Ministry for Primary Industries
Manatū Ahu Matua

Time between methyl bromide fumigation and mortality of *Musca domestica* Linnaeus (house fly) eggs

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The insects [California red scale] *that were killed by methyl bromide were very slow to develop visible characteristics of death, and in most experiments in which the mortality was approximately 50 per cent the dead and live insects could not be distinguished with any degree of certainty 2 weeks after fumigation, because of considerable delayed mortality... The mortality counts in these experiments were made not sooner than 18 days after fumigation and in most cases not until 3 or 4 weeks after fumigation. By this time a large proportion of the survivors in the tests of immature stages had developed to another stage or, in the tests of mature females, had begun reproducing... The insects killed by methyl bromide fumigation were slower to develop characteristics of death than were those killed by hydrocyanic acid fumigation* (Yust et al.1942).

To determine scale viability Japanese inspectors puncture or crush the scale, and if body fluids are present, the scale is considered to be alive. Determining scale mortality on commodities receiving quarantine fumigation treatment presents a special problem. Several factors are involved, including time elapse between treatment and inspection and temperature and RH during in-transit storage. Body decomposition and moisture loss occurred over an extended period of time under the humid conditions prevalent in Florida, and at the high RH at which fresh fruits and vegetables are best stored. At 31 days post treatment, a few insects retained some body moisture (Witherell 1984).

It is unwise to attempt to appraise the results of the fumigation immediately in terms of insect mortality. With some fumigants, such as methyl bromide ... mortality may be delayed... It is advisable, therefore, to leave the insects in a warm place (20 to 30°C) overnight before mortality counts are made and before definite conclusions are reached as to the success of the treatment (Bond 1989).

1. Abstract

When the New Zealand Ministry for Primary Industries (MPI) intercepts organisms in imported goods, it evaluates their viability and, if they are alive, makes taxonomic diagnoses. MPI has found that mortality may occur some time after treatment, and has determined that accuracy of evaluation could be improved with a better understanding of how long it takes for clinically confirmable mortality to occur after plant quarantine treatments have been applied.

This report presents a review of published literature on the time required for insects to die following methyl bromide fumigation. It then describes two experiments conducted to define the time required for *Musca domestica* L. (Diptera: Muscidae) eggs to die after fumigating them with one of two methyl bromide rates, then maintaining them under one of four post-fumigation temperature regimes. Each treatment was compared to a non-fumigated control treatment. Egg viability was determined through observations of egg hatching and use of a biochemical viability test. Post-fumigation changes in egg morphology were recorded, and correlations between morphological characters and egg viability were assessed.

No fumigated eggs hatched, while high proportions of control eggs did. Egg mortality rates and time to death showed no differences in their responses to methyl bromide applications of 25 $g.m^{-3}$ for 2 h at c. 20°C, and 45 $g.m^{-3}$ for 2 h at c. 20°C. However, time to death was strongly influenced by storage temperature, with eggs stored at either 20°C, or 7°C for 2 days then 20°C, taking 8 – 9 days to die, while those stored at either 10°C, or 7°C for 5 days then 20°C, taking 2 – 3 weeks to die. The implications of these long time lags to death, and some possible approaches to dealing with them, are discussed.

Key conclusions were:

1. Fumigated eggs took from 7.8 – 27.5 days to die.
2. At a post-fumigation storage temperature of 10°C, time to death was increased 1.5 – 2.5 times compared to storage at 20°C.
3. Different fumigation rates of 25 $g.m^{-3}$ and 45 $g.m^{-3}$ had no effect on time to death. The lowest rate was sufficient to cause 100% egg mortality.
4. As importing fresh produce from Australia usually takes 1 – 7 days, it is possible that methyl bromide fumigated insects from Australia will still be alive when they arrive at New Zealand's border, despite having been subjected to an effective fumigation treatment which will lead to death.
5. Standard morphological indicators of viability are unreliable when applied to fumigated eggs because normal coloration and turgidity can be maintained for many days after death, probably because microbe populations contributing to egg decomposition are suppressed by methyl bromide. However, fumigated eggs are less likely to exhibit normal embryonic and larval development.
6. There may be potential to refine the biochemical viability test so it registers application of a lethal treatment at an earlier stage in a treated organism's progression towards death.

Additional MPI Comment

MPI procured the research described in this report to obtain an improved understanding of the relationship between methyl bromide treatment and being able to confirm the death of regulated invertebrates found in fresh produce importation pathways, particularly fruit fly eggs, but also scale insects, mealy bugs and other sessile life forms. Because the research

was conducted in New Zealand, house fly eggs were used as a proxy to provide a preliminary indication of how other regulated pests might behave. House flies can be readily reared and induced to produce eggs of known age for experiments. The research was therefore a preliminary investigation into this issue, and the results report a significant time lag between application of methyl bromide and being able to confirm mortality. They highlight that there may be an opportunity to maintain biosecurity while reducing duplication of methyl bromide treatment. MPI acknowledges that care is required regarding extrapolating these findings to regulated pests.

2. Introduction

New Zealand's Ministry for Primary Industries (MPI) inspects imported goods to check compliance with import health standards. Any organisms found are subjected to MPI's diagnostic procedures, which may include sending specimens and host material to one of MPI's Investigation and Diagnostic Centres (IDC). For fresh produce, procedures include identifying intercepted specimens and evaluating their viability. If they are found to be alive, then MPI may require the infested consignment to be retreated. Sessile organisms including fly eggs, scale insects and mealy bug eggs are particularly challenging to deal with, and current practice to determine if they are alive or dead involves painstaking morphological examination according to a defined procedure. Appendix 2 provides the MPI procedure for eggs. MPI is seeking to refine this system to increase the accuracy of conclusions, make faster responses to importers, and reduce unnecessary re-treatment.

Improving the accuracy of viability evaluations requires a better understanding of how long it takes for clinically confirmable mortality to occur after treatments have been applied. For treatments such as methyl bromide fumigation, there is evidence that clinically confirmable mortality may occur some time after treatment, so evaluations of treatment effectiveness need to take this lag into account. Therefore, the research described in this report was commissioned to better define the time required for insect eggs to die after they have been fumigated with methyl bromide.

Dipteran eggs were used in the experiments because this family includes major quarantine pests such as fruit flies (Tephritidae) for which particular treatments have been specified. Conducting experiments on exotic pests not present in New Zealand was impractical, so eggs of the house fly, *Musca domestica* L. (Diptera: Muscidae), were used instead. This species is easily cultured to provide eggs of known age and quality for experiments, and experimental results from *M. domestica* will support further work on particular quarantine species if it is deemed necessary.

The research was facilitated by the recent development of a biochemical viability test (Iline et al. 2010; www.agresearch.co.nz/bvt) which offers improvements in reliability, accuracy and timeliness over the standard practice of making viability assessments based on morphological criteria. To compare the performance of the biochemical viability test with MPI's standard morphological procedure, both methods were used to evaluate mortality following treatment.

This research was conducted under the auspices of one of MPI's constituent organisations, the New Zealand Ministry of Agriculture and Forestry (MAF). MAF is referred to in this report whenever provision of historical context is appropriate.

3. Literature review – viability assessment methods, time to death, and the effect of temperature

The published literature was searched for information on viability assessment methods, time to death following methyl bromide fumigation, and sources of variation in methyl bromide efficacy. The effects of fumigation temperature and post-fumigation storage temperature were of particular interest because different types of fresh produce are imported into New Zealand under differing temperature regimes, and these may influence the state of any treated organisms present in consignments on arrival.

The literature was searched via CAB Abstracts, Biosis, GoogleScholar, Delphion patents, and Dialog NewsRoom using the following search terms; methyl bromide, efficacy, mode of action, time to kill, lethal time, disinfestation, dose response, quarantine, fumigation, fumigant, efficacy, effectiveness. The objective was to use a broad search to maximise the number of publications identified that might be useful in this review. Additional publications were obtained by screening the literature cited in the papers initially identified.

The frequency of use of different mortality assessment methods was recorded from 27 published methyl bromide dose-response studies. Many other publications did not present their mortality assessment methods. The studies that described their mortality assessment methods investigated species of Acarina, Coleoptera, Diptera, Hemiptera, Homoptera, Lepidoptera and Thysanoptera. No methyl bromide dose-response studies used biochemical or other indirect approaches for evaluating mortality, and only Witherell (1984) attempted to evaluate or defend the accuracy of its methods. The procedures used involved maintaining the treated organisms and observing them for:

- Hatching of eggs.
- Microscopic examination of larval development within egg chorions.
- Development of non-egg immature stages.
- Movement of larvae and adults, sometimes while stimulating them with a pin.
- Turgidity of beetle larvae and of dissected late-stage scale insects.

Witherell (1984) compared turgidity and movement for scoring mortality of *Hemiberlesia lataniae* (Signoret) (Hemiptera: Diaspididae) following methyl bromide fumigation and concluded that turgidity could underestimate mortality due to the extended period required for scale insects to become desiccated. A patent (Maier and Porterfield 2009) noted that some fumigated eggs hatched, but the larvae subsequently died, raising the possibility that egg hatching could also underestimate overall mortality.

Data for post methyl bromide fumigation observation periods (i.e. maximum time between applying a fumigation treatment and assessing mortality) were obtained from 23 of the 27 studies from which mortality assessment methods were recorded. They investigated various life stages from eight arthropod orders, 14 families, 20 genera, and 21 species. In all 23 studies, post-fumigation observation periods always exceeded the recommendation to leave insects overnight (Bond 1989) and ranged from 1 day (d) to 42 d (Table 1). Observation periods were highly variable and mean values ranged from 2 d to 22 d (Table 1). Excluding a treatment from one study (Witherell 1984) that evaluated the efficacy of methyl bromide fumigation followed by cool storage, the temperatures at which fumigated insects were maintained for mortality observations after fumigation ranged from 22°C to 27°C.

Table 1. Mean postfumigation observation periods in days (range[1]) by taxonomic order and life stage from 23 published studies.

	Mite	Nematode	Insect						Mean
	Acarina	Aphelenc.	Coleop.	Diptera	Hemip.	Homop.	Lepidop.	Thysan.	
Adult	1	2	6 (5-7)		1	35 (1-42)		1	21
Egg	5		22 (16-28)	11 (1-28)	5	7	11 (6-21)	7 (4-10)	11
Larva	1		5	16 (14-28)	1		3	1	14
Nymph	1				1	6 (1-31)			4
Pupa			5	22 (14-30)		1		10	20
Mean	2	2	7	15	2	22	11	5	
n*	2	1	6	9	2	7	5	3	

[1]No range is given where there was no variation. *n = number of unique observation periods.

Examination of the post-fumigation observation periods used in methyl bromide dose-response studies (Table 1) suggests organisms probably take at least 24 hours (h) to die following fumigation, and could sometimes take much longer. More precise data on time to death have been provided by only a few studies. Witherell (1984) found that *H. lataniae* died within 3 d of being fumigated at a range of methyl bromide doses, durations and temperatures, but many of those fumigated at the lowest dose and temperature (16 $g.m^{-3}$ for 2.5 h at 18.3°C) were still alive after 31 d. *Rhagoletis completa* Cresson (Diptera: Tephritidae) pupae removed from peaches fumigated with methyl bromide became black within 5 d and were therefore considered dead (Yokoyama et al. 1992). The black color was probably caused by a bacterial septicaemia. MacDonald (1993) noted methyl bromide can be a slow acting poison and concluded that making mortality assessments of *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) after only 1 d may underestimate total mortality. Studies of time to death of mammals following laboratory-controlled inhalation of methyl bromide have shown broadly similar patterns to those seen in arthropods, with delays of up to 150 d before mortality, and with time to death reduced by longer exposure times and/ or higher fumigant concentrations (reviewed by Alexeef and Kilgore 1983).

The view that methyl bromide can be slow acting has been reinforced by studies other than dose-response mortality experiments. Cheetham (1990) used light microscopy and transmission electron microscopy to observe development of *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) eggs following methyl bromide fumigation at 8, 24 and 48 $g.m^{-3}$ for 2 h at 21°C. The eggs were all < 24 h old when fumigated. Absence of mitotic activity indicated that most of those treated with the highest dose had died after 20 h, and all were certainly dead after 96 h (Table 2). However, many of those treated with the two lower doses may still have been alive after 96 h, though they showed abnormalities indicating mortal damage (Table 2). The duration of the 'prolonged abnormal development' of the treated eggs therefore exhibited a negative relationship with methyl bromide dose. Yokoyama et al. (1988) monitored *C. pomonella* egg development using a stereomicroscope and found that 1.35% of 1 d old embryos continued development for 15 d to 16 d after methyl bromide fumigation, but these never hatched. Boczek et al. (1975) studied the effect of methyl bromide fumigation (40 g/ m^3 for 24 h) on embryonic development of *Acarus siro* L. (Acarina: Acaridae). Eggs of

various ages were sectioned, dyed and microscopically examined 24 - 48 h post-fumigation, and a wide range of abnormalities was observed.

Table 2. Observations of cellular development of *Cydia pomonella* eggs at intervals following methyl bromide fumigation at three doses for 2 h at 21°C (Cheetham 1990).

Hours after fumigation	8 $g.m^{-3}$	24 $g.m^{-3}$	48 $g.m^{-3}$
1		2 of 14 embryos showed mitotic activity.	Minimal mitotic activity.
1.5	All embryos showed mitotic activity.		Minimal mitotic activity.
20			3 of 70 embryos showed mitotic activity from 1-20 h, but with abnormalities. General cell lysis evident by 20 h.
96	Embryos showed some development, but all malformed.	Few embryos showed development, all abnormal.	No embryos developed, all died.

Forney et al. (1991) compared the rates of decline of adenosine triphosphate (ATP) in eggs of *Asynonychus godmani* Crotch (Coleoptera: Curculionidae) after three different lethal treatments, one of which was fumigation with 80 $g.m^{-3}$ methyl bromide for 2 h at 20°C. ATP concentrations in eggs treated by freezing in liquid N for 30 s declined by 95% after 2 h, but those in the fumigated eggs had only declined by 50% after 24 h (Table 3). The rate of decline seen in fumigated eggs was broadly similar to that observed in eggs treated with hot water. These data show the rate of ATP degradation varied with treatment, rather than providing direct evidence that time to death varied with treatment. Nevertheless, the observed relatively slow rate of decline of ATP in the fumigated eggs (Forney et al. 1991) is consistent with the possibility that fumigated organisms take > 24 h to die.

