing the insect. During hydrothermal treatment of *A. grandis* larvae-infested squashes at every exposure time, there was a decrease in the survival of *A. grandis* as a function of increase in temperature. Except at 42 °C and 44 °C, no difference in the number of *A. grandis* adults was obtained when the fruits were immersed for 10 and 20 minutes (Tab. 7). The number of *A. grandis* adults was similar among the four exposure times at respective temperatures ≥47.0 °C.

A. grandis larvae were slightly more susceptible to hydrothermal treatment than eggs in squashes (Tabs. 6 and 7). Eggs were also more tolerant than larvae subjected to hot water submersion in *A. suspensa* (Sharp & Chew 1987), *C. capitata* (Lurie *et al*. 2004) and *Bactrocera cucurbitae* (Coquillett) (Jang 1986). Corcoran *et al.* (1993) obtained 100% mortality of third-instar larvae of *Bactrocera cucumis* (French) in zucchini fruit subjected to a hot water bath at 45 °C for 5 minutes, while 22-h-old eggs under the same conditions exhibited only 32% mortality.

Gould & Sharp (1992) estimated a lethal time of 32.7 minutes (Probit-9) for third-instar *A. suspensa* larvae in guava immersed in hot water at 46.1 °C. Hallman (1996) estimated 28.1 minutes (LT95) as the time required for third-instar *A. suspensa* larvae in grapefruit juice treated in hot water at 43.0 °C, which was comparably lower than the immature when treated in water.

Our results of the hot water immersion for squash infested by *A. grandis* larva (Tab. 7) are similar with the test *in vitro* (Tab. 3). It is likely that the higher water content in squash (> 90%) is favourable to heat transfer in pulp. Internal heat resistance in fruit during water heating was a more significant factor in controlling the heat transfer rates (Wang *et al*. 2001). However, when we increase the temperature of the water occur a reduction of required time for non-emergence of *A. grandis* (Tab. 6; Fig. 4). The highest value of Probit-9 was estimated to be 20 minutes (52.53 °C) and the lowest value was estimated to be 30 minutes (49.32 °C). Overall, when we tested squash, the estimated Probit values for larvae were lower than those estimated for *A. grandis* eggs. It is likely that squashes cv. Atlas exposed to hot water for 30 minutes achieve necessary quarantine disinfestation, because the results on that time were found to be similar with at 60 minutes of exposure (Tab. 7).

The 'Oroblanco' hybrid citrus infested by first-instar medfly larvae and exposed to bath water for 60 minutes at 44.0 °C is enough to provide 100% mortality; however, a temperature of 43.0 °C resulted in 93.4% mortality (Lurie *et al*. 2004). The mortality of first-instar larvae of *C. capitata* was 100% after 30 minutes of bath submersion at 46.1 °C (Hernández *et al*. 2012). In our case, the documented temperatures avoid emergence at 47.0 °C and 47.5 °C, at 60 and 30 minutes, respectively.

Conclusion

There is an increase of mortality *in vitro* of *A. grandis* eggs and larvae as a result of the increase in temperature and exposure time in hot water. *A. grandis* eggs are more tolerant to hot water than larvae, probably due to egg size and shape that influence the thermal death kinetic. There was no emergence of *A. grandis* adults when squashes infested with eggs or larvae were treated with hot water immersion at 48 °C for 20 minutes. The lowest dose observed for the non-emergence of adults from squash infested by *A. grandis* eggs and larvae when subjected to hydrothermal treatment was 48 °C for 20 minutes and 47 °C for 60 minutes, respectively. No visual external or internal damages was detected on squashes treated at 46-50 °C for 30-60 minutes. Hot water treatment is an effective phytosanitary treatment of squashes infested with eggs and larvae of *A. grandis.*

Table 7. Average number of *A. grandis* adults obtained per fruit when squash cv. Atlas infested by *A. grandis* larvae was subjected to hydrothermal treatment. / Número promedio de adultos de *A. grandis* obtenidos por fruto cuando la calabaza cv. Atlas infestada por larvas de *A. grandis* se sometió al tratamiento hidrotermal.

Temperature (°C)	Time (min)			
	10	20	30	60
Control	58.75 aA	58.75 aA	58.75 aA	58.75 aA
42	54.12 aA	48.50 BC	40.25 bB	41.62 bB
44	54.37 aA	28.50 bB	8.87 cC	33.25 bB
45	26.75 bA	29.12 bA	6.25 cB	2.50 cB
46	18.37 bcA	17.50 bcA	1.62 cB	0.62 cB
46.5	13.75 bcdA	9.25 cdAB	0.25 cB	0.12 cB
47	7.37 cdA	1.37 dA	0.12 cA	0.00 dA
47.5	2.37 dA	0.37 dA	0.00 dA	0.00 dA
48	0.25 dA	0.00 dA	0.00 dA	0.00 dA
49	0.00 dA	0.00 dA	0.00 dA	0.00 dA
50	0.00 dA	0.00 dA	0.00 dA	0.00 dA

DMS for Columns = 14.8300; DMS for lines = 11.8197. Means followed by the same uppercase letter in the row and the same lowercase letter in the column do not differ from each other by the Tukey test ($p < 0.05$). Control plots were kept at 25 °C.

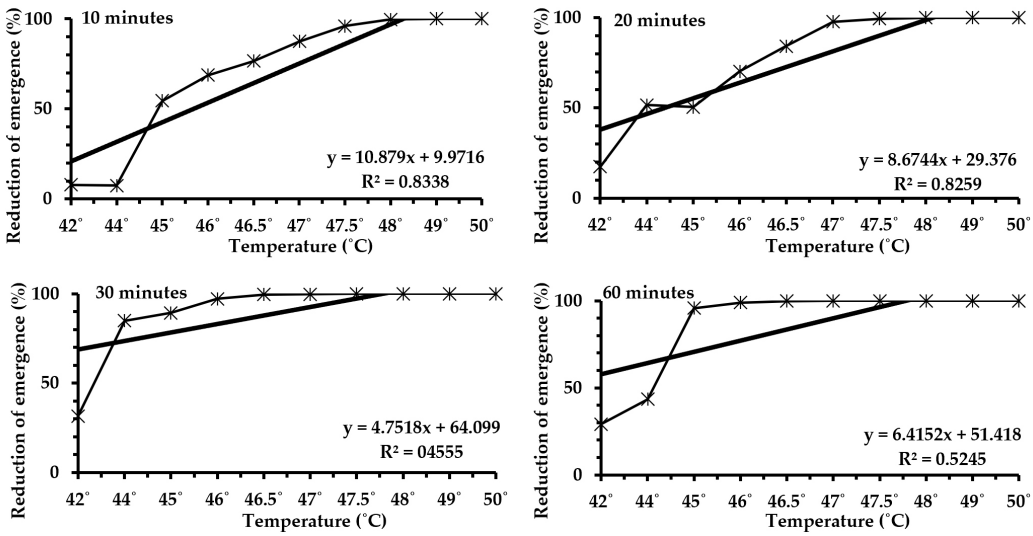

Figure 4. Percent reduction of adult emergence of *A. grandis* when Atlas squashes infested by larvae was subjected to hydrothermal treatment at different temperatures and times of exposure. / Reducción porcentual de la emergencia de adultos de *A. grandis* cuando las calabazas Atlas infestadas por larvas se sometieron al tratamiento hidrotermal a diferentes temperaturas y tiempos de exposición.

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