International market access for Australian tablegrapes through cold treatment of fruit flies with a review of methods, models and data for fresh fruit disinfestation

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Abstract

Background and Aims: International quarantine rules prohibit exports of fruit from areas with fruit flies. Cold storage was tested from 1989 to 2012 as a postharvest treatment against the Mediterranean fruit fly (Medfly) in nine cultivars of Australian grown tablegrapes. The paper shows how market access was obtained. It reviews the methods, models and science that underpin fruit fly disinfestation.

Methods and Results: Tablegrapes were infested with immature stages of Medfly for large scale tests seeking 99.99% mortality at the upper 95% confidence level. Infested berries were exposed to 1, 2 and 3°C for 12, 13, 14, 15, 16, 18 and 20 days. No survivors were found in fruit held at 1°C for 16 days, 2°C for 18 days and 3°C for 20 days.

Conclusion: The data presented satisfy international requirements for access to quarantine-restricted markets. Several decades of research are reviewed to show how science and phytosanitary regulation can be used to harmonise cold disinfestation treatments.

Significance of Study: This work has enabled Australia's tablegrape industry to gain access to several international markets. The science used and presented is expected to benefit disinfestation research.

Keywords*: binomial model, calibration, cold temperature × time, quarantine standards, refrigeration, verification trials*

Introduction

Tablegrapes cannot be exported from Australia because of fruit flies. Two species, the Queensland fruit fly (Qfly) *Bactrocera tryoni* (Froggatt) and the Mediterranean fruit fly (Medfly) *Ceratitis capitata* (Wiedemann), found on the mainland are prohibited in major overseas markets (White and Elson-Harris 1992, Dominiak and Daniels 2012). Grapes are harvested at a summer temperature >25°C and must be cooled to −1 to 2°C to preserve quality (Crisosto et al. 1994). Cold temperature is appropriate for disinfestation (De Lima 2015). The Australian Government funded disinfestation research for tablegrapes for many years (1973–1988). Laboratory infestation methods used for citrus (Hill et al. 1988) were unsuitable for tablegrapes. New techniques of artificial infestation for Medfly and Qfly (De Lima 1995, Jessup et al. 1998) enabled the necessary data to be obtained for tests at 1°C. In 2003, the tablegrape industry funded research at 1, 2 and 3°C. Red Globe, Crimson Seedless and Thompson Seedless were tested as representative of types grown in Australia (De Lima et al. 2011).

Australia and its major trading partners mandate protocols for quarantine treatments that ensure 99.99% mortality at the 95% confidence level (CL). To satisfy this standard, data must be supplied in a series of steps to prove that no survivors are found when >30 000 insects are treated. The first step requires data on fruit fly life history (LH) showing the course of development of eggs and larvae in each host cultivar. This provides information on the susceptibility of fruit cultivars to insects, the time required for development of each stage and the productiveness of hosts. The second step must supply data defining the response of each infesting stage to the treatment. At least five doses below 100% mortality are required. Applying five to seven doses to obtain responses between 15 and 99% mortality (Finney 1971) will provide three to five degrees of freedom to test for goodness of fit. The third step assesses mortality data by weighted probit regression analysis to determine the most tolerant stage (MTS) at the LD50 and higher mortality responses such as LD95 and LD99. Data around the LD50 point will have the most weight. The fourth step selects the treatment required for large scale tests. The fifth step is to conduct large scale tests on the MTS to demonstrate 99.99% mortality at the 95% CL. The sixth step identifies the most susceptible cultivar. Where two or more species are tested, the seventh step selects the most tolerant species. All test data must be supplied to the importing country for review and approval before trade can commence. The assessment of efficacy is based on testing sufficient insects in fruits. At least 200 insects per stage per replicate are required in the MTS tests and >10 000 MTS insects per replicate in large scale tests. Every dose must be tested in a minimum of three replicates. The end-point efficacy of each treatment is based on the number of pupae found in treated and untreated fruits. Data obtained to this stage are equivalent to Japan's Ministry of Agriculture Forestry and Fisheries (MAFF) test protocol. The majority of Australia's trading partners accept the body of work supplied to this level. After MAFF reviews and accepts the data, however, it specifies new tests for verification and validation. Data on the effectiveness of treatments at 1, 2 and 3°C against Qfly and Medfly were sent to Japan in 2008 (De Lima 2008). In 2011, MAFF accepted the work and selected Medfly as more tolerant than Qfly. As required by Japan's quarantine law,

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MAFF proposed a series of verification trials. These trials included new methods to validate the large scale data. Insight into the mortality effect of cold storage can be gained by examining larvae and comparison can be made with the mortality inferred from counting pupae in control fruit. Such data are provided where relevant.

Some countries including the USA (United States Department of Agriculture 2015) specify a 'probit 9' standard (Baker 1939) as the basis for market access. This requires approximately 100 000 insects to be treated with no survivors, that is, 99.99683% mortality. It is an arduous process. Despite efforts over several decades in many countries, research methodology and analysis remains ambiguous. International bodies such as the Food and Agriculture Organisation of the United Nations (FAO) seek to elucidate and harmonise methods and reduce quarantine barriers to trade (Food and Agriculture Organisation of the United Nations 2013). The work reported here contains substantial data to give confidence in assessing treatments. It presents methods for experimental design and analysis and is relevant for broader discussion. The treatments reported here have been used for bilateral certification of Australian tablegrape exports to Japan and to many other countries including the USA (Australian Government 2015, 2016).

Materials and methods

Tablegrapes

Nine cultivars were tested. Cultivar characteristics in brief are as follows: (1) Olympia, large berries, black-seeded Japanese cultivar; (2) Calmeria, pale-green berries, large, cylindrical and seeded; (3) Red Emperor, red berries, medium sized, egg shaped and seeded; (4) Flame, bright red berries, large to medium in size, round and seedless; (5) Queen, red berries, large, oval and seeded; (6) Ruby, red berries, small to medium, oval and seedless; (7) Red Globe, pinkish-red berries, very large, round and seeded; (8) Thompson Seedless, yellow-green berries, small to medium, oval and seedless; and (9) Crimson Seedless, bright red berries, large, cylindrical-oval and seedless. Eight cultivars (1 to 8) were tested from 1989 to 1994 (De Lima 1995). Three cultivars (7 to 9) were tested from 2004–2007. In 2012, only one cultivar (Red Globe) was tested. Tablegrapes for infestation were grade 1 export quality (http://agriculture.vic.gov.au/agriculture/horticulture/wine-and-grapes/production-guidelines-for-australian-table-grape-varieties. Accessed 10-06-2016).

Fruits were sourced from pesticide-free organic vineyards in the Swan Valley (31°76′S, 116°03′E) 50 km north of Perth in Western Australia. Lesser quality produce was used as filler fruit. All fruits were received packaged in perforated poly-liner film in 10 kg cartons but without sulfur dioxide pads and held at 1°C in storage.

Insects

Medflies used in tests were obtained from a standard colony of ~1.2 million adult flies. The colony is kept at 26 ± 1°C, 60–65% RH and maintained by artificially breeding a new stock of adults from eggs every week. Fruits were infested with adult flies between 8 and 15 days old after emergence from puparia. These flies were fertile and produced eggs of high (85–90%) viability. The eggs were placed on media containing a paper pulp matrix with added nutrients including protein and sugar (De Lima et al. 2007, 2011). Quality control tests were recorded every week. The colony is renewed with wild flies every year from November to March.