Methyl bromide fumigation of *M. domestica* adults for 1, 2, 5 or 10 minutes at 30°C (rate not given) caused ATP to decline by 37% to 87%, with the extent of decline correlated with duration of exposure (Winteringham 1958). The flies exposed for 1 or 2 minutes collapsed, then recovered after being provided fresh air and resumed respiration at normal or slightly supranormal rates for 1 - 3 h. Later, their respiration gradually declined over a period exceeding several hours, and they died.

Table 3. Percent reduction in ATP concentration in 6 – 7 d old ***Asynonychus godmani*** eggs compared with untreated controls at intervals following three different lethal treatments (Forney et al. 1991).

	Treatment		
Time after treatment	30 s in liquid N, then held at 26°C	3 min. in 55°C water, then held at 26°C	80 $g.m^{-3}$ methyl bromide for 2 h at 20°C, then 30 min at 20°C, then held at 26°C
2 h	95	40	0
6 h			0
1 d	95	40	50
7 d			50
9 d	no data	no data	97

None of the reviewed studies investigated the influence of post-fumigation storage temperature on time to death. Witherell (1984) compared mortality of fumigated *H. lataniae* following 7 d of storage either in a refrigerator (4.4°C to 7.2°C) or in a greenhouse; all insects in both storage treatments had died. In general, however, conditions that promote insect respiration such as warm temperatures (Bond 1975, 1989; Cotton 1932), slightly elevated CO_2 (Cotton 1932, Jones 1938) and reduced O_2 (Cotton 1932) are thought to increase methyl bromide efficacy.

More information was available on the effect of fumigation temperature on treatment efficacy. Efficacy usually increases with fumigation temperature, probably due to increased insect respiration rates (Armstrong et al. 1988; Barak et al. 2006; Barak et al. 2009; Bond 1989; Mortimer and Powell 1988), although the effect might decline above 25°C (Armstrong and Whitehand 2005). The mechanism may also involve differences in chemical reactivity of the fumigant at high and low temperatures, and differences in the rate and degree of sorption (Bond 1975).

To further elucidate temperature effects on fumigant efficacy, we compiled data from published methyl bromide dose-response studies of Tephritidae (Armstrong and Couey 1984, Armstrong et al. 1988, Armstrong and Whitehand 2005, Hallman 1998, Rippon et al. 1982, Tanaka et al. 1986, Williamson et al. 1986, Yokoyama et al. 1992), then evaluated the effect of life stage (eggs, larvae and pupae), fumigation temperature, and exposure time on response to methyl bromide concentration using probit analysis (Agresti 2002). Fumigation temperature was categorised as low (10 - 19°C), medium (20 - 29°C) and high (> 29°C). Exposure time was categorised as short (1 - 1.5 h) and long (2 - 2.5 h). Significant effects on the survival probability of Tephritidae (n = 133 cases) were attributable to life stage, fumigation temperature and exposure time. Eggs had the highest survival probability, followed by pupae, then larvae ($P < 0.001$). The survival probability of all three stages increased as fumigation temperature ($P < 0.001$) and exposure time ($P < 0.001$) declined. Examination of deviance values indicated that fumigation duration had the greatest effect (deviance 8868.156, 1 df, $P < 0.001$), followed by fumigation temperature (deviance 5427.362, 2 df, $P < 0.001$) and life stage (deviance 248.638, 2 df, $P < 0.001$). Individual studies have also recorded relatively higher survival of eggs (Armstrong and Whitehand 2005; Rippon et al. 1982; Tanaka et al. 1986), though in one study late 3rd instar larvae showed greater tolerance of methyl bromide (Yokoyama et al. 1992).

In summary, the literature supported MAF's initial observation that there can be considerable lags between methyl bromide fumigation and insect mortality. However, there were few published data, either on time to death following methyl bromide fumigation, or on the factors that influence it, to answer MAF's question about the precise time required for mortality to occur following fumigation. Based on the limited information available, it appeared the minimum time would be in the order of 24 h, while the maximum might range from 3 – 14 d. It was also clear from the literature that fumigation temperature has a strong influence on insect mortality rates, but no detailed information was available about the effects of post-fumigation storage temperature on time to death. Broadly, the literature suggested time to death should decline with increasing storage temperature.

4. Methods for experiments

Three experiments were conducted to better define time lags between methyl bromide fumigation and insect mortality, and to evaluate the effect of post-fumigation storage temperature on time to death. The first experiment (Experiment 1) made preliminary evaluations of the effects of one methyl bromide fumigation rate and two post-fumigation storage temperatures on time to death. Another similar experiment was only partially successful due to high mortality of non-fumigated control insects, so is not presented in the main part of this report, but rather as 'Experiment 1b' in Appendix 1. Experiments 1 and 1b helped to refine methods and identify parameters in preparation for a more comprehensive experiment (Experiment 2) which measured time to death following two fumigation treatments and four post-fumigation storage treatments. Two of the storage treatments in Experiment 2 were designed to simulate importation of rock melons to New Zealand from Australia, one by air cargo and the other by sea.

Table 4 lists the treatments for Experiments 1 and 2, and provides the abbreviation used for each treatment in the remainder of this report. All fumigations were to be conducted for 2 h at 20°C. Other specifications for each experiment are summarised in Table 4.

Table 4. Summary of treatments, treatment abbreviations, and specifications for Experiments 1 and 2.

Experiment	Fumigation rate, $g.m^{-3}$	Post-fumigation storage,	Abbreviation
[a]1	40	10	40g.10C
		20	40g.20C
[a,b]2	40	10	45g.10C
		20	45g.20C
		7°C for 2 d, then 20°C	45g.7C-2d
		7°C for 5 d, then 20°C	45g.7C-5d
	15	10	25g.10C
		20	25g.20C

[a]All fumigations to be conducted for 2 h at 20°C.
[b]The reason the fumigation rates shown in the abbreviations differ from those specified is explained in the Results.

In Experiment 2, the 45g.7C-2d treatment aimed to simulate air freight of rock melons between Australia and New Zealand, while the 45g.7C-5d treatment aimed to simulate sea freight.

4.1. SOURCES OF INSECTS

For Experiment 1, *M. domestica* eggs were purchased from a commercial producer. The eggs were delivered in a polystyrene box containing a frozen cooler pad, transferred to Petri dishes lined with moistened filter paper, then maintained for a maximum of 12 h at 10°C before being used in experiments.

For Experiment 2, a laboratory culture of *M. domestica* was established in October 2010 at AgResearch, Lincoln, using flies obtained from the commercial supplier who had provided the eggs for Experiment 1. The Lincoln culture was established so that the age and quality of eggs used in Experiment 2 could be more effectively managed than occurred both in Experiments 1 and 1b (Appendix 1). In February 2011, the culture was refreshed by introducing 50 wild flies captured at Tikao Bay, Banks Peninsula, Canterbury, to 50 of the laboratory reared flies. At the start of the experiment on 13 May 2011, the culture consisted of 4th generation descendents of these wild and laboratory-reared *M. domestica*. No evidence of disease or parasitism was ever observed. The flies were reared using previously published methods (Harrison 1949, Sawicki 1964, Shipp & Osborn 1967, Wilkes 1948). Oviposition and larval development occurred on paper tissues soaked in a solution containing bran (Harrison 1949, Wilkes 1948) and milk powder (Shipp & Osborn 1967). The timing of oviposition was managed by withholding protein from the flies' diet, and by preventing access to a suitable oviposition substrate, until eggs were required. The culture typically produced 7,000 – 10,000 eggs within 12 h of providing it with oviposition substrate, thus producing ample eggs with a suitably small range of known ages for experiments. Eighty five percent of 240 eggs hatched in a test conducted 14 d before the start of the experiment, and 83.5% of 200 eggs hatched in another conducted 4 d beforehand.

4.2. PREPARATION OF EGGS FOR FUMIGATION

In Experiment 1, eggs were kept in Petri dishes (90 mm diameter by 15 mm deep) containing moistened cotton rolls to maintain humidity.

For Experiment 2, Petri dishes (60 mm diameter by 14 mm deep) were prepared c. 12 h before the experiment began by labelling them with a treatment, lining them with three circular filter papers (Whatman grade 4, 50 mm), then pipetting 1 ml of sterile water onto the paper. Over 250 Petri dishes were prepared to make provision for all subsequent subsampling. To impede contamination by microorganisms, dishes were prepared in a laminar flow cabinet and sterile water was used to moisten the filter papers. Dishes were stored at 10°C until required. Eggs less than 6 h old were collected from the culture, rinsed in 10°C sterile water, then stored at 10°C to impede their development. Batches of the < 6 h old eggs were then retrieved from the 10°C cabinet, and 30 randomly selected eggs were transferred to each dish using a size 0 fine art paint brush. Petri dishes, each containing 30 eggs, were returned to the 10°C cabinet pending transport to the fumigation facility. The maximum time any egg was stored at 10°C prior to transport was 12 h, and the maximum time eggs were kept at room temperature while transferring them to Petri dishes was approximately 1 h. To assist in maintaining the high humidity required for egg survival, Petri dishes were stored in larger sealed containers lined with three sheets of chromatography paper (Whatman, grade 1). Approximately 15 ml of sterile water was used to initially saturate the container liners, and these were then kept moist throughout the experiment by applying 1 – 5 ml of sterile water as required. Filter papers within the Petri dishes were also regularly checked, and 0.1 ml of sterile water was applied whenever they began to dry.

4.3. TRANSPORT TO AND FROM FUMIGATION FACILITY

In Experiments 1 and 2, *M. domestica* eggs were transported in insulated cabinets, and data loggers (Gemini Tinytag Ultra 2) were used to record temperatures within the cabinets every 10 minutes during transport. In Experiment 1, all eggs were transported in a single cabinet, while in Experiment 2, two cabinets were used to transport the eggs to the fumigation facility, and three were used to transport them back. For Experiment 2, which involved a longer period of transport, temperature fluctuations in the cabinets were moderated by loading them with bottles of water preconditioned to the required temperatures. Experiment 2 Petri dishes were randomly allocated to the cabinets for transport to the fumigation facility.

In Experiments 1 and 2, to ensure treated and control populations were subjected to identical conditions, control eggs were transported with the treated eggs to and from the fumigation facility, but without exposing the control populations to fumigant.

4.4. FUMIGATIONS

For Experiment 1, a commercial fumigation company in Christchurch, located approximately 20 minutes drive from AgResearch at Lincoln, was contracted to treat the eggs. Treated eggs were fumigated in a single chamber, temperature was recorded every 10 minutes, but fumigant concentration was not monitored.

For Experiment 2, fumigations were performed by Plant and Food Research at its fumigation research facility in Palmerston North. Each fumigation treatment was split across three separate fumigation chambers. Chambers and eggs were maintained at 20°C for approximately 20 minutes immediately before starting the fumigations. Fumigant concentrations in each of the six chambers were measured at approximately 12 minute intervals. Temperatures were recorded at intervals of approximately 9 minutes in one of the three chambers in which 25 $g.m^{-3}$ of fumigant was applied, and in two of the three 45 $g.m^{-3}$ chambers. While treated eggs were being fumigated, untreated control eggs were kept in a controlled temperature cabinet set at 20°C. Photographs of the fumigation chambers are shown in Appendix 3.

4.5. POST-FUMIGATION STORAGE

After being transported back to AgResearch, eggs were kept in controlled temperature cabinets, one cabinet per temperature (7°C, 10°C and 20°C; Table 4), and a data logger within each cabinet was used to record temperatures every 10 minutes throughout the storage periods. The positions of Petri dishes containing eggs within the cabinets were rotated at approximately 24 h intervals to reduce the effect of any temperature stratification within cabinets.

4.6. VIABILITY ASSESSMENT METHODS

Data on the condition of the eggs were obtained using a combination of three approaches:

- Rearing to observe egg hatching.
- Conducting biochemical viability tests (BVT; Iline et al. 2010, www.agresearch.co.nz/bvt) and digitally photographing the results to enable the colour of each test reaction to be quantified using image analysis software (ImageJ v. 1.44, U. S. National Institute of Health, http://rsb.info.nih.gov/ij/).
- Using a morphological assessment protocol developed by MAF (Appendix 2). This involves following a checklist, both for preparing the egg prior to assessing its viability, and for evaluating movement within the egg, the extent of decomposition, egg colour, turgidity, and development and condition of the embryo within the egg. Before the experiment, MAF provided training to those who conducted these experiments to ensure its protocol was applied proficiently.

Eggs were morphologically assessed immediately before conducting biochemical tests. They were scored as alive either if they hatched, or if they produced a biochemical test reaction with a blue/ green (b/ g) colour ratio > 0.97. Eggs were scored as dead only if they produced b/ g ratios ≤ 0.97. The b/ g threshold for viability was adjusted from 0.95 (Iline et al. 2010) to 0.97 because microtubes with a slightly different transparency were used for conducting the biochemical tests. Eggs were not classified as alive or dead on the basis of morphological assessments.

4.7. SUBSAMPLING OF EGGS FOR VIABILITY ASSESSMENT

In Experiment 1, the first subsamples of eggs for making viability assessments were taken approximately 3 h after fumigation, then every 24 – 48 h. Subsamples for viability testing each consisted of ≥ 7 eggs, while additional eggs (n ≥ 100) were maintained to record any hatching.

In Experiment 2, eggs were subsampled every 24 h, except on days 4 – 8 when they were subsampled twice every 24 h. Sampling frequency was increased on days 4 – 8 to maximise data resolution during the period when mortality rates were expected to change most quickly based on results from the most effective treatment in Experiment 1. Subsampling involved removing one Petri dish containing 30 eggs from each treatment, counting the number of eggs that had hatched, recording the morphological condition of up to 15 unhatched eggs, then subjecting those same unhatched eggs to biochemical viability tests. Subsampled dishes and insects were then removed from the experiment.

On each Experiment 2 sampling occasion, if no survivors were found in the initial subsample of 15 eggs, then all of the eggs remaining in the same dish were also biochemically tested. If none of these was alive, then testing was continued using further dishes from the same treatment until 100% mortality had been confirmed in a total sample size of at least 50 eggs.

If a low rate of survival was detected (e.g. one survivor from 40 eggs), then further biochemical testing of that treatment was postponed until the following day when the process was repeated. Daily subsampling from each treatment continued either until no survivors were detected during testing of at least 50 eggs, or until all eggs had hatched.

