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Israeli Acute Paralysis Virus Heat Inactivation Research

IAPV thermal inactivation in honey

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Contents

Page

Executive Summary **1**

1 Introduction **2**

2 Methods **4**
2.1 Preparation of inoculation material 4
2.2 Harvesting honey bee pupae 5
2.3 Heat inactivation of IAPV in honey 5
2.4 IAPV amplification bioassay 6
2.5 Analysis of results 7

3 Results **8**

4 Discussion **12**

5 Acknowledgements **12**

6 References **13**

Executive Summary

Israeli acute paralysis virus (IAPV) is currently not known to be present in New Zealand, and the Ministry of Primary Industries (MPI), New Zealand, are keen to take the necessary steps to ensure that this virus does not arrive and become established. The draft Import Health Standard (IHS) for the importation of bee products from Australia into New Zealand requires a range of heat treatments for the inactivation of *Melissococcus plutonius*, the causative agent of European Foulbrood (EFB). MPI deemed it necessary to conduct further research to identify whether these heat treatments would effectively inactivate IAPV in honey. The inactivation of IAPV in honey by heating can be tested by the injection of honey bee pupae with a solution that contains treated honey. If viable IAPV is present, it replicates in the pupae and can be detected at elevated levels by a molecular based method. If the IAPV is inactivated then no replication occurs, and it is detected within a similar range as the original inoculum only. Honey containing IAPV was thermally treated (with various treatment conditions), and following dilution with Ringers solution, injected into white eyed honey bee pupae. All samples treated at 65ºC for 6 hours or more showed the effectiveness of thermal inactivation of IAPV. The heat treatments tested provide evidence of thermal inactivation of IAPV. Further work may be required to demonstrate reliable deactivation of virus at larger scales.

Keywords: Israeli acute paralysis virus (IAPV); injection; honey; real-time PCR; quantification;

1 Introduction

The European honey bee (*Apis mellifera*) provides essential pollination services to field, horticultural and vegetable crops (Arbia & Babbay 2011), as well as providing important hive products like honey, wax, pollen and propolis. A recent review estimated that pollination services provided by managed honey bees contribute a crop production value of nearly $\$3{,}000\ ha^{-1}$ (Kleijn et al 2015). The health and vigour of honey bee colonies are threatened by numerous parasites and pathogens (Al-Abbadi et al. 2010) and honey bees have been reported to host at least 18 viruses, with individual bees often harbouring more than one species of virus (Maori et al. 2007). Diagnosis of honey bee viruses is difficult as they are often present as covert infections, causing no signs of disease. However, honey bee viruses can sometimes cause severe symptoms resulting in the collapse of entire colonies (Morse & Calderone 2000).

Israeli acute paralysis virus (IAPV) was first described infecting adult honey bees in 2004 in Israel (Maori et al. 2007), where individuals presented with shivering wings that progressed to paralysis and ultimately death (Cox-Foster et al. 2007). IAPV infects a wide variety of bees, and also wasps (Singh et al 2010). IAPV has since been detected in numerous countries around the world including Argentina, Australia, Brazil, China, France, Japan, Poland, Russia, South Korea, Spain, Italy and the USA (Pohorecka et al. 2011, Kojima et al. 2011, Teixeira et al. 2012, Reynaldi et al. 2011, Cox-Foster et al. 2007, de Miranda et al 2010, Formato et al. 2011). IAPV has been shown to cause persistent infection in some honey bee populations and infects all development stages and both sexes (Chen et al 2014). The virus has been detected in honey and pollen and the highest titre has been recorded in gut tissue, suggesting horizontal oral transmission may be important in viral epidemiology (Chen et al, 2014). Based on homology and genomic structure, IAPV is classified as a member of the Family Dicistroviridae (Christian et al. 2005). IAPV is closely related to both Kashmir bee virus (KBV) and acute bee paralysis virus (ABPV). Whilst being sufficiently different to be

discerned by PCR and serology (Maori et al. 2007), some regard these three closely related viruses as a species complex (de Miranda et al. 2010). Prior to the recent characterisation of this virus, IAPV may well have been previously classified as a variant of KBV, particularly in Australia where IAPV is prevalent (de Miranda et al. 2010). Most dicistroviruses persist at low titres, with no symptoms at the individual or colony level, however when injected into pupae or adults they are extremely virulent requiring less than 100 particles to cause death within a few days (*Ribière* et al. *2008)*. IAPV has been suggested as a risk indicator of Colony Collapse Disorder (CCD), however no cause and effect has been demonstrated (Cox-Foster et al. 2007) and the most recent work has, amongst other pathogens, implicated KBV rather than IAPV (Cornman et al. 2012).