Life history

Berries were artificially infested with <6 h-old eggs to minimise the overlap between immature stages. A 0.5–0.6 mL cavity was created by removing berry pulp and seeds (in seeded cultivars). Thereafter, ~0.5 mL paper rearing medium containing 250–300 eggs was inserted by forceps (De Lima et al. 2011). The cavity was plugged with more paper medium. Infested berries were arranged 25 each in shallow polystyrene trays (22 × 12 × 1.5 cm) placed in 13 L (38 × 29 × 12 cm) ventilated plastic boxes and incubated at 26 ± 1°C and 60–65% RH. A test batch of 500 berries was infested to record development of life stages. Thereafter, at 24 h intervals, a sample of 10–30 berries was examined. The number of live and dead immature stages per berry was recorded. Each berry was dissected over a set of four sieves (Endecotts, London, England) with aperture sizes 125, 250, 500 and 2000 μm and washed under a gentle stream of tap water to separate eggs and larvae from the berry pulp and media. This gives a broad but not definitive classification based on size. Most of the berry and media remain in the (2000 μm) first sieve. Late second and third instars are mainly collected in the 500 μm sieve. Most eggs, first and early second instars, are found in the 250 μm sieve, and the few that pass through are retained in the 125 μm sieve. Smaller live larvae are tenacious, cling to the sieve mesh wire and hide in crevices. Each sieve is examined under the binocular microscope (×40 magnification), with the gentle warmth of a 25 watt incandescent light bulb to stimulate movement. Probing crevices with a small sable paint brush and systematically checking the underside of the mesh enables more accurate counting. The mouthparts and posterior spiracles of every larva are examined to determine instar stage (White and Elson-Harris 1992). The number of pupae per berry was determined by setting aside and incubating 100 un-dissected berries for pupation. Adult eclosion was assessed in 100 pupae.

Infestation and setup for large scale tests and verification trials

Large numbers of fruit were treated in every replicate of large-scale tests and verification trials. To do this, <6 h-old eggs were collected continuously from the 26 ± 1°C, 60–65% RH colony room. Staff worked in an adjacent laboratory at 22°C. The time between the first and last berry infested for a test batch was ~6 h. Trays of infested berries (25 per tray, ~300 g) for cold treatment and control were selected at random immediately after infestation and placed in labelled paired rows in the incubation room to develop to the correct stage for treatment. Berries were infested over 2 days because of the heavy workload. Thus, eggs in berries were incubated for 7 or 8 days to obtain >50% second instar in large scale tests. Every replicate test contained half the berries that were 7 days old and half 8 days old. On the day of loading into the cold room, infested trays were placed in cartons containing ~9.7 kg of cold un-infested berries. The total mass made ~10 kg, which is the normal mass of a full carton. A few cartons (ten in large scale tests and 12 in verification trials) were supplied with an extra ~300 g warm (26°C) un-infested berries for temperature sensor probes (thermistors). These were placed at pre-determined positions where cooldown is slowest in the cold room. The remaining cartons with infested berries were placed at random in eight stacks. In all tests, control berries were incubated for emergence of pupae. After every treatment except for the verification trial in cardboard cartons, berries were incubated for 4–6 weeks for survivors to emerge as pupae. In 1989–1994 tests, 100 treated berries per replicate were examined for larvae after treatment. In every test, extra berries were infested to determine the course of development of life

stages and to replace those that became mouldy. The LH methods were used to determine the number of live and dead insects per instar per berry on the day of loading every trial replicate into the cold room and to correct the number treated. The data from the MTS trials conducted in 1989–1994 (De Lima 1995) and in 2002–2007 (De Lima et al. 2011) showed that the second instar was more tolerant than eggs, first and third instars at the LD50 level at 1, 2 and 3°C. As required in MAFF's test protocol, the second instar became the focus for large scale and verification trials. In large scale tests, ventilated double-walled cardboard cartons were used throughout. In verification trials, cardboard cartons were used first and ventilated polystyrene cartons were used later. In both types of packaging, the berries were wrapped in thin micro-perforated poly-liner film before covering with the lid as in commercial practice.

Large scale tests

There were three replicates per treatment per cultivar for cultivars 1 to 8 shown earlier. In the 1989–1994 large scale tests (De Lima 1995), 1600 berries were infested per replicate, of which 400 were Control and 1200 were treated (1:3 ratio). This made 64 trays of 25 infested berries per replicate, giving 48 trays for treatment and 24 for Control. In 32 trays, larvae were 7 days old and in the remaining 32 they were 8 days old. Infested berries were placed in 48 cardboard cartons and distributed six per stack in eight stacks per cold room replicate containing 448 cartons. In this way, each cold room per cultivar per replicate was set up. A total of 38 400 berries (1536 trays of 25 infested berries) were tested at 1°C for 14 days. Red Globe and Thompson Seedless (one replicate only, 400 Control : 1200 treated) were also tested for 12, 13 and 15 days at 1°C. To determine the yield of adults in Controls, 100 pupae per replicate per cultivar were set aside for incubation at 26 ± 1°C and 60–65% RH. Similar methods were used in the 2004–2007 large scale tests, but each replicate had 3000 infested berries of which 2000 were treated and 1000 were Control. There were three replicates per cultivar per treatment temperature making a total of 9000 berries infested per cultivar, of which 6000 were treated and 3000 were Control held for pupation. The Control : treated ratio was 1:2. This made 120 trays of 25 infested berries per tray. In 60 trays, larvae were 7 days old, and in the remaining 60, they were 8 days old. Forty trays were Control and 80 were treated. In the three cultivars tested (Red Globe, Thompson Seedless and Crimson Seedless), 27 000 berries were infested of which 18 000 were treated and 9000 were Controls at each temperature. Three temperature values, 1, 2 and 3°C, were tested giving 81 000 infested berries in total.

Verification trials

Verification trials (1, 2 and 3°C for 16, 18 and 20 days, respectively) were conducted from March to September 2012. The MAFF selected Medfly as the most tolerant species and second instar as the MTS. Red Globe was selected as the most susceptible cultivar. Red Globe for infestation (~500 kg) was harvested from a single organic vineyard on 20 March 2012. The MAFF required only one replicate test in cardboard cartons of >10 000 live second instar Medfly for each temperature and exposure period. From 26 April 2012, 11 000 Red Globe berries (440 trays) were artificially infested with Medfly eggs and incubated for 7 days to obtain second instar. For tests in polystyrene cartons, a further 11 000 berries were infested from 8 August 2012 and incubated for 8 days. For each trial, 3200 berries were infested with 1600 placed in the cold room and 1600 set aside as Control to obtain pupae. Thus 9600 berries were infested for three cardboard carton trials at 1, 2 and 3°C and 9600 for three polystyrene carton tests taking the total to 19 200 infested. The average mass of a Red Globe berry was 12.2 g (range 11.3–14.7 g) giving ~820 berries/10 kg carton. Each cold room replicate had 564 cardboard cartons or 560 polystyrene cartons and each test had ~462 480 berries of which 1600 were infested. The Control : treated ratio was 1:1. This made 64 trays of 25 infested berries/tray/cold room. Control berries were 64 trays incubated for emergence of pupae. The berries for cold treatments were distributed in 64 cartons, one per layer in eight layers/stack in the eight stacks in each cold room. Control berries for all tests were incubated at 26 ± 1°C and 60–65% RH for pupae that were removed every day from 14 to 21 days after infestation and thereafter checked every 3–4 days for a further 3 weeks. Treated berries for cardboard carton tests were dissected to recover larvae. For polystyrene carton tests conducted 12 weeks later from 8 August 2012, the treated berries were retrieved and incubated for 3 weeks to obtain survivors as pupae.

The MAFF requirement for tests in cardboard cartons was to cut up every treated berry, identify and count all larval stages and record the numbers alive and dead. Thus, the 1600 cold-treated berries/test were retrieved and incubated for 48 h at 26°C. Dissection of berries was required to be completed within 72 h after warm up. A total of 140 specially trained technicians (mainly university graduate students) were employed. Training was given for up to 10 days/person over a period of 3 months. Technicians were considered suitable if they achieved (i) >95% recovery from spiked berries containing known instars; and (ii) >90% recovery and instar identification from berries with unknown numbers. There were 110 sets of four sieves (125 μm to 2.0 mm) and 110 binocular microscopes (magnification ×40) accommodated in five adjacent laboratories. This allowed rest periods for 30 staff at any time. The teams dissected berries from each treatment in sequence beginning with 1.1°C on 25 May 2012.