Once mortality of 100% of eggs in a treatment had been confirmed, biochemical testing ceased, but any eggs remaining from that treatment were maintained at their designated storage temperature so scoring of their morphological attributes could continue. These morphological assessments generally occurred once each day until further assessment became uninformative due to eventual decomposition of the eggs. Unlike the earlier morphological and biochemical assessments, where subsampled Petri dishes were removed from the experiment, the postmortem morphological assessments involved repeated observations of the same eggs.

4.8. DATA ANALYSIS

4.8.1 Alignment with temperature specifications

To evaluate how closely each experiment met its temperature specifications, we modified the standard calculation of degree days (°d) for insect development (Nietschke 2007) by summing each experiment's absolute deviations from its specified temperatures, rather than the actual deviations from an insect's development threshold. The experimental deviation from the temperature specification was calculated as days times (actual temperature minus specified temperature). For example, in Experiment 1, the actual fumigation was at 19.4°C for 2 h, while 20°C for 2 h was specified. Two hours is 0.083 days, so the deviation is (19.4°C - 20°C) x 0.083 days equals -0.05°d, and the absolute deviation is 0.05°d. To obtain the total deviation for each experiment, we summed the absolute values of the deviations from across all treatments and phases of the experiment (i.e. fumigation, transport back to laboratory and post-fumigation storage) up to the time at least 95% of fumigated eggs had died. Finally, we expressed this as a percentage of the °d that would have accumulated if all temperature specifications had been perfectly met.

4.8.2 Survival analysis

Two statistical approaches were used to estimate survival times. The first was the Kaplan-Meier method which is a standard non-parametric statistical method for survival time analysis (Crawley 2007). We compared Kaplan-Meier survival curves arising from different treatments using the standard non-parametric Log-rank test. The Kaplan-Meier method is suitable for comparing the effects of different treatments on survival but, in situations where some organisms survive the experiment, it does not predict beyond the last observation and cannot be used for extrapolation (Crawley 2007). Similarly, where there are long time intervals between observations, the Kaplan-Meier method does not estimate mortality rate changes within the intervals.

The second approach involved parametric survival analysis which is useful for prediction in situations either where some organisms survive the experiment, or where there are long intervals between observations (Crawley 2007). With time to death data, the variance in age at death usually increases with the mean, so standard models which assume constant variance and normal errors are inappropriate. Choice of error distribution is therefore critical, and the best approach is to try several distributions and select the one that produces the minimum error deviance. Generalized linear models (GLM) are also useful when the response variable is not normally distributed and when a transformation is undesirable or impossible (Crawley 2007).

For the parametric analysis, different parametric curves (Exponential, Gaussian, Poisson, Log Normal, Logistic & Weibull) were fitted to the data and the best fitted curve was determined using either the Anderson-Darling (AD) statistic, or the Akaike information criterion (AIC). To analyse Experiment 2, a GLM with Poisson errors was also fitted and compared its residual deviance and AIC to the other parametric models.

The Results present Kaplan-Meier analyses for both experiments, and add the parametric analyses only where they provide pertinent additional information.

4.8.3 Experiment 2 variation between fumigation chambers

To verify that the three fumigation chambers in which temperatures were not monitored provided equivalent results to those from which temperature data were recorded, survival analyses were conducted as described above to compare survival of eggs from different chambers that had been subjected to the same treatment. Therefore, results from the three 25 $g.m^{-3}$ chambers were compared within each of the 25g.20C and 25g.10C treatments, while those from the three 45 $g.m^{-3}$ chambers were compared within each of the 45g.20C, 45g.7C-2d, 45g.7C-5d and 45g.10C treatments. The parametric model showing the best fit was then chosen for further analysis. Where this was a GLM, the significance of fumigation chamber was tested using chi square to compare models with and without fumigation chamber as a factor (Crawley 2007).

5. Results

5.1 ALIGNMENT WITH TEMPERATURE AND FUMIGANT SPECIFICATIONS

Table 4 summarises specifications for each experiment, while Table 5 summarises the fumigant concentrations and temperatures recorded during each experiment.

In Experiment 1, temperatures were generally close to those specified, with the most notable deviation being 20 minutes during transport from the fumigation facility back to AgResearch where eggs specified for storage at 20°C travelled at a mean temperature of 17.3°C (Table 5). However, the short period of this deviation compared to the overall interval from the start of fumigation to eventual ≥ 95% mortality of fumigated eggs, and the good precision of the controlled temperature cabinets used to store the insects post-fumigation (Table 5), meant there was only a moderate overall deviation from the specified temperatures of 5.3%.

In Experiment 2, temperatures were generally very close to those specified, with the most notable deviation being a 4 h interval during transport from the fumigation facility back to AgResearch where eggs specified for storage at 10°C travelled at a mean temperature of 13°C (Table 5). The overall deviation from the specified temperatures in Experiment 2 was 1.1%.

In Experiment 2, methyl bromide concentration was measured ten times during the 2 h fumigation in each of the six fumigation chambers and mean concentrations in each chamber exceeded specifications (Table 5). The overall mean (± SD) for the 15 $g.m^{-3}$ treatment was 24.6 ± 2.7 $g.m^{-3}$, and for the 40 $g.m^{-3}$ treatment was 45.6 ± 17.1 $g.m^{-3}$. These treatments are therefore referred to as the 25 $g.m^{-3}$ and 45 $g.m^{-3}$ treatments (as per the abbreviations listed in Table 4). The possible effects of these deviations from specifications are considered in the Discussion.

Table 5. Summary of fumigant concentrations and temperatures recorded during Experiments 1 and 2.

	Experiment 1		Experiment 2						
Transfer to fumigation									
Duration, h	0.3		4						
Mean temperature ± SD, °C	20.1±0.9		2 containers: 12.6±0.8 and 12.0±0.5						
During fumigation	1 chamber		6 chambers						
Specified rate, $g.m^{-3}$	40		40	40	40	15	15	15	
Measured rate, $g.m^{-3}$	-		47.1±16.9	42.1±14.6	47.7±20.5	21.5±0.7	25.7±2.2	26.7±1.1	
Mean temperature ± SD, °C	19.4±2.1		-	20.8±0.1	19.2±0.4	-	-	20.9±0.1	
Controls (not fumigated)									
[1]Mean temperature ± SD, °C	17.7±0.2		19.7±0.5						
Transfer back to laboratory									
Duration, h	0.3		4						
Mean temperature ± SD, °C	17.3±0.3		3 containers: 7.8±3.1, 13.0±1.4 and 19.6±0.3						
Post treatment storage, °C	10	20	20	7, 10 and 20					
Mean temperature ± SD, °C	10.7±0.2	20.8±0.7	20.0±0.5	7.2±0.3, 9.9±1.1 and 20.0±0.3					

[1]During the 2 h period the treated eggs were being fumigated.

5.2. Experiment 1 survival times

Experiment 1 involved fumigation at 40 $g.m^{-3}$ and 20°C, followed by storage at either 10°C or 20°C. Overall, 546 eggs were scored for viability, either through biochemical tests, or by observing hatching. These comprised 200 eggs from the nil.10C controls, 200 from the nil.20C controls, 90 from the 40g.10C treatment, and 56 from the 40g.20C treatment.

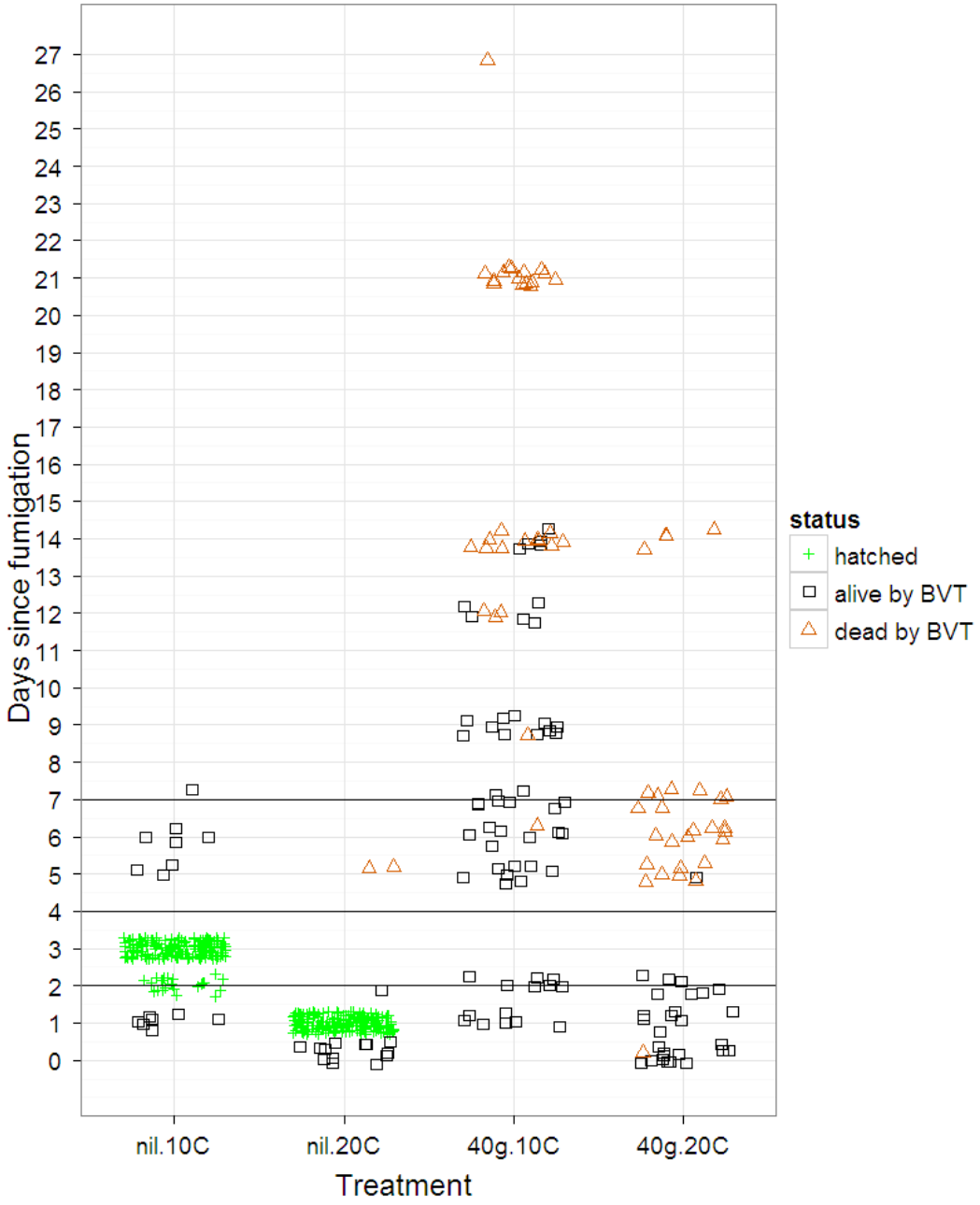
Figure 1 shows the status of Experiment 1 eggs at different intervals after fumigation, with each point corresponding to a single egg. Eggs that hatched were only observed in the controls. Qualitative assessment of Figure 1 suggests egg hatch and egg mortality were both delayed by storage at 10°C compared to storage at 20°C.

For control eggs, all of those stored at 10°C hatched, so the survival probability was 1.0 and a 95% confidence interval could not be calculated. Those stored at 20°C also had very high survival probabilities with a Kaplan-Meier 95% confidence interval (95% CI) of 0.974 to 1.0.

Kaplan-Meier estimates for survival times of fumigated eggs from Experiment 1 are shown in Figure 2 and summarised in Table 9, while those for control eggs are not shown in Figure 2 due to their low mortality. The mean survival probability of fumigated eggs stored at 20°C declined to 0.15 (95% CI of 0.01 – 0.36) after 7 d, and to zero after 14 d. The mean survival probability of fumigated eggs stored at 10°C declined to 0.04 (95% CI of 0.01 – 0.25) after 21 d, and to zero after 27 d. The survival curves for these two treatments were significantly different (Log-rank test $p < 0.001$). Control eggs survived significantly longer than fumigated eggs stored at the same temperature (Log-rank test $p < 0.001$).

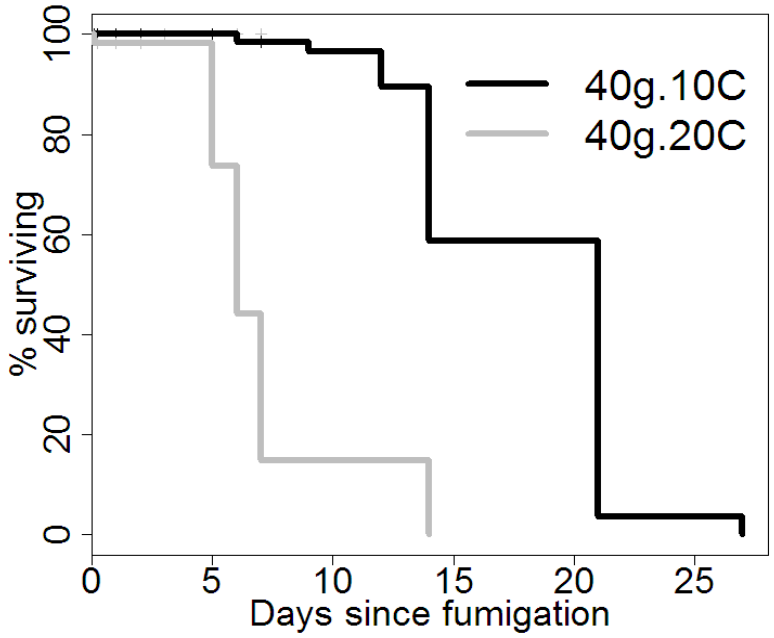
In summary, the 40 $g.m^{-3}$ fumigation was completely effective, though slow-acting, while the lag between treatment and death was strongly influenced by storage temperature, with the cooler temperature causing a longer lag.

Figure 1. Experiment 1 biochemical viability test results and hatching observations versus days since fumigation*, grouped by treatment.



*Horizontal lines at days 2, 4 and 7 show when MPI inspections of Australian air and sea cargo would usually occur.

Figure 2. Experiment 1 Kaplan-Meier survival curves.