A large proportion (80%) of adult honey bees orally infected with the virus die within a week (Maori et al. 2009), and *Varroa destructor* is thought to be an effective vector of the virus (Prisco et al. 2011). There is a suggestion that IAPV has been moved via international trade in packaged bees imported from Australia, royal jelly sourced from China, and pollen passed between pollinator species in North America (Cox-Foster et al. 2007, Singh et al. 2010). However, IAPV has subsequently been identified in honey bee samples in North America that predate documented importation of bees from Australia, casting some doubt on the link to viral movement via packaged bees (Chen & Evans 2007).

Following an independent review of the draft Import Health Standard (IHS) for the importation of specified bee products from Australia into New Zealand, MPI deemed it necessary to conduct research to address evidence gaps on the measures required to manage risks associated with the potential introduction of IAPV, which is not known to be present in New Zealand. Currently the draft IHS for the importation of honey bee products from Australia requires a range of heat treatments for the inactivation of *Melissococcus plutonius*,

the causative agent of European Foulbrood (EFB). Therefore, MPI wished to determine whether any such heat treatments would effectively deactivate IAPV in honey and limit the likelihood of viable virus entering New Zealand and becoming established.

IAPV replicates readily in pupae (Maori et al. 2007), therefore white eyed pupae were selected for the bioassay (Powell and Budge 2012). A bioassay was necessary to show amplification of virus within a biological system, rather than relying solely on a molecular test, in order to distinguish between viable or non-viable virus. A previous study at Fera demonstrated that IAPV can be inactivated following heat treatments in Ringers solution (Powell and Budge 2012), a buffer suitable for injecting into honey bee pupae and monitoring virus viability. A pilot study was then commissioned to demonstrate that honey could be diluted with Ringers solution and injected in honey bee pupae with no detrimental effect on pupal survival (Tomkies, Pietravalle and Budge 2014). The current study was commissioned by MPI to study the effectiveness of different heat treatments inactivating IAPV in honey.

2 Methods

2.1 PREPARATION OF INOCULATION MATERIAL

The initial IAPV homogenate contained 10 adult honey bees (*A. mellifera*) homogenised in 5 ml Ringers solution (125 mM NaCl, 1.5 mM $CaCl_2$, 5 mM KCl; pH 7.31) and mixed to homogeneity (kindly provided by Judy Chen at the USDA Beltsville laboratory in Maryland, USA). A fresh batch of infected pupal material was generated by injecting 80 white-eyed pupae each with 1 µl of homogenised IAPV solution. After 96 hours incubation at 33°C and 60 % R.H, infected pupae were homogenised in 5 ml of Ringers solution, filtered through two layers of linen, and frozen at -80^{o}C in 100 µl batches. Viability of the frozen virus was confirmed before experimental work started in 2015 by injecting the thawed homogenate into

honey bee pupae. Following incubation for 96 hours at 33ºC, 60% RH, viral amplification was demonstrated.

2.2 HARVESTING HONEY BEE PUPAE

Brood frames were removed from strong honey bee colonies headed by sister queens. For each of 4 large experiments, approximately 200 white-eyed pupae (10 days old) were gently removed from capped cells using fine-tipped forceps. In each experiment, all pupae were removed from the same side of the same brood frame. To ensure that the pupae had not been damaged during removal from the comb, all were left overnight in individual wells of a humidified 24 well plate, in an environmental chamber (33°C and 60 % R.H).
Bruised/damaged pupae were not included in the experiment.

2.3 HEAT INACTIVATION OF IAPV IN HONEY

IAPV negative honey (confirmed by RT-PCR) collected from Yorkshire in 2014 was pre-warmed at 50°C overnight in a laboratory oven. IAPV homogenate (100 µl) was defrosted at room temperature for approximately 15 minutes before mixing with 10 ml of pre-warmed honey by vortexing Aliquots (50 µl) of honey containing IAPV was added to individual 200 µl PCR tubes and exposed to specified temperature/time treatments in an Applied Biosystems 2720 thermocycler (Table 1). Results from each experiment were provided to MPI NZ who decided which treatments to include in subsequent replicate experiments.