Cold rooms

Six cold rooms each 34.90 m^3 were used for trials. Two additional cold rooms and two refrigerated containers were available to hold berries and maintain quality at 1°C. Refrigeration for each of the six treatment cold rooms was supplied by a Patton (Model CCH 250) air-cooled condensing unit (R22 refrigerant) and a Patton BL 38 induced draught evaporator with refrigeration capacity of 5090 W at 1°C (Patton, Osborne Park, WA, Australia). In each cold room, two fans (300 mm five blade propeller type) circulated air across the evaporator at an air flow averaging 960 L/s measured at various points in the room. The fans automatically switched off during defrost. A forced air-cooling unit, externally controlled, was available (variable speed 500 mm five blade propeller type) in each cold room. The unit was placed under the evaporator to draw in cold air through the vents in the cartons and to speed up the rate of cooldown if required.

Produce load in cold rooms

Cartons of grapes for large scale tests in cardboard cartons were loaded on custom built slatted pallets in eight stacks per cold room replicate. To provide for good air circulation, stacks were placed three each along the two side walls with two stacks in the centre below the height of the evaporator fans. There was a 2-3 cm gap around each stack. The cardboard cartons were arranged on each pallet to create a chimney in the centre for air circulation, while the polystyrene cartons were placed flush

against each other. Each cardboard carton or polystyrene carton contained 10 kg of berries. For the 1989–1994 and 2004–2007 trials, there were 448 cardboard cartons/cold room making 4480 kg tablegrapes giving a treated mass load of 132 kg/m^3 and a volume load of 39.5% in each of the three cold room replicates. For the 2012 verification trials, there were 564 cardboard cartons/replicate in the first set of tests, giving a treated volume of 48.12% and a mass load of 165.9 kg/m^3 in each cold room. The three trials (1, 2 and 3°C) were run within 2–4 days of each other and a total of 1692 cardboard cartons (16.92 tonnes) were used. In the second set of verification trials using 560 polystyrene cartons, the load in each cold room was similar: 48.7% by volume and 164.7 kg/m^3 by mass.

Temperature monitoring

Temperature was automatically logged at 60 min intervals throughout the trial and stored in the logger memory. Each cold room was supplied with a Grant Squirrel meter/logger with 16 channels (Model 2020, Cambridge, England) connected to 16 'U type' mini thermistor probe sensors (accuracy ±0.01°C) by factory-built and calibrated cables of 10 to 34 m length. Probes were placed in the centre of single berries in cartons. In large-scale tests, ten probes were placed in warm un-infested berries (held at 26 ± 1°C and 60–65% RH) in eight stacks while six thermistors measured air temperature in six locations in the cold room: (1) inlet; (2) outlet; (3) centre of the cold room; (4) top corner at door entry; (5) left side wall; and (6) right side wall. In verification trials, air positions 1 to 4 were used, while 12 probes were placed in warm un-infested berries in different layers in eight stacks. At least half the fruit probes were placed in previously identified positions that were slower to cool down. Before and after each trial, all thermistor probes were calibrated in melting ice against a certified mercury glass thermometer to verify their accuracy and to apply the necessary correction to logged temperature values. Thereafter, each cold room, 24–48 h before loading, was calibrated with the 16 probes to assess equilibration of temperature and uniformity of airflow. Data were viewed in real time via software (SquirrelView V5.1) on a computer and downloaded from the logger memory every 24 h as an EXCEL (Microsoft, Richmond, WA, USA) readable file and supplied to quarantine authorities monitoring the trials. Data accumulated every hour, and the file increased each day. A separate EXCEL file containing the calibration data taken before each trial enabled data correction each day. Final correction was applied following re-calibration of the 16 probes after every trial.

Timing of cold treatments

After each cold room was loaded, the door was sealed and timing of cooldown commenced. Treatment was timed in hours. In the large scale tests, treatment time started when the last of the ten fruit probes in each cold room replicate recorded the upper limit for each specified treatment, that is, 1.5, 2.5 and 3.5°C for 1, 2 and 3°C, respectively. The required exposure time was counted in hours, that is, 288, 312, 336, 360, 384, 432 and 480 h for 12, 13, 14, 15, 16, 18 and 20 days, respectively. In large scale tests, the forced air-cooling unit was run until the last fruit probe recorded the upper temperature limit for treatment timing to start. Thereafter, the unit was turned off and cooling was maintained under normal air circulation. For the verification trials, forced air cooldown was not allowed. The MAFF required treatments to start when half the fruit sensors (six out of 12) recorded 1.5, 2.5 and 3.5°C or after 72 h of cooldown, whichever was earlier. The MAFF set the lower temperature limits as 1.1, 2.1 and 3.1°C.

Analysis of data

The equations set out later describe the analysis used. The theory is described in more detail in Abbott (1925), Finney (1971, 1978), Couey and Chew (1986) and Food and Agriculture Organization of the United Nations (2016b). Liquido and Griffin (2010) supply an online aid to assist analysis (http://cqtstats.cphst.org/index.cfm). The true population responding is given by P^* of which P is an estimate. The true proportion surviving is normally given as Q. This forms the basis for the binomial expansion $(P + Q)^n$ that underpins the subsequent equations. Treatments are expected to deliver few survivors, that is, 0, 1, 2 and 3, assuming large numbers are present. Therefore, the Poisson distribution provides a more convenient model for estimating survivors. The proportion of larvae responding to cold treatment doses was corrected based on mortality in berries before treatment as (Equation 1):

$$M = (P - C)/(1 - C) \quad (1)$$

where the corrected proportion killed by the dose is M, the proportion observed responding in treatments is P and the proportion responding (dying) in Controls is C.

The true population treated 'n' is estimated by calculating 'N'. Where no survivors (pupae) are found in replicated large scale tests, the numbers expected to have been treated are obtained from the corresponding Control replicates and corrected as (Equation 2)

$$N = \mu - [\mathrm{SD} \times \sqrt{(1 + 1/R)}] \quad (2)$$

where N represents the corrected numbers treated, μ represents the Control mean, SD is the standard deviation and R is the number of Control replicates.

Treatment precision is assessed by estimating the CL. When large numbers of insects are treated and none survive, the CL is estimated as (Equation 3)

$$\mathrm{CL} = 1 - (1 - q)^N \quad (3)$$

where N is the corrected number of insects treated from Equation 2 and q is the probability of failure (hence presence of survivors) and an estimate of Q that is the unknown true number of survivors. Where no survivors are found, the minimum numbers of insects that must be treated at specified CL and % mortality is obtained as (Equation 4)

$$N^* = [\log (1 - \mathrm{CL})/\log (1 - q)] \quad (4)$$

where N^* is the minimum numbers of insects that must be treated, CL and q are given by quarantine authorities (e.g. 95% CL with 99.99% mortality, q = 0.0001). When one or more insects survive (S) a specified treatment, the additional number of insects required to be tested with no survivors is calculated using Equation 5:

$$N\hat{} = m/q \quad (5)$$

where $N\hat{}$ is the additional number required to be tested and q is the probability of failure (survivors). The value of 'm' for number of survivors (S) (S = 0, 1, 2, 3 … 5) are obtained using the 'gaminv' function in MATLAB R2016a, version 9.0 (MathWorks, Natick, MA, USA). At 95% CL, these are

S =	0	1	2	3	4	5
m =	3.0	4.7	6.3	7.8	9.2	10.5

Calculations were done in EXCEL 2013. Analysis of variance and regression analysis tests were made on $\log_{10}$ transformed data using GenStat release 11.1 (VSN International, Rothamstead, England).

Results

Medfly life history in tablegrapes

The data taken over several years in nine tablegrape cultivars from 45 LH data sets are summarised in Figure 1. Day 0 is the day of egg inoculation in berries; only eggs are present on day 1–2 because none have hatched; first instar is >50% from day 3–5; second instar is >50% from day 6–8; and third instar is >50% from day 9–12. The last viable day for 95% of each stage is eggs day 3; first instar day 7; second instar day 10; feeding third instar day 13 and non-feeding third instar day 14. Pupation begins from day 12, and adults emerge from day 21. The between cultivars comparisons for 45 LH tests were not significant ($F = 0.083$, df 44, 176; $P = 1$), whereas the day when each larval stage was ≥50% in the berry was highly significant for the five stages, that is, eggs, three larval instars and pupae ($F = 6.06$, df 4, 176; $P = 0.00014$). Red Globe berries infested for cardboard carton tests were used within 4 weeks of harvest while berries from the same harvest that had been >12 weeks in cold storage (1°C) were used for polystyrene carton tests. The LH data show, however, that the berries in both tests were equally suitable for development of immature stages. The LH data (Figure 1) show that at 26°C and 60% RH after 5 days there were >50% first but <40% second instars and after 8 days there were >50% second and <40% third instars.