5.3. Experiment 2 survival times

Overall, 3791 eggs were scored for viability, either through biochemical testing, or by observing hatching. The number of eggs scored per treatment is shown in Table 6.

Table 6. Number of eggs for which viability was determined, either by BVT or hatching, shown by treatment.

Rate, $g.m^{-3}$	nil	nil	nil	nil	25	25	45	45	45	45	SUM
Storage, °C	10	7C-2d	7C-5d	20	10	20	10	7C-2d	7C-5d	20	
Observations	309	501	482	271	515	234	482	282	389	326	3791

5.3.1 Within-treatment variation between fumigation chambers in Experiment 2

Days to < 0.05 survival of eggs fumigated in chambers without temperature data were very similar to those of eggs fumigated in chambers with temperature data, and no systematic bias in days to < 0.05 survival was apparent between chambers (Table 7).

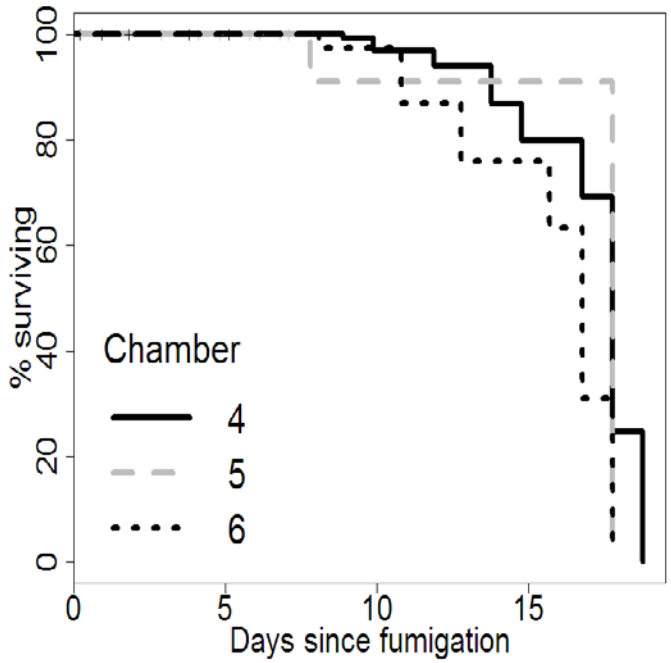
Table 7. Days to ≤ 0.05 survival for eggs from different chambers within each treatment estimated by Kaplan-Meier analysis.

	Fumigation chamber					
	25 g.m^{-3}			45 g.m^{-3}		
Treatment	1	2	3*	4	5*	6*
25g.20C	7.8	7.8	6.8			
25g.10C	19.8	19.9	19.9			
45g.20C				8.9	8.1	7.0
45g.7C-2d				8.8	8.9	8.8
45g.7C-5d				12.9	12.9	14.7
45g.10C				18.8	17.8	17.8

*Chambers in which temperatures were recorded

For each parametric analysis of within-treatment variation between chambers, a generalised linear model (GLM) with Poisson errors gave the best fit. The parametric analysis showed no significant differences between chambers in any treatment except 45g.10C, where survival of eggs from chamber 6 exhibited a slightly different relationship with time since treatment to eggs from chamber 5 ($p = 0.004$; Figure 3). These were the two 45 g.m^{-3} chambers for which temperature data were recorded (Table 7).

Figure 3. Kaplan-Meier survival curves for 45g.10C eggs fumigated in different chambers.



5.3.2 Variation between treatments in Experiment 2

Figure 4 shows the status of Experiment 2 eggs at different intervals after fumigation, with each point corresponding to a single egg. Eggs hatched only from the controls. Qualitative assessment of Figure 4 suggests storage temperature had a strong influence on time to death, with numerous dead eggs occurring within 10 d of fumigation in the 45g.20C and 25g.20C treatments, while longer times of 15 – 21 d elapsed in the 45g.10C and 25g.10C treatments before high mortality was observed. Fumigation rate, however, appeared to have much less influence, with only minor variation evident between the 45g.20C and 25g.20C treatments, and between the 45g.10C and 25g.10C treatments. Figure 4 also suggests storage regime influenced survival of the controls, with more dead eggs apparent in the nil.7C-5d treatment than in the controls stored at higher temperatures. These patterns are evaluated more rigorously in the quantitative analyses that follow.

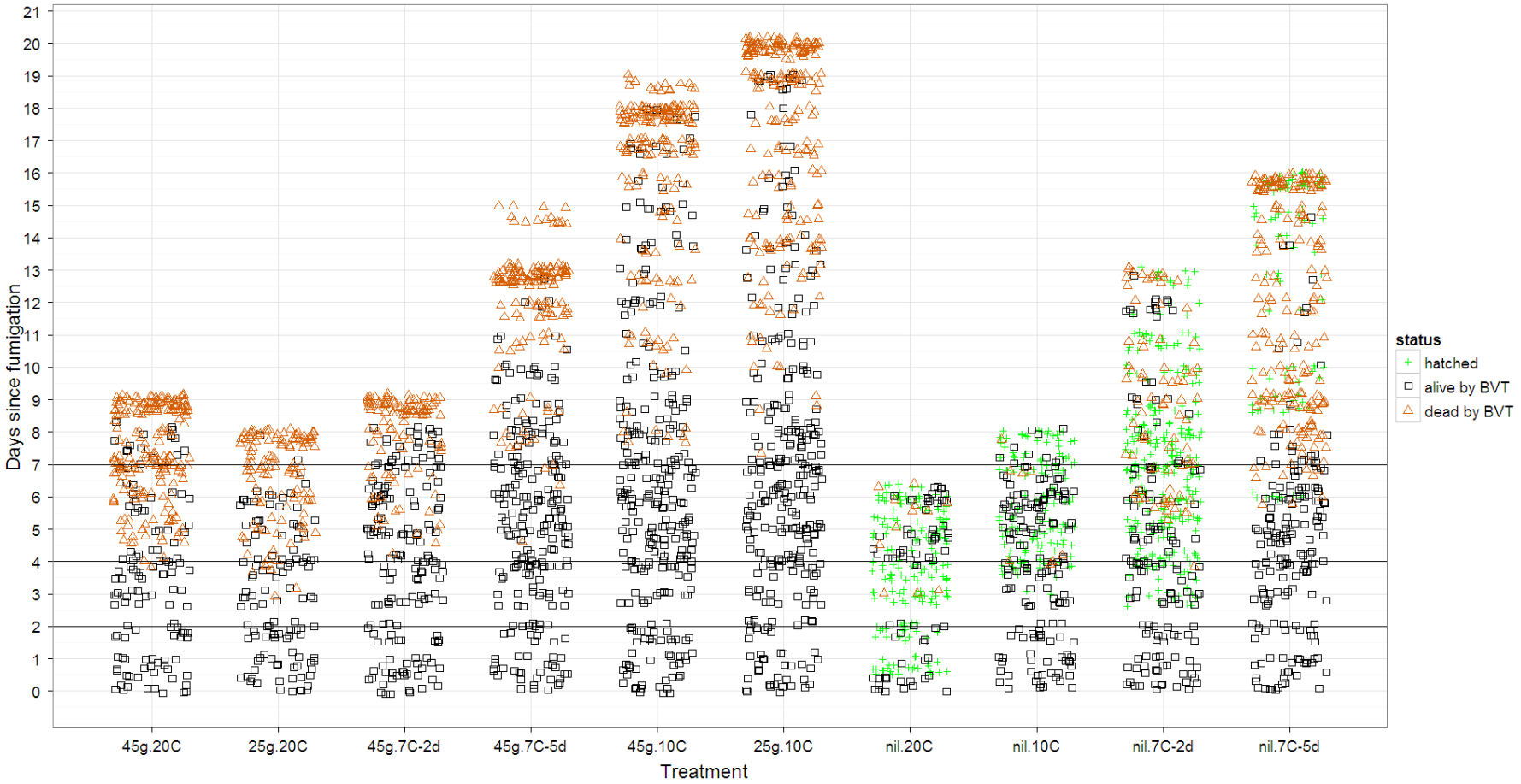
The Kaplan-Meier survival curves calculated for eggs from each treatment in Experiment 2 are shown in Figure 5, and survival times are summarised in Table 9.

Control eggs stored at 10°C and 20°C had high mean survival probabilities (95% CI) of 0.91 (0.84 - 0.99) and 0.81 (0.71 - 0.94), respectively. These curves have therefore been omitted from Figure 5. Control eggs stored at 7°C for 2 d also had a relatively high mean survival probability of 0.30 (0.20 – 0.45), while those stored at 7°C for 5 d had a mean survival probability of 0.15 (0.11 - 0.21).

For fumigated eggs, the mean survival probability of eggs from the 25g.20°C treatment took 7.8 d to decline below 0.05; 45g.20°C eggs took 8.9 d; 45g.7°C-for-2-d eggs also took 8.9 d; 45g.7°C-for-5-d eggs took 14.7 d; 45g.10°C eggs took 18.8 d; and 25g.10°C eggs took 19.9 d (Figure 5).

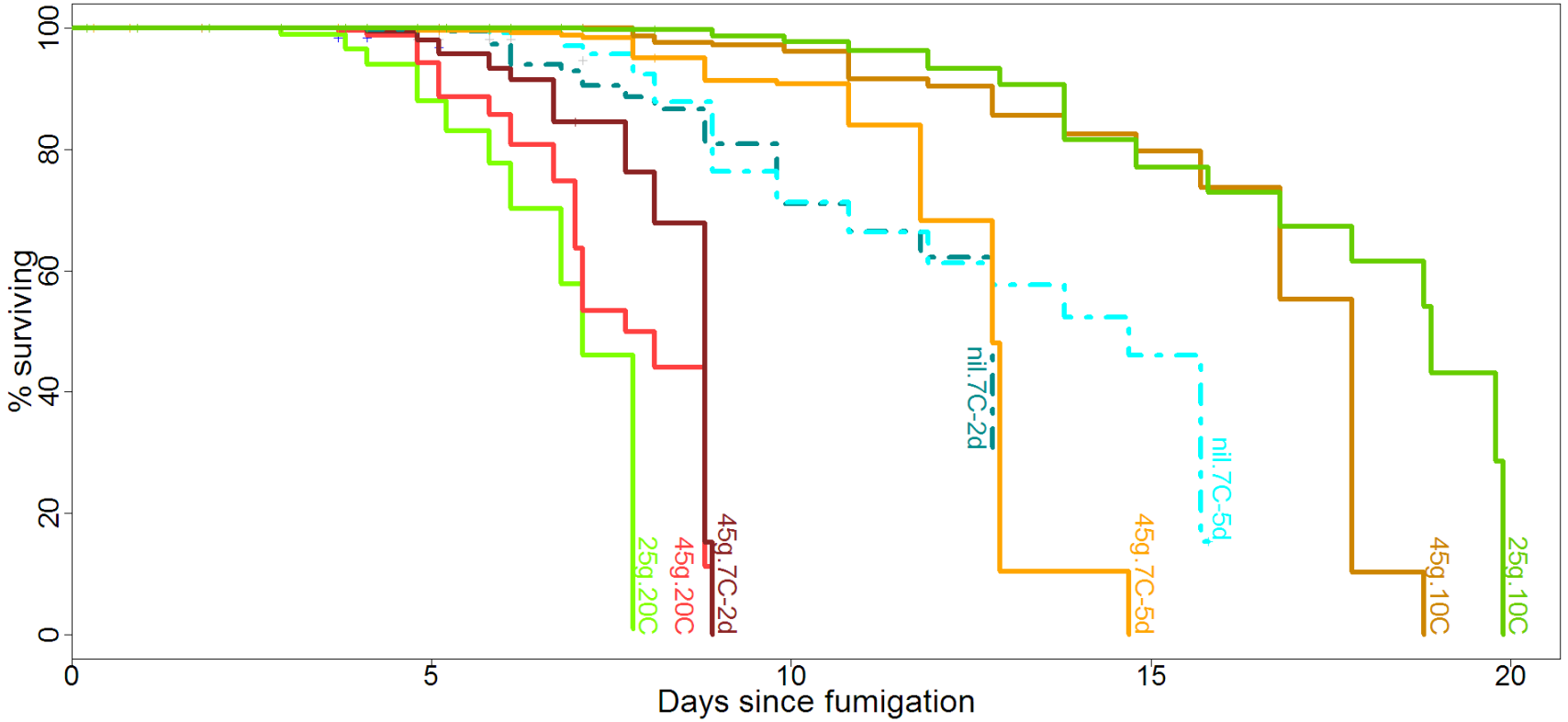
The log-rank test indicated there were significant differences between some of the curves ($p < 0.001$, Chi square = 2309, 9 df). These differences are further examined in the following parametric analysis.

Figure 4. Experiment 2 biochemical viability test results and hatching observations versus days since fumigation*, grouped by treatment.



*Horizontal lines at days 2, 4 and 7 show when MPI inspections of Australian air and sea cargo would usually occur.

Figure 5. Experiment 2 Kaplan-Meier survival curves. Blue lines show controls*, green show 25 g.m^{-3} fumigations, and red show 45 g.m^{-3}



*Nil.10C and nil.20C controls not shown because survival probabilities remained very high (0.91 and 0.81, respectively).

Of the five parametric curves tested for goodness of fit to the survival data, the Weibull curve gave both the smallest absolute log likelihood and the smallest Akaike Information Criterion (AIC) (-3172 and 6366, respectively). However, a generalised linear model (GLM) with Poisson errors was also evaluated and this had a residual deviance of 1525.7 (3781 df) and an AIC of 4181.7 (log likelihood = –2080). The GLM was therefore used for further analysis.

The GLM indicated that most treatments were significantly different from one another ($p \leq 0.05$; Table 8). The four treatments that were not significantly different from one another are shown as red cells in Table 8. Two of the non-significant comparisons were: 25g.10C *c.f.* 45g.10C ($p = 0.85$) and 25g.20C *c.f.* 45g.20C ($p = 0.98$). This confirms the qualitative indication from Figure 4 that there was no effect of fumigation rate on egg survival.

A third non-significant comparison (Table 8) was between the nil.7C- 5d and 45g.7C-5d treatments ($p = 0.49$). As inferred from Figure 4, this indicated that low temperature negatively influenced survival of control eggs to the extent that they died at similar rates to fumigated eggs stored at the same temperature. Nevertheless, hatching occurred in some nil.7C-5d eggs, but not in any fumigated eggs (Figure 4).