Table 1: Duration and temperature of each treatment with corresponding number of honey aliquots for each of the four replicate experiments

	Treatment conditions		# pupae in each replicate experiment			
Treatment	Temp (ºC)	Time (hh:mm)	1	2	3	4
T2	60	10:00	20	20	-	-
T3	65	04:00	20	-	25	25
T4	70	01:48	20	20	-	-
T5	90	00:05	20	-	-	-
T6	65	06:00	-	20	25	25
T7	65	08:00	-	20	25	25
NC*	RT	00:00	20	20	25	25
P0**	RT	00:00	20	20	25	25
P96***	RT	00:00	20	20	25	25

*NC - No virus control: Honey amended with ringers containing no IAPV.
**P0 - Positive control: Honey containing IAPV injected into pupae and frozen immediately after injection.
***P96 - Positive control: Honey containing IAPV injected into pupae and frozen 96 hours after injection.

2.4 IAPV AMPLIFICATION BIOASSAY

Each aliquot of 50 µl spiked honey was diluted into 200 µl of pre-warmed Ringers solution, mixed thoroughly and 1 µl injected into the abdominal intersegment space between the 4th and 5th tergite of an individual white eyed pupa (Tomkies et al 2014). Injected pupae were placed in individual wells of a humidified 24 well plate, in an environmental chamber for 96 hours (33°C and 60 % R.H), with the exception of P0 pupae which were frozen at -80ºC immediately after injection. After 96 hours pupae were killed by transferring to -80ºC for a minimum of 36 hours.

RNA was extracted from each pupa using the Qiagen RNeasy Mini Kit following the manufacturer's instructions. Throughout the project, due to the large number of samples processed, three different batches of Qiagen RNeasy Mini Kits were used. In addition, extraction blanks, containing no pupae, were prepared in parallel to monitor for extraction contamination. No IAPV contamination was detected in these. Each RNA extraction was tested for the presence of IAPV and *A. mellifera* 18S ribosomal RNA (Powell & Budge, 2012). Real-time RT-PCR was conducted in 25 µl reactions comprising 1 µl RNA extract, 10X Buffer A, 5.5 mM of $MgCl_2$, 0.025U AmpliTaq Gold® (Applied Biosystems), 0.2 mM of each dNTP (Web Scientific) and 300 nM of each primer and 100 nM probe. (Martin et al 2012, Budge et al 2015).

Reactions were run on the ABI Viia7 Sequence Detection System using generic cycling conditions of 48°C for 30 min for reverse transcription, 95°C for 10 min as an initial polymerase activation step, followed by 40 amplification cycles of 95°C for 10 s and 60°C for 1 min. All samples were tested in duplicate, with each result being analysed separately.

Known positive RNA samples and plasmid controls were used on each run to validate both the reverse transcription and PCR stages of the assay.