Large scale tests

In the 1989–1994 tests, cooldown time for berries in individual replicates ranged from 29 to 45 h with means of three replicates ranging from 34 to 44 h (Table 1). The data show that once treatment started (when the last fruit sensor recorded 1.5°C), the average daily berry temperature (ten probes × 24 h records per day) ranged from a low of 0.8°C to a high of 1.5°C but the gap between lowest and highest temperature narrowed to 0.4°C and the average over 14 days (3360 records per cultivar; 26 880 records in eight cultivars) was 1.1 ± 0.1°C. The air

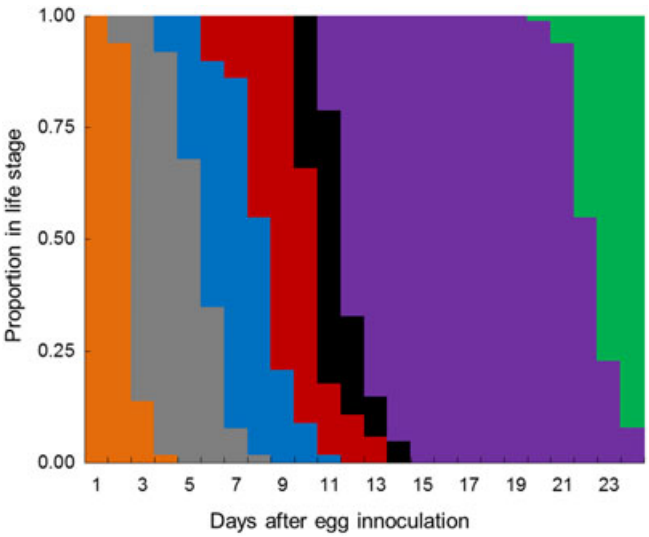

Figure 1. Life history of the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) in tablegrapes: egg (■), first instar (■), second instar (■), third instar (■), non-feeding third instar (■), pupa (■) and adult (■).

records (six probes × 24 h × 14 days = 2016 readings per cultivar; 16 128 in eight cultivars) were consistent with the thermostat setting required to maintain berries at 1°C.

Counts of immature insects treated in every replicate were corrected where required. The total number of live larvae was corrected using Equation 1. The estimated totals of three replicates per cultivar on the day of treatment at 1°C for eight cultivars were 783 747 (Table 1). The number in each stage was 67 382 first instars, 530 980 second instars and 185 385 third instars. No pupae were found in the 1°C 14-day treatment. The total number found in Control berries was corrected using Equation 2 giving 398 622 pupae. The corrected number of adults (Equation 2) estimated was 359 280. To satisfy MAFF's requirement for 99.99% mortality (0.0001% survival) due to treatment at the 95% CL, Equation 3 was used for each cultivar. The corrected number of pupae (Table 1) is >30 000 in Control berries/cultivar per treatment. No pupae were obtained from treated berries. Thus, MAFF's condition was met. More than 29 956 insects were killed in every cultivar; therefore, estimated N^* obtained from Equation 4, the prerequisite for 99.99% control at 95% CL, was satisfied for all cultivars using 14 days at 1°C.

In other tests set up as single replicates for 12, 13 and 15 days (Table 1), cooldown to 1.5°C took 44 h before treatment timing was started. Fruit temperature averaged 1.1 ± 0.1°C. Corrected estimates (Equation 1) show that 31 993 live larvae were treated in Red Globe and 31 105 in Thompson Seedless for each exposure period at 1°C. The corrected numbers of pupae (Equation 2) found in untreated Red Globe Controls were 19 855 yielding 17 856 adults, while in Thompson Seedless there were 15 571 pupae giving 13 692 adults. After 12 days at 1°C, there were two pupae in Red Globe and one in Thompson Seedless giving a total of three survivors out of 35 426 treated insects. Although 99.99% mortality was achieved, the CL estimate using Equation 3 was only 47.3%. After 13 days, only one pupa was found in Thompson Seedless giving 99.99% mortality with CL of only 86.5%. The number of additional insects that must be treated to achieve 99.99% mortality with 95% CL without finding any more survivors is obtained using Equstion 5. The value of m is 4.7 for one survivor and 7.8 for three survivors. A further 47 579 insects must be treated for 12 days and a further 17 475 insects must be treated for 13 days. Only one adult (female) emerged from Thompson Seedless after 12 days (Table 1) while no adults were found for Red Globe. No survivors as pupae were found after 15 days at 1°C.

From 2004 to 2007, three cultivars were tested at 1, 2 and 3°C for 16, 18 and 20 days, respectively (Table 2). Treatment time was started when the last berry temperature sensor recorded 1.5, 2.5 or 3.5°C. The mean cooldown times for three cold room replicates per cultivar ranged from 33–43 h for 1.5°C, 54–57 h for 2.5°C and 37–52 h for 3.5°C. The average daily fruit temperature (ten probes × 24 h records) of three cold room replicates per cultivar were stable at 1.1°C (11 520 records), 2.1°C (12 960 records) and 3.1°C (14 400 records). The average air temperature (six probes × 24 h records) of three cold room replicates per cultivar were also stable giving 6192 readings at 1.1°C, 7776 at 2.1°C and 8640 at 3.1°C. Relatively high (5 to 12°C) instantaneous air records logged during defrost cycles did not affect daily averages. The number of live larvae corrected (Equation 1), for each stage and replicate on the day of loading, shows that the total in the target stage, that is the second instar, in all three cultivars per temperature was >300 000. The total larvae alive in all three instars, in all three cultivars and all three temperature values before cold storage

Table 1. Effect of time and a temperature of 1°C on the survival of the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) in large scale disinfestation tests (1989–1994) with eight tablegrape cultivars.

Cultivar	Treatment time (days)	Larvae found in treated fruits (No.)				Surviving pupae and adults (No.)	Pupae from Controls (No.)	Adults from Controls (No.)	Cooldown time to 1.5°C (h)	Fruit temperature (°C)	Air temperature (°C)
		1st†	2nd	3rd	Total						
Olympia	14	8429	62 559	22 293	93 281	0	44 430	41 364	33 ± 5	1.0 ± 0.1	0.5 ± 0.7
Red Emperor	14	11 252	70 954	21 658	103 864	0	53 476	47 664	36 ± 6	1.1 ± 0.1	0.6 ± 0.6
Red Globe	14	8256	55 768	27 949	91 973	0	57 075	52 164	41 ± 3	1.1 ± 0.1	0.6 ± 0.6
Thompson Seedless	14	7954	63 290	19 026	90 270	0	40 671	36 540	40 ± 6	1.2 ± 0.1	0.6 ± 0.6
Flame	14	10 023	67 156	22 315	99 494	0	42 745	37 764	43 ± 3	1.2 ± 0.1	0.6 ± 0.7
Calmeria	14	7649	71 481	26 289	105 419	0	55 033	49 320	39 ± 7	1.0 ± 0.1	0.5 ± 0.6
Queen	14	7861	65 943	22 317	96 121	0	54 138	47 628	43 ± 3	1.1 ± 0.1	0.6 ± 0.8
Ruby	14	5958	73 829	23 538	103 325	0	51 054	46 836	42 ± 5	1.2 ± 0.1	0.6 ± 0.6
Total	14	67 382	530 980	185 385	783 747	0	398 622	359 280	–‡	–	–
Red Globe	12	2144	18 990	10 859	31 993	2 P (1 A, F)	19 855	17 856	44	1.1 ± 0.1	0.6 ± 0.7
Thompson Seedless	12	2573	16 572	11 960	31 105	1 P (0 A)	15 571	13 692	–	–	–
Total	12	4717	35 562	22 819	63 098	3 P (1 A, F)	35 864	30 528	–	–	–
Red Globe	13	–	–	–	–	0	–	–	44	1.1 ± 0.1	0.6 ± 0.7
Thompson Seedless	13	–	–	–	–	1 P (0 A)	–	–	–	–	–
Total	13	4717	35 562	22 819	63 098	1 P (0 A)	35 864	30 528	–	–	–
Red Globe	15	–	–	–	–	0	–	–	44	1.1 ± 0.1	0.6 ± 0.7
Thompson Seedless	15	–	–	–	–	0	–	–	–	–	–
Total	15	4717	35 562	22 819	63 098	0	35 864	30 528	–	–	–

For 14 day tests, $n = 3$; for 12, 13 and 15 day tests, $n = 1$. A, adult; F, female adult (no M, male adults were found); P, pupae. †Instar. ‡No data.