The final non-significant comparison was between the nil.20C and nil.10C treatments ($p = 0.07$) which reflects the high hatching rates and correspondingly low mortality rates recorded in both treatments (Figure 4).

Table 8. Probabilities* that treatments are the same as estimated by the generalised linear model.

	nil.20C	nil.10C	25g.20C	45g.20C	45g.7C-2d	nil.7C-2d	nil.7C-5d	45g.7C-5d	45g.10C
nil.10C	0.073								
25g.20C	< 0.001	< 0.001							
45g.20C	< 0.001	< 0.001	0.98281						
45g.7C-2d	< 0.001	< 0.001	0.002	0.0006					
nil.7C-2d	0.027	< 0.001	< 0.001	< 0.001	< 0.001				
nil.7C-5d	< 0.001	< 0.001	< 0.001	< 0.001	0.001	< 0.001			
45g.7C-5d	< 0.001	< 0.001	< 0.001	< 0.001	0.012	< 0.001	0.490		
45g.10C	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.049	0.012	
25g.10C	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.026	0.006	0.852586

*Comparisons showing no significant difference ($p > 0.05$) indicated in red. Those showing significant differences indicated in blue ($0.001 > p \leq 0.05$) and clear ($p \leq 0.001$).

Table 9. Summary of days to ≤ 0.05 egg survival in each experiment and treatment estimated by Kaplan-Meier analysis.

Experiment	nil.10C	nil.20C	nil.7C-2d	nil.7C-5d	15g.20C	25g.20C	45g.7C-2d	40g.20C	45g.20C	45g.7C-5d	15g.10C	25g.10C	40g.10C	45g.10C
1	*	*						14					21	
3	*	*	*	*		7.8	8.9		8.9	14.7		19.9		18.8

*Survival probability did not decline to 0.05.

5.4. Morphological variables

In Experiments 1 and 2, an egg's morphological attributes were recorded immediately before biochemically testing it. In these cases, it was possible to directly link an egg's morphological data with its BVT result, and such eggs were classified either as 'alive by BVT' or 'dead by BVT'.

In Experiment 2, subsampling of eggs for assessment both by morphology and BVT continued until mortality had been observed in 100% of at least 50 eggs. After this time, biochemical testing ceased, while the morphology of any remaining untested eggs was repeatedly assessed until the eggs decomposed. These eggs were known to be dead because they came from the same treatments in which 100% mortality had already been observed, but, because they were not biochemically tested, we classified them as 'dead by inference' rather than as 'dead by BVT'.

Photographs of various egg morphologies are shown in Appendix 4.

5.4.1 Egg colour

Most eggs were scored as either white, yellow, grey or brown. Other colours such as pink or red were occasionally observed and were also noted. Healthy eggs of *M. domestica* are white so, to simplify description and analysis, all egg colours other than white are here classified as 'abnormal'. Eggs that exhibited obvious signs of fungal infection such as mycelia were also classified as 'abnormal'.

Figure 6 shows the frequencies of white and abnormally coloured eggs in living and dead eggs, as determined biochemically. In Experiments 1 and 2, greater proportions of white eggs were observed in the 'alive by BVT' groups and greater proportions of abnormally coloured eggs occurred in the 'dead by BVT' groups. However, Figure 6 clearly illustrates that the correlation between egg colour and viability was imperfect, and both colour classes occurred in the living and dead groups.

Figure 7 shows egg colour by viability, treatment and time since fumigation for Experiment 2. It includes 7727 observations of 'dead by inference' eggs that are additional to those shown in Figure 6. Qualitative assessment of Figure 7 indicates that white eggs generally predominated, irrespective of viability, treatment and time since fumigation. Indeed, white eggs that were 'dead by inference' occurred up to 44.9 d after fumigation in the 45g.10C treatment. It also suggests the frequency of abnormally coloured eggs was negatively correlated with both fumigation rate and temperature, and positively correlated with time. For example, control eggs shown to be 'dead by BVT' exhibited high frequencies of abnormally coloured eggs within 5 – 15 d, while such eggs did not become frequent in the 45 $g.m^{-3}$ fumigation treatments until 15 – 20 d after fumigation (see 'dead by inference' section of Figure 7). Similarly, abnormally coloured eggs generally became evident more quickly in the 20°C treatments than in the 10°C treatments.

Figure 6. Frequency of egg colour by life status in Experiments 1 and 2. Numbers within columns are counts of observations.

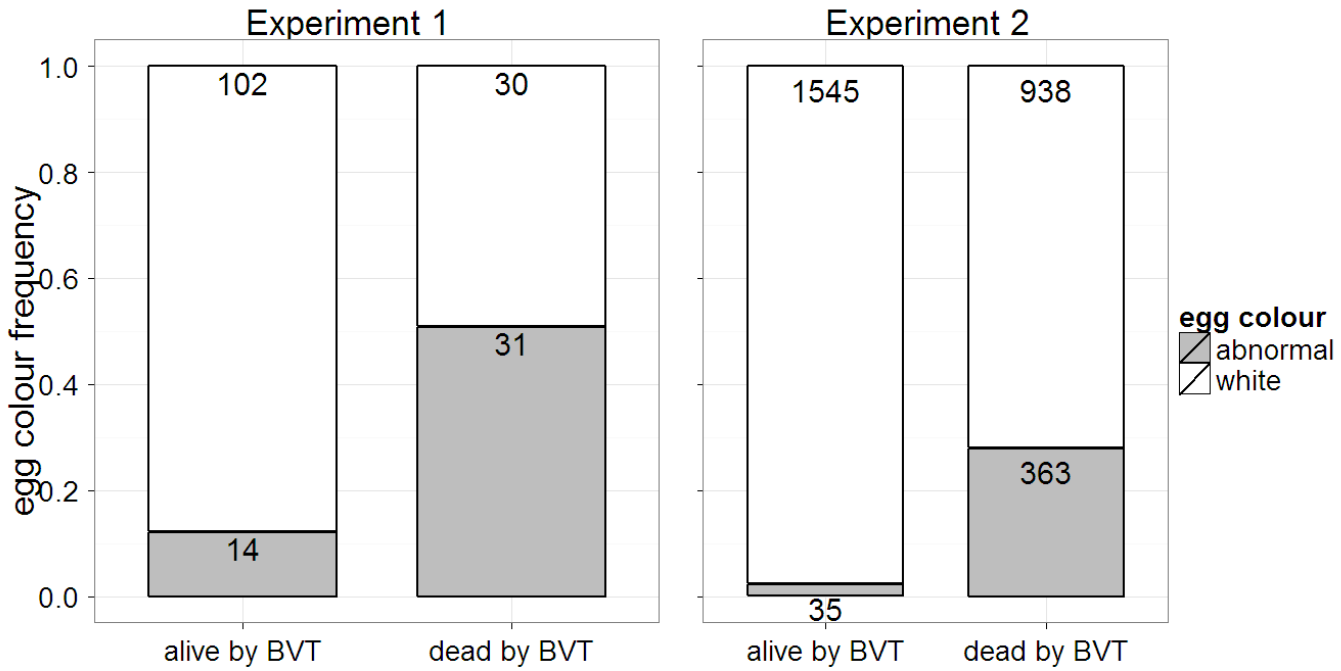
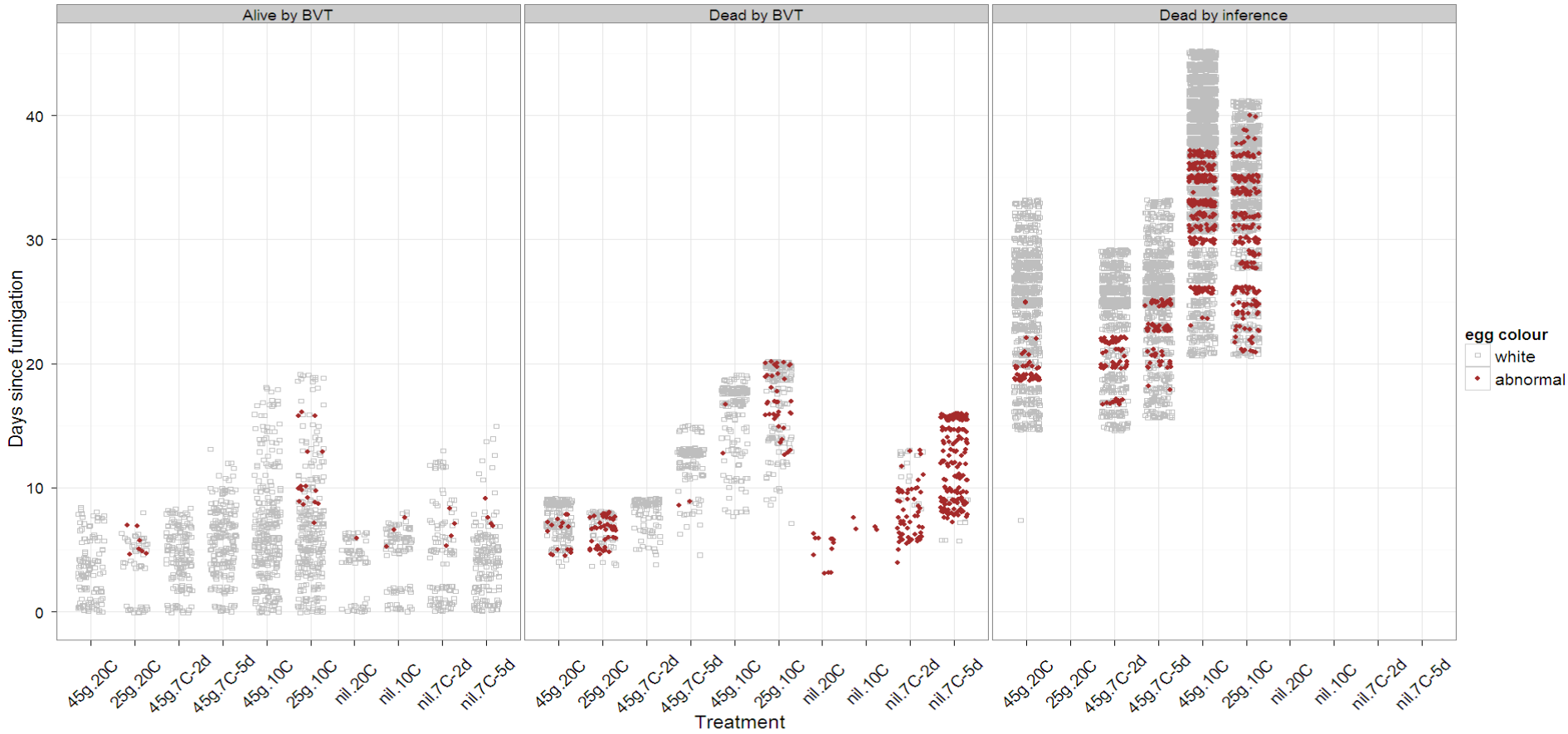


Figure 7. Egg colour by viability, treatment and days since fumigation in Experiment 2



5.2.1 Egg turgidity

The numbers of eggs classed as having low, intermediate or normal turgidity were compared to BVT results from the same eggs (Figure 8). Except in Experiment 1 where the number of observations was low, there was a trend for live eggs to have normal turgidity, and dead eggs to have intermediate or low turgidity.

Figure 9 shows egg turgidity by viability, treatment and time since fumigation for Experiment 2. It includes 7727 observations of 'dead by inference' eggs that are additional to those shown in Figure 8. Visual inspection of Figure 9 indicates that eggs with normal or intermediate turgidity predominated in the 'alive by BVT' and 'dead by BVT' classes of eggs. It was not until at least several days after the eggs had died that gross changes in turgidity started to become evident. These changes are shown in the 'dead by inference' section of Figure 9 where there are markedly higher frequencies of eggs that either had low turgidity, or had completely collapsed, or showed other abnormalities such as split chorions and the presence of gas bubbles within the chorions.

5.2.2 Visibility of larvae within eggs

The numbers of eggs containing visible larvae were compared to BVT results from the same eggs (Figure 10). In general, there were greater proportions of eggs containing visible larvae in 'dead by BVT' eggs than in 'alive by BVT' eggs. In Experiment 2, this pattern arose mainly because many control eggs that died in cold temperature treatments (i.e. nil.7C-2d and nil.7C-5d) contained visible larvae (Figure 11). Relatively few fumigated eggs contained visible larvae, whether alive or dead, and none of the 7728 'dead by inference' eggs did (Figure 11).

Figure 8. Frequency of different egg turgidity classes by life status in Experiments 1 and 2. Numbers within columns are counts of observations.

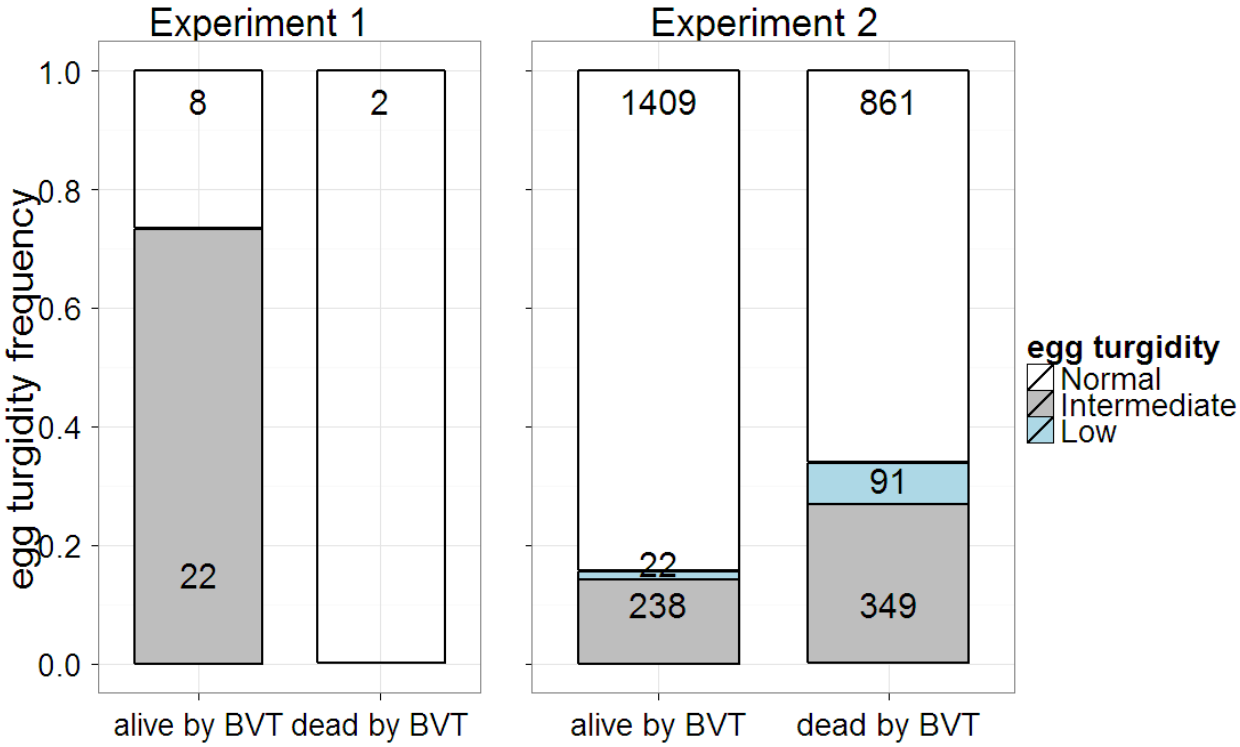


Figure 9. Egg turgidity by viability, treatment and time since fumigation.