2.5 ANALYSIS OF RESULTS

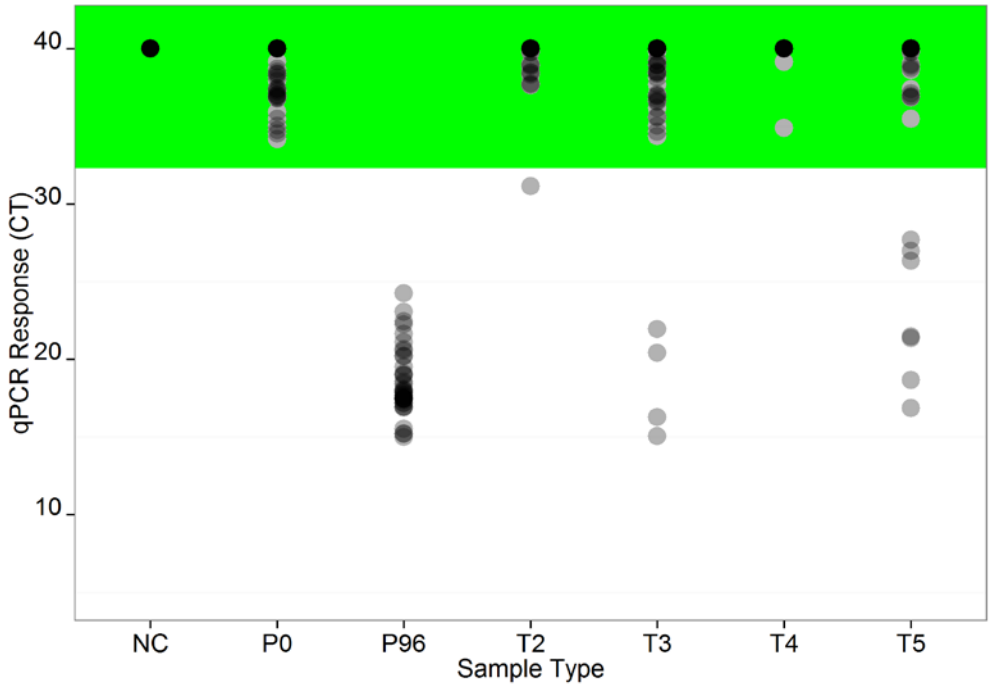
Deactivation of IAPV was treated as an event that could either happen or not happen each time that a treatment was applied. The aim of the experiment was to estimate the probability of IAPV deactivation occurring each time that a treatment was applied to a sample of honey that contained the virus. If the C_T response for IAPV produced by a treated sample was larger (larger means numerically lower) than an upper limit estimated from the observed responses of P0 samples (samples derived from pupae into which honey containing IAPV had been injected and then immediately frozen) then this was treated as evidence of IAPV replication and the treatment was deemed to have failed to deactivate the virus in that sample. Each sample was analysed twice. If either of the pair of IAPV analyses undertaken on each sample produced a response that was larger than the P0 threshold then this was treated as evidence of virus replication. The value of threshold was derived from the 99th percentile of a normal distribution fitted by maximum likelihood to each replicate result (two per sample) (Delignette-Muller 2015). The utility of the fit was checked by comparing the fitted cumulative distribution function to the empirical cumulative distribution, in particular checking that the fitted distribution at the largest response (lowest $C_{T;}$ the end of the distribution in which we are interested) was consistent with the empirical distribution. A threshold was derived for each of the four experiments the results of each treatment within each experiment were compared to the threshold for that experiment. The 99th percentile was chosen as an appropriate quantile for the threshold to reduce the expected rate of false positives for viral growth.

3 Results

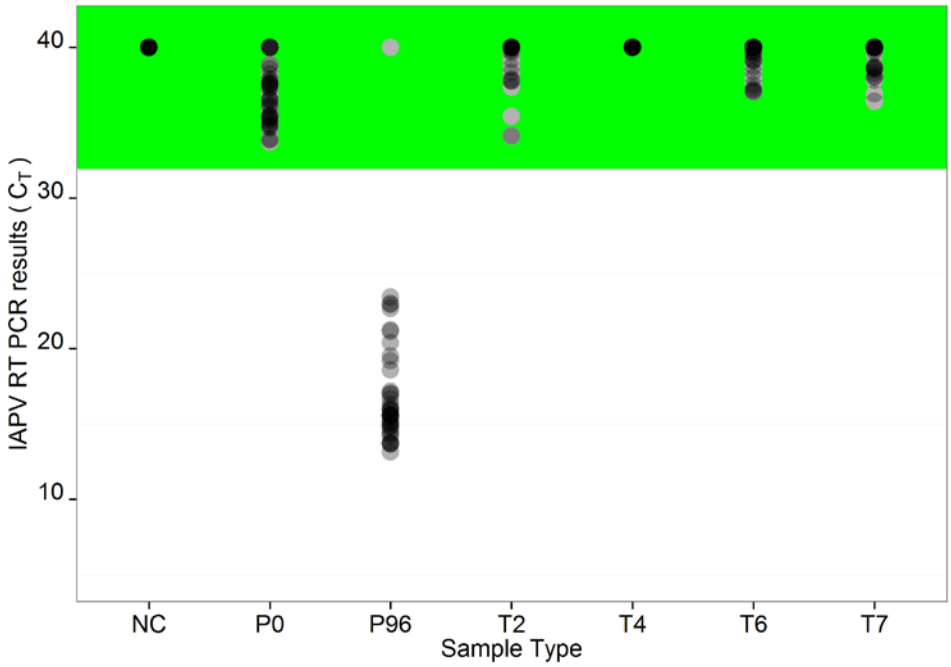
All injected pupae survived to 96 hours. All negative control samples (NC) returned a C_T of 40 for IAPV, indicating the experimental system was robust with respect to virus contamination and sample handling. The *A. mellifera* 18S controls were positive (C_T <40) for all but 14 out of 1160 tests and no RNA extraction consistently tested negative. In total, 179 out of 180 tests for the positive control group (P96) produced a C_T lower than the P0 cut-off, indicating IAPV amplification in every pupa. The probability of a sample giving duplicate C_{Ts} of 40 as a result of failed PCR, based on observed single test failures in 18S control (14/1160) and IAPV (1/180), was estimated to be approximately 1 in 8,000 i.e. (15/1340)^2. Assuming a similar rate applies to the detection of virus RNA across treated groups, then this effect is unlikely to lead to a failed treatment being misclassified as successful. Heat treatments showed a range of abilities to reduce the viability of IAPV in honey (Table 2). Each of the following figures demonstrate the individual IAPV RT PCR results (C_T) for all control and test samples for each experiment. The green area represents the interval within which results produced by inactivated virus samples are expected to lie.