Table 2. Effect of time and temperature on the survival of Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) in large scale disinfestation tests (2004–2007) with three tablegrape cultivars.

Cultivar	Treatment (°C/days)	Larvae found in treated fruits (No.)				Surviving pupae (No.)	Pupae from Control fruits (No.)	Cooldown time to 1.5, 2.5 and 3.5°C (h)	Fruit temperature (°C)	Air temperature (°C)
		1st†	2nd	3rd	Total					
Red Globe	1/16	53 184	114 140	32 531	199 855	0	67 827	43 ± 4	1.3 ± 0.1	0.4 ± 0.1
Thompson Seedless	1/16	44 836	97 297	36 497	178 630	0	78 104	33 ± 9	1.1 ± 0.1	0.4 ± 0.1
Crimson Seedless	1/16	38 378	100 651	35 990	175 019	0	77 592	36 ± 3	1.0 ± 0.1	0.4 ± 0.1
Total	–‡	136 398	312 088	105 018	553 504	–	223 522	–	–	–
Red Globe	2/18	47 860	109 082	36 114	193 056	0	78 189	55 ± 1	2.2 ± 0.1	1.8 ± 0.4
Thompson Seedless	2/18	39 018	96 835	31 892	167 745	0	73 875	54 ± 1	2.1 ± 0.1	1.6 ± 0.7
Crimson Seedless	2/18	61 320	98 452	36 246	196 018	0	75 125	57 ± 1	2.2 ± 0.1	1.5 ± 0.6
Total	–	148 198	304 369	104 252	556 819	–	227 190	–	–	–
Red Globe	3/20	50 524	115 872	38 883	205 279	0	76 255	37 ± 2	3.1 ± 0.1	3.1 ± 0.3
Thompson Seedless	3/20	45 267	97 529	37 214	180 010	0	73 386	52 ± 2	3.1 ± 0.1	3.0 ± 0.4
Crimson Seedless	3/20	35 360	103 075	31 640	170 075	0	68 240	46 ± 9	3.2 ± 0.1	3.1 ± 0.4
Total	–	131 151	316 476	107 737	555 364	–	217 882	–	–	–

$n = 3$. †Instar. ‡No data.

were >500 000. The corresponding corrected total pupae using Equation 2 were >200 000. Using Equation 3, the total number treated satisfied MAFF's requirement for 99.99% mortality at the 95% CL for individual cultivars and temperature. The minimum numbers expected using N^* in Equation 4 were satisfied as no survivors were found in treatments and actual corrected numbers found in controls were >30 000.

Verification trials

On the day of treatment in cardboard cartons, the allocation of live insects 7 days after infestation (Figure 2a) shows that the second instar was predominant but there were more first instars than third instars. In polystyrene carton tests 8 days after infestation (Figure 2b), the distribution of live insects was mainly second instars, but third instars were more than first instars. The average of live and dead insects present on the day when berries were loaded into the cold room for cardboard carton tests showed the following: first instar 7.7% alive, 0.8% dead; second instar 77.1% alive, 6.0% dead; and third instar 7.9% alive, 0.5% dead. In polystyrene carton tests, it was as follows: first instar 1.2% alive, 1.5% dead; second instar 59.6% alive, 5.1% dead; and third instar 32.3% alive, 0.3% dead. The data on live instars were used in Equation 1 to correct the number of insects killed by the treatment in each stage at the end of every trial (Table 3).

Treatment temperature for cardboard carton tests is summarised in Table 3. The 1.1°C treatment was started after 72 h of cooldown when the average of 12 probes was 1.8°C. Three probes recorded 0.8, 1.1 and 1.3°C while nine probes recorded >1.5°C. The average of 1.5°C was achieved for all 12 probes 40 h after the trial started, that is, the berries were treated at 1.5°C for only 344 h, which is 1.7 days less than the required duration of 16 days. Only two probes achieved 1.1°C while the average of the 12 fruit probes remained at 1.4°C throughout the trial. The first sensor achieved 1.5°C within 16 h, but the last sensor never dropped down to 1.5°C. The 2.1°C treatment started after 72 h of cooldown when the average temperature of 12 probes was 2.6°C. Three probes recorded 1.7, 2.1 and 2.4°C while nine probes were >2.5°C. The average of 2.5°C was achieved for all 12 probes 2 h after trial started, that is, on average the berries were treated at 2.5°C for only 2 h less than the 18 days required. Six probes achieved 2.1°C 52 h after cool-down, while the average of the 12 fruit sensors remained at 2.2°C throughout the trial. The first sensor achieved 2.5°C in 26 h, while the last sensor required 289 h. The results for 3.1°C tests are based on 11 sensors

Figure 2. Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) in tablegrapes before treatment: (a) after egg incubation for 7 days, the proportion alive as first instar (■), second instar (■) and third instar (■) stages before tests commenced in cardboard cartons; and (b) after egg incubation for 8 days, the proportion alive as first instar (■), second instar (■) and third instar (■) stages before tests commenced in polystyrene cartons.

Table 3. Effect of time and temperature on the survival of Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) in verification trials (2012) with Red Globe tablegrapes packed in cardboard and polystyrene cartons.

Carton type	Treatment (°C/days)	Larvae found in treated fruits (No.)				Surviving larvae and pupae (No.)	Pupae from Control fruits (No.)	Cooldown time to 1.5, 2.5 and 3.5°C (h)		Fruit temperature (°C)	Air temperature (°C)
		1st†	2nd	3rd	Total			First sensor	Last sensor		
CC	1/16	17 793	42 870	7834	68 497	(L) 0	27 196	16	344	1.4 ± 0.1	0.9 ± 0.5
PC	1/16	3860	40 901	24 398	69 159	(P) 0	32 112	45	94	1.2 ± 0.1	0.4 ± 0.5
Total	–	21 653	83 771	32 232	137 656	–	59 308	–‡	–	–	–
CC	2/18	11 074	38 703	7500	57 277	(L) 0	27 177	26	289	2.2 ± 0.1	1.4 ± 0.5
PC	2/18	2344	40 773	25 582	68 699	(P) 0	21 097	55	74	2.2 ± 0.1	1.2 ± 0.4
Total	–	13 418	79 476	33 082	125 976	–	48 274		–	–	–
CC	3/20	15 020	39 729	7068	61 817	(L) 0	29 876	12	216	3.3 ± 0.1	2.5 ± 0.6
PC	3/20	2875	37 592	28 725	69 192	(P) 0	23 397	27	51	3.2 ± 0.1	2.1 ± 0.5
Total	–	17 895	77 321	35 793	131 009	–	53 273		–	–	–

CC, cardboard cartons; L, larvae; P, pupae; PC, polystyrene cartons. †Instar. ‡No data.

because data from one probe were rejected after it was found to be faulty during post-treatment calibration. The trial was started 21 h after cooldown when real time records of 12 probes showed that the average of 3.5°C had been achieved, but the corrected average temperature obtained from 11 fruit probes was 3.7°C. Five probes recorded an average >3.2°C and two sensors were at 3.5°C. Four probes recorded >3.5°C for the entire treatment period. The 3.5°C average was achieved for all 11 probes 9 h after treatment started. Thus, on average the berries were treated at 3.5°C for only 9 h less than the 20 days. Six probes recorded 3.1°C within 36 h, while the average of the 11 fruit sensors remained at 3.2°C throughout the trial. The first sensor took 12 h to reach 3.5°C and the last sensor required 216 h. For polystyrene carton tests (Table 2), the 1.1°C treatment started after 55 h of cooldown when the average of 12 probes was 1.5°C but only six were ≤1.5°C. At the end of the treatment, no fruit average was <1.1°C while only one probe was >1.5°C at 1.7°C. The first sensor required 45 h to drop down to 1.5°C, the last 94 h but one sensor was always >1.5°C. The 2.1°C treatment started after 65 h cooldown when the average of 12 probes was 2.5°C but only seven probes were ≤2.5°C. At the end of the treatment, no fruit sensor average was <2.1°C and no sensor was >2.5°C. The first sensor took 55 h to reach 2.5°C, the last took 74 h. The 3.1°C treatment started after cooldown for 42 h when the average of 12 probes was 3.5°C but only eight were ≤3.5°C. At the end of the treatment, the average of one fruit probe was 2.9°C while no probe was >3.5°C. The first sensor required 27 h to drop to 3.5°C while the last required 51 h. To sum up in all tests, the last fruit sensors always recorded shorter treatment times at 1.5, 2.5 and 3.5°C. Thus, the verification trials became more a test of effectiveness in the $>1.1 < 1.5$, $>2.1 < 2.5$ and $>3.1 < 3.5$°C intervals.