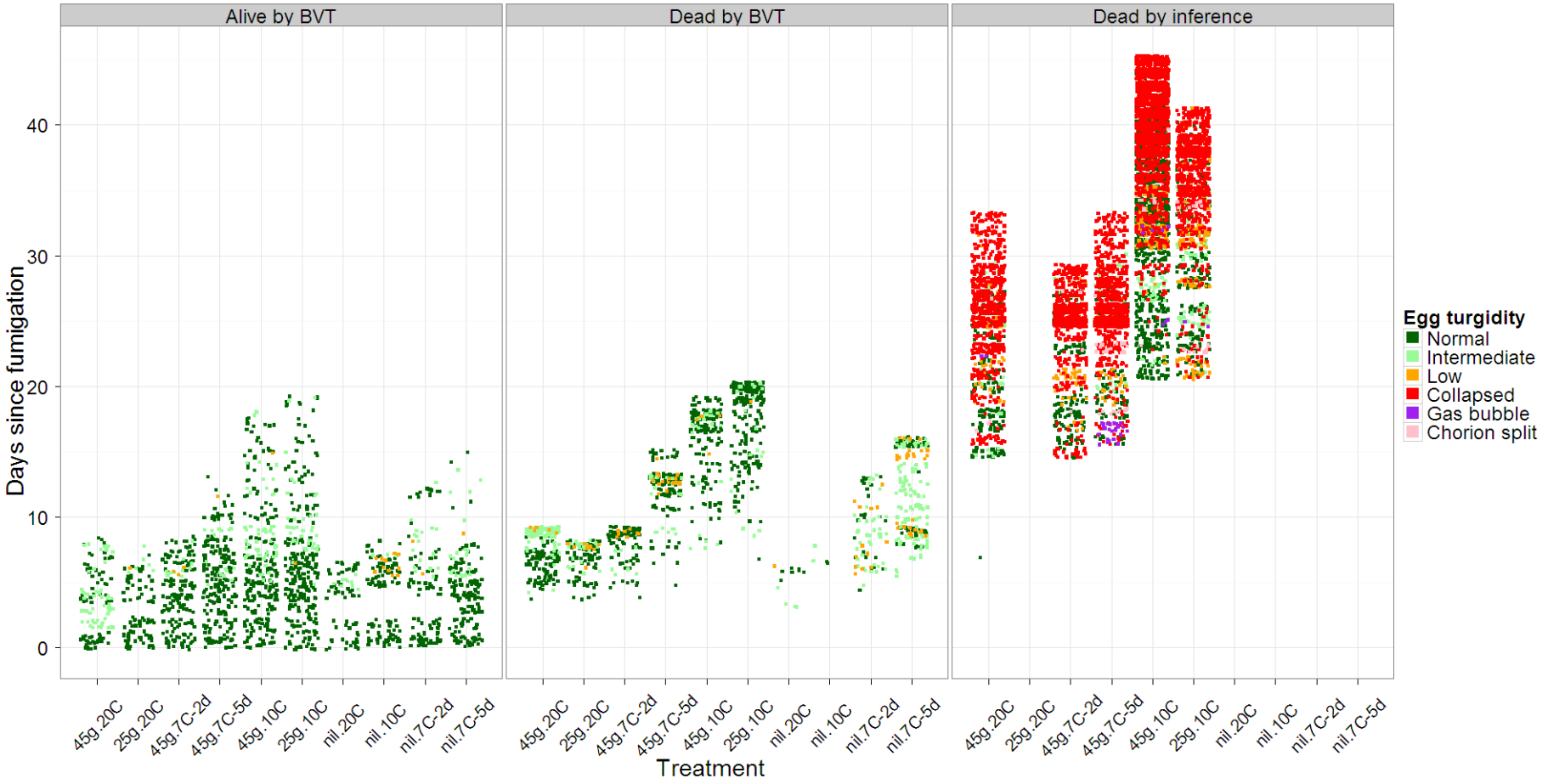


Figure 10. Frequency that larvae were visible within eggs by life status in Experiments 1 and 2. Numbers within columns are counts of observations.

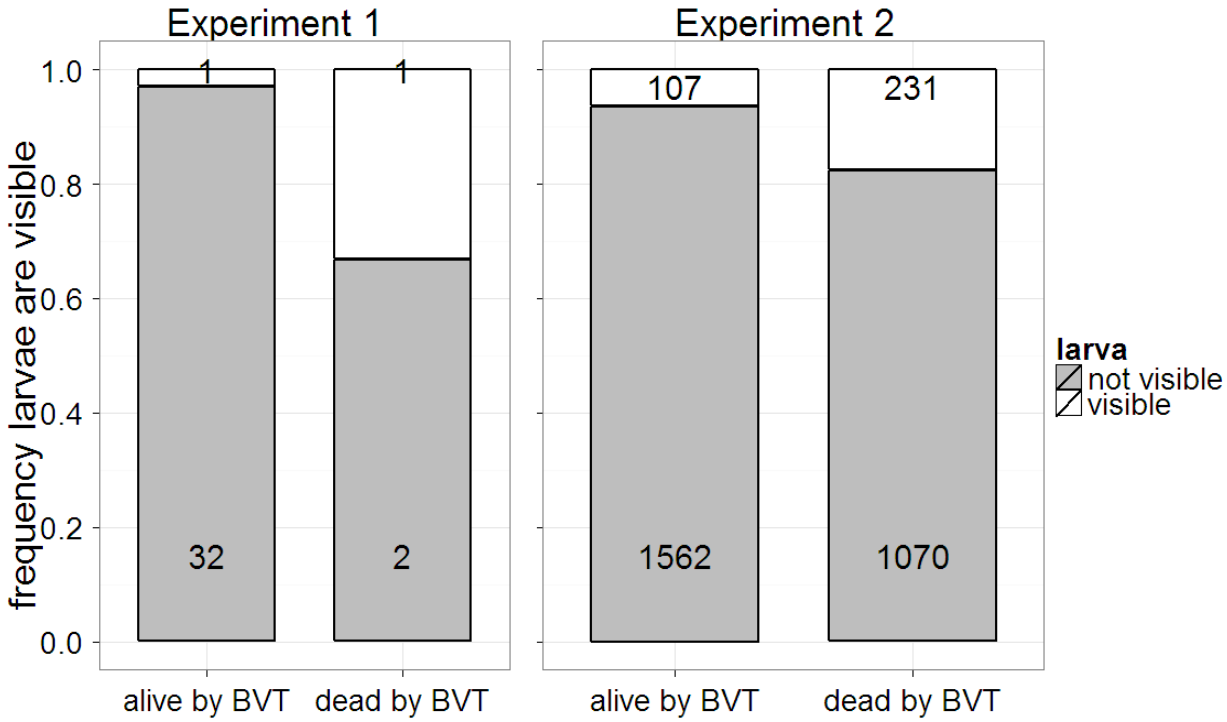
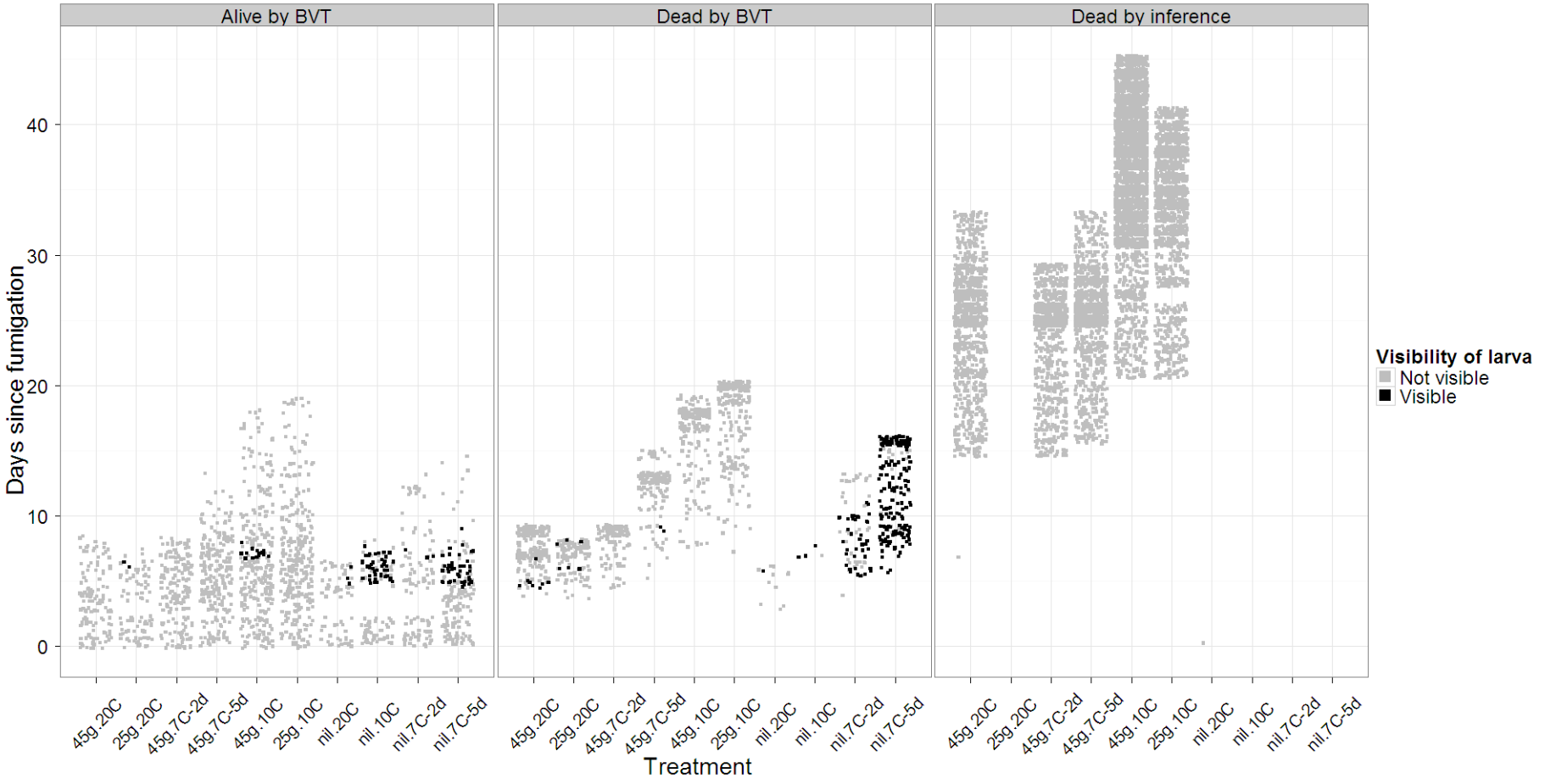


Figure 11.Visibility of larvae within eggs by viability, treatment and time since fumigation.



6. Discussion

First, data quality are considered, then the results are discussed in the context both of initial expectations and of the published literature. Finally, the significance of the results for MPI are evaluated and possible solutions are considered.

6.1. Data quality

The higher-than-specified fumigant concentrations in Experiment 2 initially caused concern, but it transpired that the two fumigation rates which were applied (25 $g.m^{-3}$ and 45 $g.m^{-3}$) both caused 100% mortality, with no difference in time to death. This indicated that application of the specified 15 $g.m^{-3}$ rate would either have given the same results as the 25 $g.m^{-3}$ and 45 $g.m^{-3}$ rates, or caused less mortality, possibly with a longer time to death. Application of the lower rate would not, therefore, have altered the main conclusion that significant time lags occur between treatment and death, and that organisms fumigated offshore with lethal doses may still be alive when they reach New Zealand's border. For this reason, it was agreed with MAF that conducting additional experiments with lower fumigation rates was not warranted.

In Experiment 2, while temperature data were not obtained from three of the six fumigation chambers, an analysis of variation showed that none of these chambers gave results significantly different to those in which temperatures had been recorded. It was therefore concluded that the temperature specifications of Experiment 2 had been met. The reason chamber 5 initially showed delayed mortality compared to chamber 6 (Figure 5) was that eggs from chamber 5 did not occur in the randomly chosen subsamples tested between days 8 and 16, so no chamber 5 mortality was recorded during this period, even though it almost certainly would have occurred, as it did in Chamber 6.

6.2. Initial expectations versus results

Based both on MAF's observations and our literature review, we expected that:

i) Following fumigation, there would be delays in egg mortality in the order of 1 – 14 d.
ii) Time to death following fumigation would decline as storage temperature increased.
iii) Time to death following fumigation would decline as fumigation rate increased.
iv) Egg mortality rates would increase with fumigation rate.

6.3. Delays in egg mortality

The results of Experiments 1 and 2 were consistent with our first expectation, except the observed delays between fumigation and mortality were longer than predicted, with lags ranging from 7.8 – 27.5 d. Nevertheless, this range corresponds with observations of *C. pomonella* embryos, some of which continued development for 15.5 d following methyl bromide fumigation before dying (Yokoyama et al. 1988), and it accords with a 97% decline in ATP in eggs of *A. cervinus* measured 9 d after methyl bromide fumigation (Forney 1991). Our measurements were also within the range exhibited by *H. lataniae*, some of which were still alive 31 d after fumigation (Witherell 1984). Our results confirm earlier suggestions that methyl bromide is a slow acting poison (Alexeef and Kilgore 1983, Cheetham 1990, Forney 1991, MacDonald 1993, Witherell 1984, Yokoyama et al. 1988), and indicate a clear need to revise the recommendation to leave insects overnight before assessing mortality from methyl bromide fumigation (Bond 1989).