The efficacy of the treatment was expressed as an inhibition of IAPV growth when compared to a control in which virus was present but growth has been inhibited by freezing immediately after injection (P0). The threshold C_T for expected results produced by samples that contain inactivated virus varied between experiments because, whilst each replicate experiment was undertaken using a fresh aliquot of the same IAPV preparation, batches of honey bee pupae and RNA extraction kits differed between replicate experiments. Hence, the C_T responses produced by 'P0' samples, which provided the baseline for treatment success, vary systematically between the four experiments and at random within each experiment. This required an experiment-specific threshold for determining efficacy as opposed to a single threshold across all experiments.

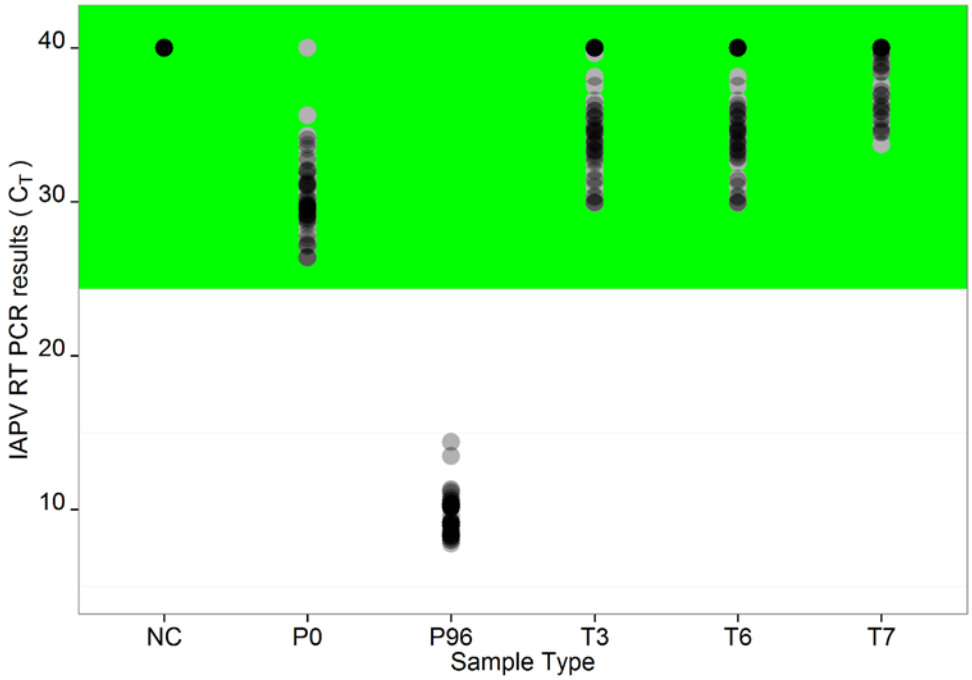
A: Experiment 1



B: Experiment 2



C: Experiment 3



D: Experiment 4

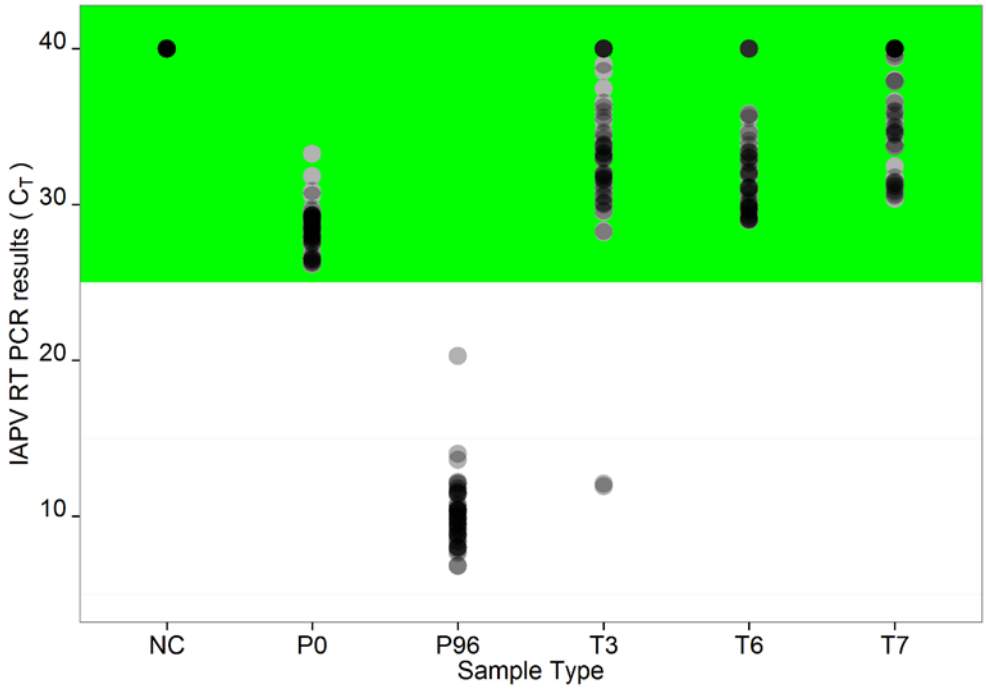


Figure 1 IAPV RT PCR results for control and treatment groups from each of the 4 experimental replicates (A-D).

*NC - No virus control: Honey amended with ringers containing no IAPV.

**P0 - Positive control: Honey containing IAPV injected into pupae and frozen immediately after injection.

***P96 - Positive control: Honey containing IAPV injected into pupae and frozen 96 hours after injection.

Table 2: Estimated percentage deactivation for each heat treatment after combining the number of tests over the four experiments for each treatment.

Treatment	Negative samples	Total samples	Deactivation (%) Estimate	5th and 95th percentile	
T2	39	40	97.5	90.6	99.6
T3	67	70	95.7	90.3	98.4
T4	40	40	100.0	92.8	100.0
T5	16	20	80.0	62.7	91.3
T6	70	70	100.0	95.8	100.0
T7	70	70	100.0	95.8	100.0

4 Discussion