In cardboard carton tests, treated fruits were not held for pupation but dissected to recover larvae. No live insects were found through dissection of 1600 treated fruits per test, but the number and stage of every dead insect per berry was recorded. This shows as '0 (L)' in the survivors column in Table 3. The data on % dead in 160 berries dissected before loading each trial enabled use of Equation 1 to correct for natural mortality before treatment. The data in Table 3 show the corrected numbers of live larvae treated. Comparisons of the corrected numbers estimated alive in 1600 berries/temperature × time before treatment with those actually found dead are given in Figure 3a. No live insects were found. The corrected number of larvae in polystyrene carton tests (Figure 3b), estimated alive in 1600 berries before treatments and found dead after treatments, is based on dissection of samples of 160 berries/test. The remaining 1440 berries treated in polystyrene carton tests (Table 3) were not dissected but held for puparia. The regressions of expected numbers treated over the observed number after treatment are significant (cardboard cartons $F = 151.8$, df 1, 7; $P < 0.001$ and polystyrene cartons $F = 106.5$, df 1, 7; $P < 0.001$). The number predicted alive from pre-treatment samples gives an over-estimate of corrected numbers actually counted dead after treatment.

Every treated berry from the cardboard carton tests was inspected, and the structure of the data was examined by plotting the frequency of numbers/instar/berry/temperature treated (Figure 4). These graphs show that although a relatively high frequency of treated berries contain less than ten first and third instars/berry, many berries contain >11 second instars/berry. The higher proportion of second instars is expected because this was the target stage for treatments. Every treated berry had evidence of artificial infestation, but five, seven and

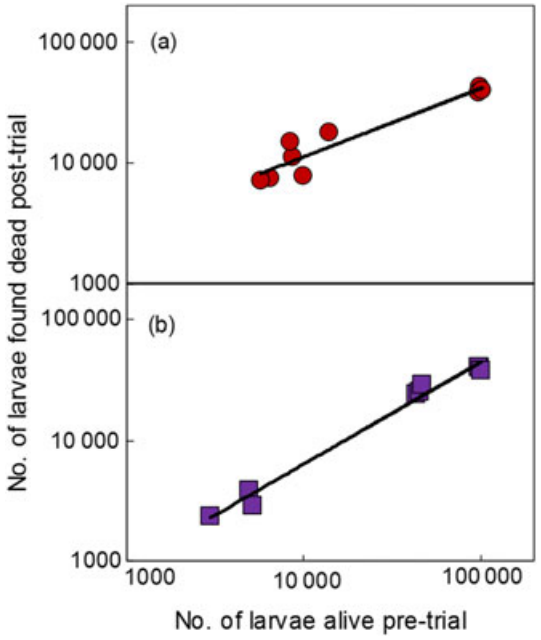

Figure 3. The number of Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) larvae found dead after treatment versus the number estimated alive before treatment. There are nine data points for each series of trials comprising a total of three instars × three temperature values. Some points overlap. Data are from tests in (a) cardboard cartons: n = 187 591; $y = 59.52x^{0.569}$, $P < 0.001$, $r^2 = 0.909$; and (b) in polystyrene cartons: n = 207 050; $y = 2.73x^{0.842}$, $P < 0.001$, $r^2 = 0.985$.

six berries in 1, 2 and 3°C, respectively, had no larvae. A further 79 berries at 1°C, 95 at 2°C and 86 at 3°C had less than five insects/berry. The number of larvae found before treatments was always more than ten per berry, and therefore, the pre-treatment population estimates are higher than actual post-treatment counts (Figure 3a,b).

The infested half (1600 berries/test) held as Control gave >10 000 pupae/replicate/test (Table 3), which satisfied MAFF's requirements. In the cardboard carton tests, the total number of pupae recovered from control berries was 1.4°C, 33 154; 2.2°C, 21 296; 3.2°C, 24 241; the estimated mortality was 1.4°C, 99.9999%; 2.2°C, 99.9859%; and 3.2°C, 99.9896%, respectively. The estimated CL (Equation 3) for 99.99% mortality is 1.4°C, 96.4%; 2.2°C, 88.11%; and 3.2°C, 91.2%. In the polystyrene carton tests, the total pupae recovered from control berries was 1.2°C, 28 295; 2.2°C, 28 173; and 3.2°C, 31 001, with estimated mortality as 99.9989, 99.9989 and 99.9990%, respectively. The estimated CL (Equation 3) for 99.99% mortality is 1.2°C, 94.1%; 2.2°C, 94.0%; and 3.2°C, 95.5%. When the number of pupae found in cardboard carton and polystyrene carton tests is combined, the CL (Equation 3) is >95% for all treatments. The comparison at 1.1, 2.1 and 3.1°C of pupae obtained from Control berries in tests with cardboard cartons versus polystyrene cartons shows no significant difference (ANOVA $F = 0.91$, df 22, 4; $P = 0.44$) even though berries infested for the polystyrene carton tests had remained in cold storage for 5 months after harvest while berries infested for cardboard carton tests were tested <1 month after harvest.

Discussion

There are concerns regarding the standards set for end point treatments in international trade (Food and Agriculture Organization of the United Nations 2013). Considerable attention has been given to the United States Department of Agriculture (USDA) 'probit 9 standard' (United States Department of Agriculture 2015) and its relationship to other standards. In this discussion, we assess the significance, appropriateness and scientific justification for standards applied to quarantine treatments. When each insect is treated as an experimental unit, the two possible outcomes are that it is either alive or dead. Such 'all or nothing' quantal responses follow the binomial distribution that underpins the mortality models presented.

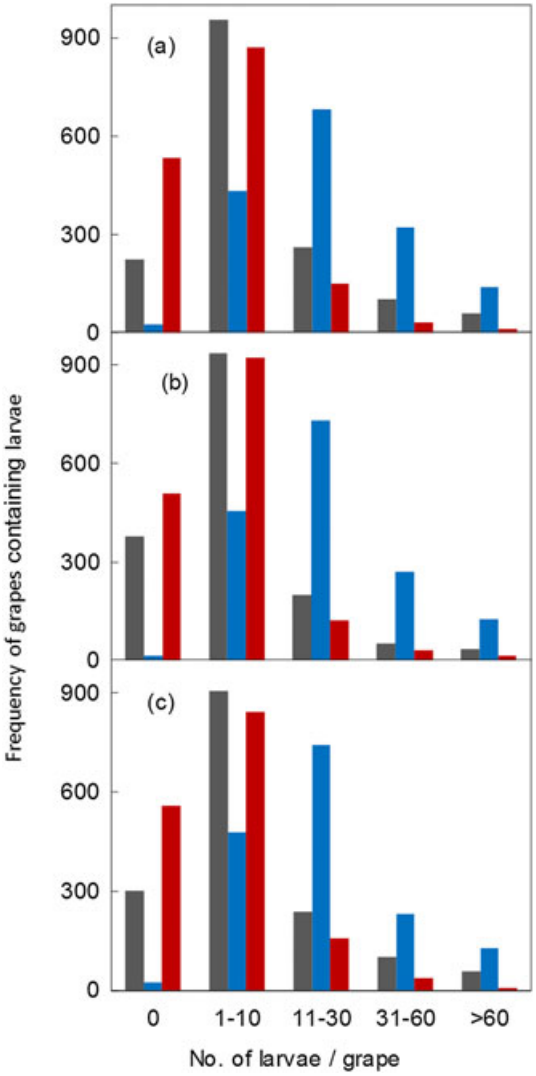

Figure 4. Effect of treatment at (a) 1°C, (b) 2°C and (c) 3°C on the frequency of grapes containing Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) larvae as first instar (■), second instar (■) and third instar (■) in 1600 grapes/test in cardboard cartons. Data are grouped in five categories showing number of grapes containing 0, that is, no larvae of the specified stage, 1–10, 11–30, 31–60 and >60/fruit after dissection.