6.4. Post fumigation storage temperature

In Experiments 1 and 2, time to death responded strongly to storage temperature, with the lags observed at 10°C storage being 1.5 to 2.5 times longer than those observed at 20°C. This

response was expected as it is broadly consistent with the supposition that temperatures which promote insect metabolic activity increase methyl bromide efficacy (Bond 1975, 1989). Our observation that post-fumigation storage temperature influences time to death is new, however, since it differs from previous observations that fumigation temperature influences overall mortality rates (Armstrong et al. 1988; Barak et al. 2006; Barak et al. 2009; Bond 1989; Mortimer and Powell 1988). Experiment 1b reported in Appendix 1 also demonstrated a strong effect of storage temperature on time to death.

Some Experiment 2 results strengthened published indications that a simple relationship between temperature and methyl bromide efficacy might occur only at moderate temperatures. For example, Armstrong & Whitehand (2005) found that increasing fumigation temperatures from 15°C to 25°C allowed shorter fumigation times to be used without compromising efficacy against two species of Tephritidae. Above 25°C, however, further reductions in fumigation time penalised efficacy. Similarly, the median lethal methyl bromide dose for *Sitophilus granarius* (L.) (Coleoptera: Curculionidae) increased as temperature declined from 30°C to 0°C, but that for *Tribolium confusum* Duv. (Coleoptera: Tenebrionidae) only increased as temperature declined to 10°C, then it decreased again between 10°C and 0°C, presumably due to the additional lethal effect of cold temperature (Shepard & Buzick 1939). In our Experiment 2, *M. domestica* mortality rates and time to death were broadly comparable between fumigated and control eggs stored at 7°C for 5 d. Leopold (2000) recorded rapidly declining hatch rates in *M. domestica* eggs after 5 d of storage at 5°C, so our results probably arose because the eggs could also only tolerate 7°C for relatively short periods. The key message from these observations is that the negative relationship we observed between post-fumigation storage temperature and time to death could break down at temperatures outside the moderate 7°C – 20°C temperature range explored in Experiments 1 and 2.

6.5. Fumigation rate and mortality rate

Contrary to our third and fourth expectations, neither time to death, nor egg mortality rates, showed any response to different fumigation rates in Experiment 2. The lowest methyl bromide rate tested of 25 $g.m^{-3}$ was sufficient to cause 100% *M. domestica* egg mortality with minimum delay, and increasing the rate to 45 $g.m^{-3}$ had no effect. The results obtained from the 40 $g.m^{-3}$ rate tested in Experiment 1 were comparable to those obtained from both of the Experiment 2 rates. We were unable to find any published data on the susceptibility of *M. domestica* eggs to methyl bromide, but variation in susceptibility both between species, and between life stages within species, is well recognised (Alexeef & Kilgore 1983, Armstrong & Whitehand 2005, Bond 1989, Williamson et al. 1986, Dentener et al. 1998, MacDonald 1993, Misumi et al. 2009, Mortimer & Powell 1984, Pike 1994). It would therefore be unsurprising if future dose-response studies showed *M. domestica* eggs to be relatively susceptible to methyl bromide.

Although our measurements of time to death of fumigated *M. domestica* eggs broadly accorded with the few available published observations for other species (Yokoyama et al. 1988, Forney 1991, Witherell 1984), previous observations of interspecific variation in susceptibility to methyl bromide (Alexeef & Kilgore 1983, Armstrong & Whitehand 2005, Bond 1989, Williamson et al. 1986, Dentener et al. 1998, MacDonald 1993, Misumi et al. 2009, Mortimer & Powell 1984, Pike 1994) indicate different species could exhibit considerable variation in post-fumigation time to death.

6.6. Egg morphology

Our study of the relationship between *M. domestica* egg morphology and viability strongly supported MAF's suspicions that morphology is a poor indicator of viability in fumigated

eggs. Delays between death and the onset of obvious signs of death in sessile insects have rarely been recorded in the literature, but Witherell (1984) compared turgidity and movement for scoring mortality of *H. lataniae* following methyl bromide fumigation and concluded that turgidity could underestimate mortality due to the extended period required for scale insects to become desiccated. We recorded considerable postmortem delays before the frequency of abnormalities in egg colour and egg turgidity increased, and many eggs failed to change colour before they eventually decomposed and collapsed.

The egg colour and turgidity data showed clear indications of an interaction between egg morphology and fumigation: Normal egg colour and turgidity appeared to be prolonged following fumigation, while abnormal colour and turgidity in control eggs became apparent more quickly. Methyl bromide has been shown to kill some fungi (Menge et al. 1978; Munnecke et al. 1959), bacteria and viruses (Harry et al. 1972; Schmittle 1955). Since some abnormalities in dead eggs are probably caused by microbial degradation, suppression of microbes by methyl bromide would explain the extended duration of normal egg morphology following fumigation. After fumigation, therefore, abnormalities arise only slowly as microbe populations become reestablished. It is possible our efforts to maintain the *M. domestica* eggs in relatively sterile environments to optimise their viability exacerbated this effect, but it is unlikely to fully account for it. For example, Petri dishes containing eggs were always placed in fumigation chambers without their lids, so eggs would have been fully exposed to airborne microbes while loading and unloading the chambers.

Data on the presence of visible larvae within the eggs showed a different pattern where larvae were rarely visible in eggs that had been fumigated, while they were only sometimes visible in control eggs. This is consistent both with fumigation impeding development of larvae, and with the appearance of larvae in control eggs being an ephemeral, seldom observed event due to the eggs' rapid development and hatching.

6.7. Implications of results and next steps

Our results relate to *M. domestica* eggs where the minimum observed time to death was 7.8 d when maintained at 20°C after fumigation. This raised the possibility that fumigated specimens from a wide range of species will still be alive upon arrival at New Zealand's border from Australia, even though the fumigations may have been adequate to provide required levels of security and the expected mortality will subsequently occur.

While storage at 7°C for 2 d, which was designed to equate to the regime applied to rock melons sent by air to New Zealand, did not significantly increase time to death of *M. domestica* eggs compared to storage at 20°C, storage either at 7°C for 5 d (which equated to the regime for rock melons sent by sea), or at constant 10°C, did significantly increase time to death. In these experiments on *M. domestica*, post-fumigation time to death at 7°C for 5 d and at constant 10°C was 2 – 3 weeks compared with 8 – 9 d for organisms at either 7°C for 2 d, or 20°C. Therefore cool storage, particularly for longer durations, may further increase the challenge of determining if any organisms that are found to be alive on inspection have been adequately treated.

The results indicate there is potential to improve on the current procedure based on morphological examination. One option would be to explore refinement of the current BVT to determine if it could accurately predict an outcome of death earlier in the dying process. We observed declines in BVT b/ g ratios as organisms approached death, but further work is required to determine if b/ g ratios declining towards the alive/ dead threshold provide conclusive evidence of an organism's impending death. There are several additional avenues for further developing the BVT for this purpose. Another possibility would be to develop a new enzyme-based test by building on published research about insect and plant metabolic

responses to methyl bromide (e.g. Price et al. 1981, Starratt and Bond 1981, Starratt and Bond 1990a, Starratt and Bond 1990b).

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9. Appendices

Appendix 1: Additional preliminary experiment, Experiment 1b

The following presents data from an additional preliminary evaluation of the effects of one methyl bromide fumigation rate and two post-fumigation storage temperatures on time to death. This work was conducted after Experiment 1 and before Experiment 2. High mortality of control insects occurred in this experiment, which influenced planning for production and management of eggs in Experiment 2. Irrespective of the high control mortalities, time to die was strongly influenced by storage temperature, which was consistent with results from Experiments 1 and 2, so the data are presented here.

Methods

The Experiment 1b methods used were identical to those of Experiment 1, except methyl bromide was applied at 15 $g.m^{-3}$ rather than 40 $g.m^{-3}$, so the Experiment 1b treatment abbreviations used are '15g.10C','15g.20C', 'nil.10C'and 'nil.20C'.

Results

Alignment with temperature and fumigant specifications

Table 10 summarises the fumigant concentrations and temperatures recorded during Experiment 1b. Temperatures were below those specified both during the 2 h fumigation and during the 20 minute return trip from the fumigation facility to AgResearch. The controlled temperature cabinets performed quite well and, despite relatively large temperature deviations during fumigation and transport, the overall deviation from the specified temperatures was 5.9%.

Table 10. Summary of fumigant concentrations and temperatures recorded during Experiment 1b.

	Experiment 1b
Transfer to fumigation facility	
Duration, h	0.3
Mean temperature ± SD, °C	16.3±2.8
During fumigation	1 chamber
Specified rate, $g.m^{-3}$	15
Measured rate, $g.m^{-3}$	-
Mean temperature ± SD, °C	12.4±0.4
Controls (not fumigated)	
[1]Mean temperature ± SD, °C	9.1±1.0
Transfer back to laboratory	
Duration, h	0.3
Mean temperature ± SD, °C	9.4±0.2
Post treatment storage, °C	10
Mean temperature ± SD, °C	11.3±0.4

[1]During the 2 h period the treated eggs were being fumigated.

Experiment 1b survival times

Experiment 1b involved fumigation at 15 $g.m^{-3}$ and 16°C, followed by storage at either 10°C or 20°C. Overall, 1494 eggs were scored for viability, either through biochemical tests, or by

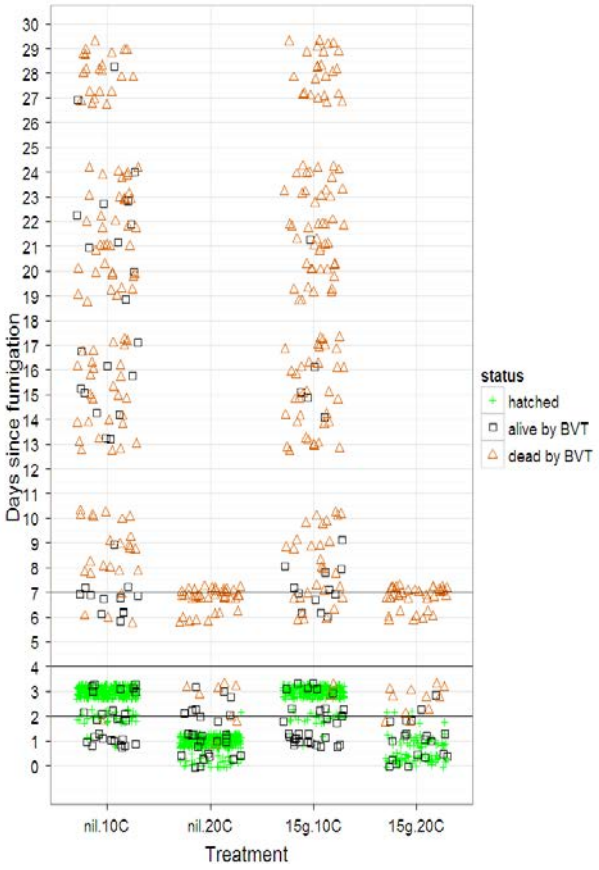
observing hatching. These comprised 496 eggs from the nil.10C control, 379 from the nil.20C control, 456 from the 15g.10C treatment, and 163 from the 15g.20C treatment.

Figure 12 shows the status of Experiment 1b eggs at different intervals after fumigation, with each point corresponding to a single egg. Eggs from all treatments hatched, and qualitative assessment of Figure 12 suggests there was minimal difference in mortality between fumigated and control eggs stored at the same temperature.

The Kaplan-Meier Estimates for survival times of fumigated and control eggs stored at 10°C and 20°C are shown in Figure 13. Survival data for fumigated and control eggs stored at the same temperature were nearly identical. The mean survival probability of fumigated and control eggs took 29 d to decline below 0.05 when stored at 10°C, and 7 d when stored at 20°C (Figure 13).

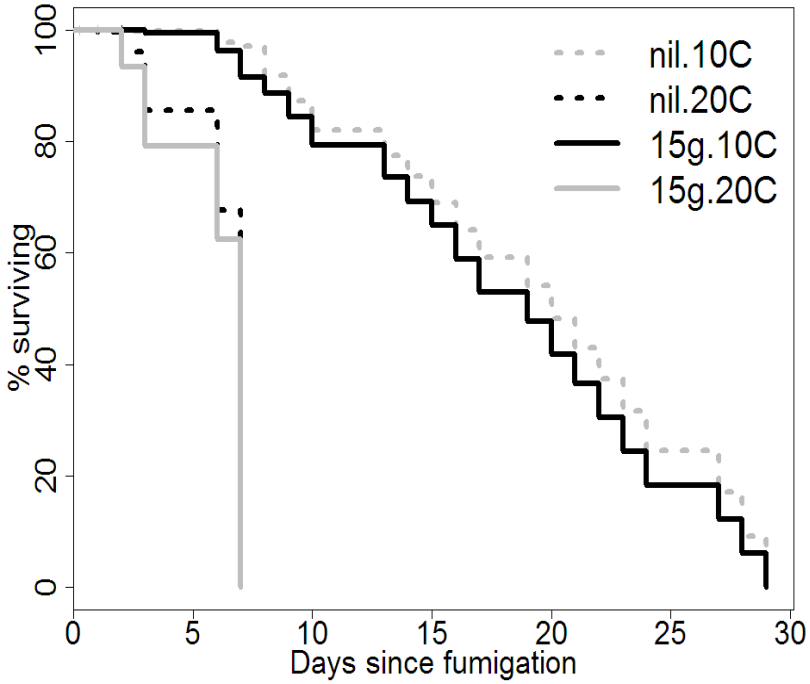
There was a significant difference in survival times between eggs stored at 10°C and 20°C (Log-rank test $p < 0.001$), but no difference between the fumigated and control groups (Log-rank test, $p = 0.562$ for 10°C, and $p = 0.466$ for 20°C).

Figure 12. Experiment 1b biochemical viability test results and hatching observations versus days since fumigation*, grouped by treatment.



*Horizontal lines at days 2, 4 and 7 show when MPI inspections of Australian air and sea cargo would usually occur.