This study was co-designed with MPI NZ. None of the 'no virus controls' (NC) tested positive using the IAPV real-time RT PCR assay, indicating no IAPV contamination above detectable limits. IAPV levels were noticeably higher for all 90 pupae in the P96 treatment group, giving IAPV C_T values which were above the threshold for pupae in the P0 treatment, indicating amplification of viable IAPV in all pupae. The probability of a sample giving duplicate low C_{Ts} after the injection of viable IAPV and 96 hr incubation was estimated to be no more than approximately 1 in 8 000.

The thermal treatments selected in this project showed a range of success at deactivating IAPV, with some treatments deactivating virus in all samples tested (Table 2): T4 (70ºC 1hr 48min), T6 (65ºC 6 hr) and T7 (65ºC 8 hr). Thermal treatments of 60ºC for 10 hr (T2), 70ºC for 1hr 48 min (T4) and 90ºC for 5 min (T5) were excluded from further replicated experiments due to evidence of IAPV replication, or because they were deemed impractical by MPI NZ for large-scale honey treatment. IAPV tended not to be deactivated when using shorter durations at 65ºC. However, a duration of six hours or greater increased IAPV deactivation (Table 2; Figure 1C and 1D). Whilst recent studies have suggested IAPV can be deactivated in pollen by gamma irradiation (Meeus at al., 2014), we present the first preliminary evidence for the effectiveness of thermal deactivation of IAPV in honey.

Further work may be required to demonstrate that similar deactivation demonstrated in this set of experiments occurs when similar treatments are, or have been, applied to real large scale batches of honey. This may be achieved by taking samples from batches of honey post-treatment, or on arrival and testing them using the approach applied during this study to detect virus growth.

5 Acknowledgements

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