Natural mortality

The equations used to correct numbers in large scale tests and verification trials show how natural mortality is assessed in larvae and pupae. The response of insects to a mortality-causing stimulus is made more complex when other unknown factors cause death. Abbott (1925) was concerned that researchers were not using proper methods to correct for natural mortality in their assessment of treatment effects. He proposed a formula (known as Abbott's Correction) to correct for causes of mortality other than the direct effect of the treatment. Abbott (1925) expressed the % corrected mortality (M) as

$M = ((X - Y)/X) \times 100$, where X is the % living in the Control and Y is the % living in the treatment. Thus $X - Y$ is the % killed by the treatment. Finney (1971) re-arranged and expressed this correction using proportions as given in Equation 1. When there is Control mortality, these equations reduce the effective numbers of insects treated per dose. Abbott (1925) stated that the means of treatment replicates should be assessed against the means of Control replicates to determine significance. Every treatment and Control must be a sample of the same population. Abbott (1925) required the 'probable error' to be computed when the variation between replicate numbers is high. He considered that treatments applied are significant if the difference between Control and treatment means are three times greater than the 'probable error'. The 'probable error' is an estimate about the population mean similar to the standard error. In large scale tests and verification trials, larval mortality was assessed (Tables 1–3) at several temperature × time points. Numbers treated/stage were corrected using Equation 1. The sample estimate of the unknown true population mean must be adjusted when immature stages are treated and mortality is assessed in a later stage such as pupae. Where zero pupae are found in treated fruits, Abbott's advice (Abbott 1925) to assess the 'probable error' must be followed. This can be done by adjusting (Food and Agriculture Organization of the United Nations 2016a) the number of pupae/treated fruit from the number found as pupae/Control fruit. This adjustment is given in Equation 2. When the number of Control fruits differ from the number treated, but tests are replicated for treatments and Controls, the mean number (μ) of insects/fruit/replicate found in Controls is adjusted downwards. This is done by subtracting the product of the standard deviation and a weight as shown in Equation 2. This weighted adjustment of the Control mean is similar to the lower estimate of the weighted standard error (Finney 1971). This value is then used to correct the mean numbers treated (N) in Equation 2 and is a better estimate of the unknown treated population mean. The corrected mean of pupae in Controls is then used to extrapolate to the total number of insects treated. In the 1989–1994 large scale tests, (Table 1) the Control : treated ratio was 1:3, while in the 2004–2007 (Table 2) tests it was 1:2. Table 2 shows the adjusted numbers for pupae found in the 2004–2007 large scale Control fruits that were previously supplied (De Lima et al. 2011) but not adjusted. These corrections do not affect their significance but satisfy proposed changes in international standards (Food and Agriculture Organization of the United Nations 2016a). In the 2012 verification trials (Table 3), the Control : treated ratio was 1:1. Hence, no extrapolation beyond the test data was required.

Efficacy tests using large numbers – 30 000 versus ~100 000

Quarantine regulations mandate no survivors in treated fruits. To satisfy this requirement within probability theory, we must specify the uncertainty by estimating the lower and upper 95% CL. In basic research, weighted regression lines (Finney 1971) are used to estimate the relationship between dose and response. The data required, however, to give accurate estimates of high mortality (e.g. 99.99%) are not readily obtained using small numbers (e.g. 200 insects per replicate) because the error is large at the extreme estimate of mortality. Thus, while the MTS can be selected by regression analysis at say LD50, LD95 and LD99, large numbers must be treated to obtain confidence in proposed LD99.99 or LD99.99683 end points. Baker (1939) presented data showing that the probit transform of mortality plotted against the logarithm of treatment time was linear. The test temperature, exposure time and number of insects killed were: 0°C, 12 days, 136 131; 0.6°C, 13 days, 78 190; 1.1°C, 14 days, 56 212; and 2.2°C, 16 days, 90 826. Baker (1939) then proposed what he called the 'probit 9' standard for fruit flies, that is, not more than 32 survivors in 1 000 000 treated insects (99.99683% mortality). Couey et al. (1984) interpreted 'probit 9' as three survivors in 100 000 treated at 95% CL. Couey and Chew (1986) showed that when 100 000 insects are treated and none live (99.9968% mortality), the likelihood of survival in the lower and upper 95 CI (confidence interval) is between 0 and 2.96 insects. When 93 613 insects are treated and all die (also truncated to 99.9968% mortality), the likelihood of survival is between 0 and 3.2 insects. Baker (1939) did not specify confidence levels or intervals but said large numbers (100 000 to 200 000) in treated and Control fruits were required to avoid extrapolation of data. Landolt et al. (1984) acknowledged that the 'probit 9' standard is rarely applied in practice and that the USDA accepts data showing no survivors when ~10 000 insects are treated; albeit, only for Hawaiian exports to the US mainland. Reliance on the 'probit 9' standard alone may not be sufficient if fruits are sourced from uncontrolled natural infestations (Landolt et al. 1984, Mangan et al. 1997). Most of Australia's trading partners have adopted the 99.99% mortality at 95% CL standard. This requires treating at least 30 000 insects (the model requires >29 956) with no survivors.

Temperature, cooldown time and in-transit treatment

Cooling time to specified temperature in the centre of fruit is termed cooldown time. In large scale tests (1989–2007, Tables 1, 2), cooldown ended and treatment time started when the last fruit sensor recorded 1.5, 2.5 and 3.5°C. In verification trials (2012, Table 3), treatments started when half the sensors in fruit had recorded 1.5, 2.5 and 3.5°C or after 72 h whichever was earlier. Therefore, the chances for survival of larvae in the 2012 verification trials were greater than in 2004–2007 large scale tests. The records show (Table 3) that the 25 infested warm berries in 64 out of 560 cartons take several days to cool down even when the bulk has been precooled to treatment temperature. Thus, some berries remained above test temperature for a long time after the trial started leading to 'hot spots' in the cold bulk; however, no larvae survived. This proved to MAFF that each temperature × time treatment has a good margin of safety.

Refrigerated shipping containers (reefers) do not have the capacity to remove 'hot spots'. If the loading process is kept short ~30 min and fruit stacks are transferred directly from cold room to cold reefer, hot spots are unlikely to develop. Because packaging impedes transfer of heat, the facilities conducting cold treatments must not load fruits packaged in cardboard cartons or polystyrene cartons unless pre-cooled to at least 0.5°C below specified treatment temperature. The USDA (United States Department of Agriculture 2015) regulatory standard mandates precooling of fruit to or below the prescribed cold treatment temperature before loading a reefer for in-transit disinfestation. The USDA accepts sensor variation of ±0.1°C between consecutive readings in calibration and variation not exceeding ±0.3°C between sensors. The standard requires that differences between consecutive hourly readings do not exceed ±0.39°C in fruit pulp.

Significance of treatments and harmonisation of standards

Quarantine authorities deal with large quantities of imported fruits potentially infested with eggs and larvae of fruit flies. They therefore require that data obtained from treatments be tested at extreme limits with high precision, to give a low

survival probability. Good experimental design improves precision by employing methods that reduce the interval or gap at these limits. Precision of the estimated mortality is further improved by testing large numbers of insects (>30 000). Thus, 95% CL equivalence in standards is obtained by finding no survivors at LD99.9968 when 93 613 are treated and LD99.99 when 29 957 are treated. This accepts a risk of 32 and 100 survivors, respectively, when in both cases fruits are infested with 1 000 000 insects. For practical purposes, these standards are broadly equivalent.