Figure 13. Experiment 1b Kaplan-Meier survival curves.



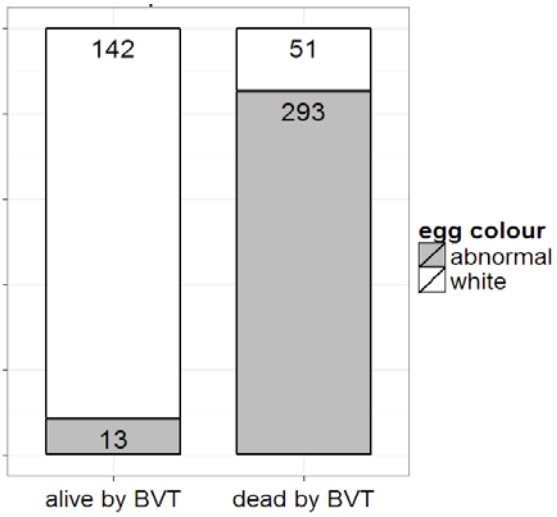
Morphological variables

Egg colour

Healthy eggs of *M. domestica* are white so, to simplify description and analysis, all egg colours other than white are here classified as ‘abnormal’. Eggs that exhibited obvious signs of fungal infection such as mycelia were also scored as ‘abnormal’.

Figure 14 shows the frequencies of white and abnormally coloured eggs in living and dead eggs in Experiment 1b, as determined biochemically. Greater proportions of white eggs were observed in the ‘alive by BVT’ group, and greater proportions of abnormally coloured eggs occurred in the ‘dead by BVT’ group. However, Figure 14 illustrates that the correlation between egg colour and viability was imperfect, and both colour classes occurred in the living and dead groups.

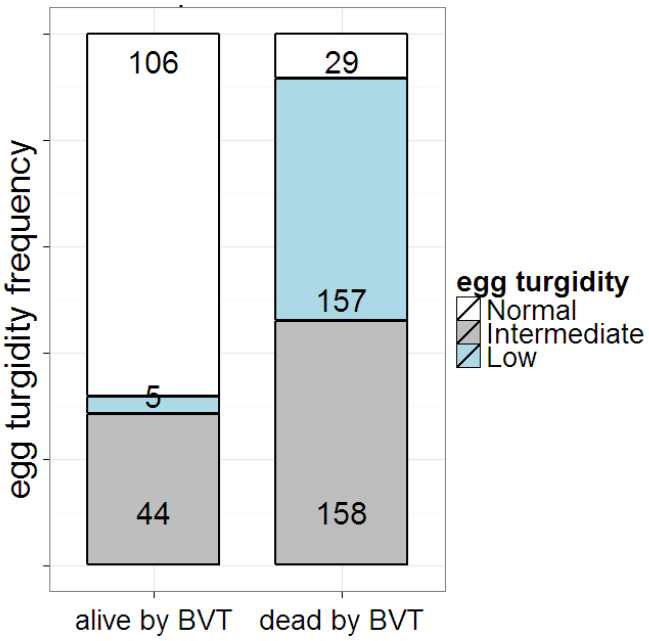
Figure 14. Frequency of egg colour by life status in Experiment 1b. Numbers within columns are counts of observations.



9.1.1. Egg turgidity

The numbers of eggs classed as having low, intermediate or normal turgidity were compared to BVT results from the same eggs (Figure 15). There was a trend for live eggs to have normal turgidity, and dead eggs to have intermediate or low turgidity.

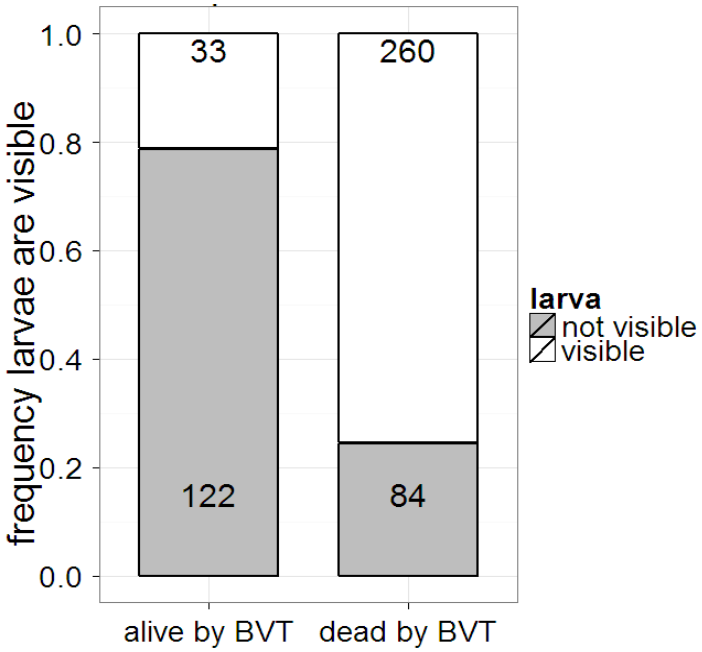
Figure 15. Frequency of different egg turgidity classes by life status in Experiment 1b. Numbers within columns are counts of observations.



Visibility of larvae within eggs

The numbers of eggs containing visible larvae were compared to BVT results from the same eggs (Figure 16).

Figure 16. Frequency that larvae were visible within eggs by life status in Experiment 1b. Numbers within columns are counts of observations.



Discussion

Experiment 1b did not meet specifications due to high mortality of control eggs, hatching of fumigated eggs, uncertainty about the fumigant concentration applied, and deviations from temperature specifications, particularly during fumigation. The Experiment 1b data were therefore of little value for defining the influence of fumigation rate on time to die, but they were helpful for planning further experimentation. Possible explanations for the observed results included the *M. domestica* eggs obtained from the commercial supplier had low viability and were too old (e.g. some could have hatched as they were entering the fumigation chamber), uncertain fumigant concentration, and the observed low fumigation temperature. Nevertheless, the Experiment 1b result that time to death was significantly longer in the 10°C treatments than in the 20°C treatments, though independent of fumigation, was broadly consistent both with the results of Experiments 1 and 2, and with our expectation that time to death following fumigation would decline as storage temperature increased.

Appendix 2: Morphological assessment protocol of MPI

Life State Determination Check List: Eggs

Note: If samples appear chilled (which may affect expression of the life state of specimens) allow sample to warm to room temperature before examination.

Egg sampling for slide mounting and examination

- Select eggs in the best condition
- If eggs are suspected Tephritidae, keep at least five eggs in good condition aside, in case it is necessary to send them for DNA testing, (refer BR-07).
- Slide mount a representative selection of good condition eggs from all submitted samples and examine using a compound microscope.

Criteria	***Live***	***Dead***
1. Movement: Slide mount egg/s in Hoyer's or waterand observe under a compound microscope to see whether there is any embryo movement.	☐ Yes	☐ No
2. Decomposition/Fungal Growth (NB: this character can only confirm death)	☐ No	☐ Yes

If uncertainty about life state remains then check the following characters

3. Colour (NB: eggs may retain good colour for several days after death	☐ Normal	☐ discoloured
4. Turgidity	☐ Turgid	☐ Flaccid
5. Embryo developed	☐ Yes	☐ No

6. Embryo condition ☐ Normal ☐ Discoloured\

Deteriorating

7. Larvae Present near eggs ☐ Yes ☐ No

If Yes determine the Life state of larvae.

If larvae is alive, this may assist egg viability assessment.

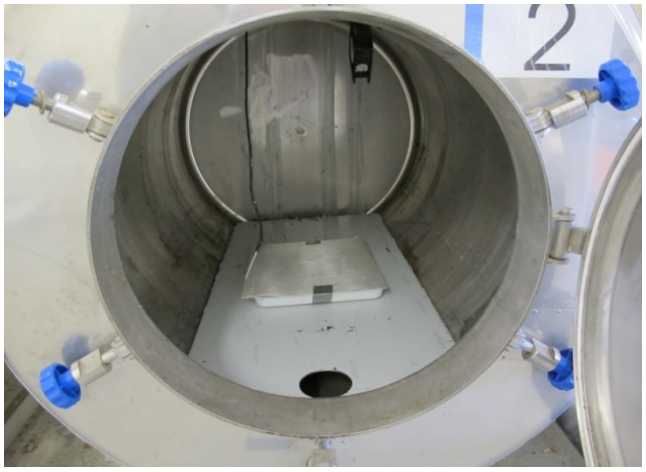
Terms to use for reporting the Life State

1. Live
2. Dead
3. Unsure cases (no movement observed but other criteria (#3-6) match as for live) record as “live”

Appendix 3: Photographs of fumigation chambers

Fumigation chambers located at Plant and Food, Palmerston North

The Experiment 2 fumigations were divided between six chambers.



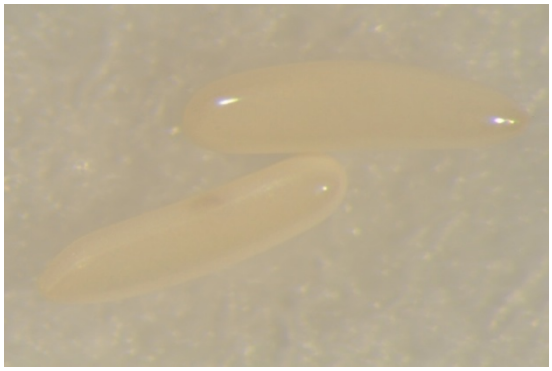
Set-up inside a fumigation chamber.

Lids were removed from the Petri dishes containing eggs before being placed on a plastic tray. Metal gauze was taped over the tray to prevent the dishes from shifting during fumigation.

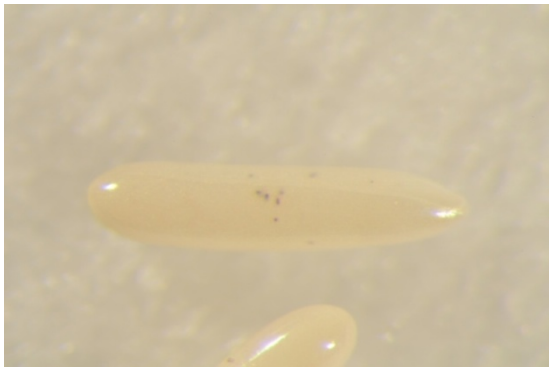
Appendix 4: egg morphology

Examples of house fly egg morphology observed during Experiments 1 and 2.

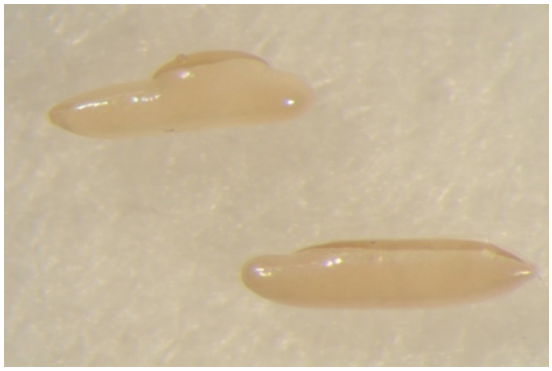
A normal house fly egg due showing typical white colour and normal turgidity. The larva is not visible within the egg.



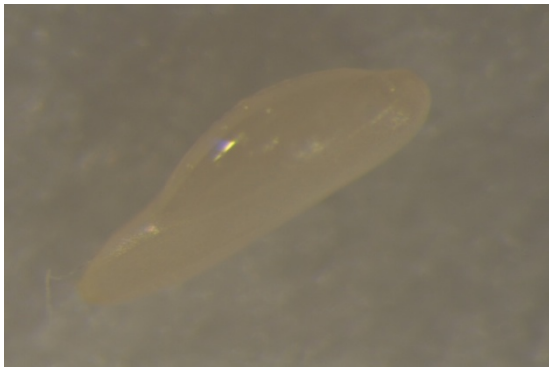
Early signs of abnormal morphology - upper egg is enlarged and the lower egg has a dark smudge on its top surface.



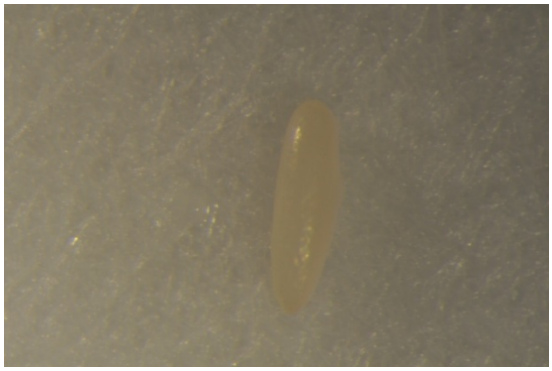
Black spots probably indicative of early microbial contamination on an otherwise normal-looking egg.



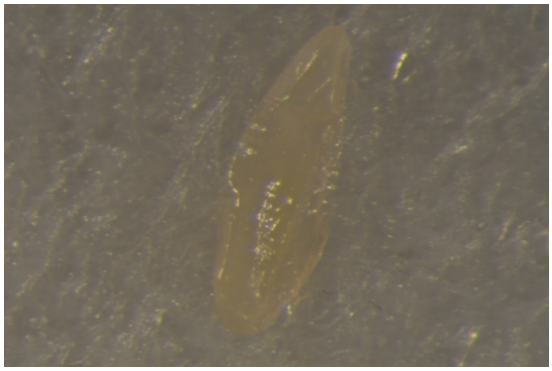
Abnormal eggs showing brown colour and split chorions.



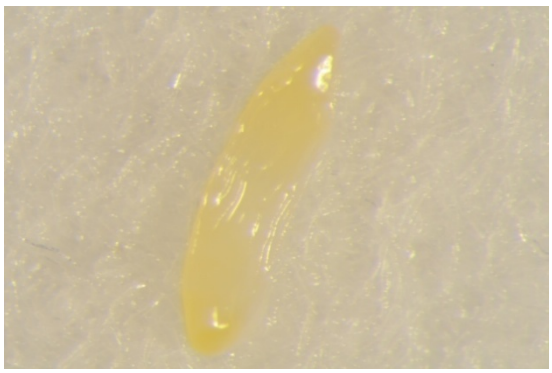
White egg with abnormal gas bubble.



Egg with abnormal yellowish colour and low turgidity.



A collapsed house fly egg showing normal white colour.



A collapsed house fly egg showing abnormal yellow colour.