The FAO (Food and Agriculture Organization of the United Nations 2013, 2016a) advocates the harmonisation of quarantine disinfestation standards against fruit flies. The costly nature of the research to obtain such data inhibits many small horticultural industries from exporting. Australia has exported citrus over a period of 25 years to Japan and other countries including the USA, on the basis of cold treatments showing 99.99% efficacy at the 95% CL. No failures have been recorded. Several annexures to the International Standard of Phytosanitary Measures ISPM 28 (Food and Agriculture Organization of the United Nations 2016b) contain cold treatments against fruit flies in citrus. Research presented in this paper will assist consideration of similar standards for tablegrapes.

Conclusions

The methods, models, data, and science used and discussed in this paper contribute to good experimental design. The essential body of required research is presented in steps: assessment of life history in hosts; defining end-point mortality of eggs and larvae as pupae; determining the MTS and the most tolerant species; selecting the most susceptible cultivar; and conducting large scale confirmatory tests and correct analysis of data. Analysis should not be extrapolated beyond the range of doses and numbers tested. The estimated means should be adjusted when numbers of treated and Control fruits are unequal. To provide confidence in data obtained at high mortality, tests should have at least three replicates, each using >10 000 insects. The dose achieving 99.99% mortality with 95% CL will provide sufficient proof of efficacy for international trade.

Red Globe, Thompson Seedless and Crimson Seedless have been exported to several countries including Japan since the November 2014 harvest using treatment schedules 1.1°C 16 days, 2.1°C 18 days and 3.1°C 20 days. This trade has been facilitated through bilateral agreements. Although data on these three cultivars (against Qfly and Medfly) were presented to MAFF Japan as a package, verification trials were required only for Red Globe as the most susceptible cultivar. Six other tablegrape cultivars were successfully tested at 1.1°C for 14 days (Table 1). The work has been comprehensive and covers seedless and seeded cultivars. Thus, new cultivars of tablegrapes intended for future export from Australia should be approved at each temperature without need for further substantial proving tests. Some regulatory bodies require testing against fruit flies in every new cultivar presented for export. When a large body of relevant information already exists on the pest and host species, this becomes an unnecessary imposition on international trade. The body of work reported here satisfies international phytosanitary standards. International standards ratified by FAO member states (Food and Agriculture Organization of the United Nations 2016b) facilitate market access beyond bilateral agreements and assist countries without the capacity to conduct disinfestation research.

Acknowledgements

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References

Abbott, W.S. (1925) A method of computing the effectiveness of an insecticide. Journal of Economic Entomology **18**, 265–267.

Australian Government (2015) http://www.agriculture.gov.au/site collection documents/ biosecurity/export/ industry-advice-notice. [Accessed 12–06-2016].

Australian Government (2016) http://www.agriculture.gov.au/export/ controlled-goods /plants-plant-products. [Accessed 12–06-2016].

Baker, A.C. (1939) The basis for treatment of products where fruit flies are involved as a condition for entry into the United States. Circular No. 551 (United States Department of Agriculture: Washington, DC, USA).

Couey, H.M. and Chew, V. (1986) Confidence limits and sample size in quarantine research. Journal of Economic Entomology **79**, 887–890.

Couey, H.M., Linse, E.S. and Nakamura, A.N. (1984) Quarantine procedure for Hawaiian papayas using heat and cold treatments. Journal of Economic Entomology **77**, 984–988.

Crisosto, C.H., Smilanick, J.L., Dokoozlian, N.K. and Luvisi, D.A. (1994) Maintaining table grape post-harvest quality for long distant markets. International symposium on table grape production; 28–29 June, 1994; Anaheim, CA, USA (American Society of Enology and Viticulture: Davis, CA, USA) pp. 195–199.

De Lima, C.P.F. (1995) Disinfestation of table grapes against the Mediterranean fruit fly for export to Japan. Project no. DF 119 (Horticulture Research and Development Corporation: Sydney, NSW, Australia) p. 85.

De Lima, C.P.F. (2008) Data packages for cold disinfestation of table grapes for export to Korea and China. Project TG04004. Final report (Horticulture Australia Ltd: Sydney, NSW, Australia) p. 22.

De Lima, C.P.F. (2015) Maintaining table grape quality by applying quarantine treatments during cooling. Acta Horticulturae **1091**, 55–61.

De Lima, C.P.F., Jessup, A.J., Mansfield, E.R. and Daniels, D. (2011) Cold treatment of table grapes infested with Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) and Queensland fruit fly *Bactrocera tryoni* (Froggatt) Diptera: Tephritidae. New Zealand Journal of Crop and Horticultural Science **39**, 95–105.

De Lima, C.P.F., Jessup, A.J., Cruickshank, L., Walsh, C.J. and Mansfield, E.R. (2007) Cold disinfestation of citrus (Citrus spp.) for Mediterranean fruit fly (*Ceratitis capitata*) and Queensland fruit fly *(Bactrocera tryoni*) (Diptera: Tephritidae). New Zealand Journal of Crop and Horticultural Science **35**, 39–50.

Dominiak, B.C. and Daniels, D. (2012) Review of the past and present distribution of Mediterranean fruit fly (*Ceratitis capitata* Wiedemann) and Queensland fruit fly (*Bactrocera tryoni*) in Australia. Australian Journal of Entomology **51**, 104–115.

Finney, D.J. (1971) Probit analysis. 3d edn (Cambridge University Press: Cambridge, England).

Finney, D.J. (1978) Statistical method in biological assay. 3d edn (Charles Griffin: London, England).

Food and Agriculture Organization of the United Nations (2013) Review of phytosanitary security based on a probit 9 treatment standard. Commission on phytosanitary measures. Eighth session. Agenda item 14. CPM 2013/CRP/07. 8–12 April 2003. Rome, Italy. Prepared by R. Griffin, USDA APHIS PPQ (Food and Agriculture Organization of the United Nations: Rome, Italy) 10 p.

Food and Agriculture Organization of the United Nations (2016a) International plant protection convention 2015–2016 procedure manual. Technical panel on phytosanitary treatments (Food and Agriculture Organization of the United Nations: Rome, Italy). 191 p.

Food and Agriculture Organization of the United Nations (2016b) ISPM 28: Phytosanitary treatments for regulated pests. Produced by the Secretariat of the International Plant Protection Convention. Adopted 2007. Published 2016. (Food and Agriculture Organization of the United Nations: Rome, Italy).

Hill, A.R., Rigney, C.J. and Sproul, A.N. (1988) Cold storage of oranges as a disinfestation treatment against the fruit flies *Dacus tryoni* (Froggatt) and *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae). Journal of Economic Entomology **81**, 257–260.

Jessup, A.J., Dalton, S.P. and Slogget, R.F. (1998) Determination of host status of table grapes to Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae), for export to New Zealand. General and Applied Entomology **28**, 73–75.

Landolt, P.J., Chambers, D.L. and Chew, V. (1984) Alternative to the use of probit-9 mortality as a criterion for quarantine treatments of fruit fly (Diptera: Tephritidae)-infested fruit. Journal of Economic Entomology **77**, 285–287.

Liquido, N.J. and Griffin, R.L. (2010) Quarantine treatment statistics (United States Department of Agriculture, Center for Plant Health Science and Technology: Raleigh, NC, USA) Available online at http://cqtstats.cphst.org/index.cfm.

Mangan, R.L., Frampton, E.R., Thomas, D.B. and Moreno, D.S. (1997) Application of the maximum pest limit concept to quarantine security standards for the Mexican fruit fly (Diptera: Tephritidae). Journal of Economic Entomology **90**, 1433–1440.

United States Department of Agriculture (2015) Treatment manual. https://www. aphis.usda.gov/import_export/plants/manuals/ports/downloads/treatment.pdf. [Accessed 11 July 2016].

White, I.M. and Elson-Harris, M.M. (1992) Fruit flies of economic significance; their identification and bionomics (CAB International: Wallingford, England).

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