Potential to exploit postmortem enzyme degradation for evaluating arthropod viability

Craig B. Phillips · Ilia I. Iline · Max Novoselov · Nicola K. Richards

Received: 23 October 2013 / Accepted: 28 March 2014 / Published online: 23 April 2014
© The Author(s) 2014. This article is published with open access at Springerlink.com

Abstract Inspecting for live organisms is the main method used to verify efficacy of phytosanitary treatments. Evaluating whether small, immobile organisms such as eggs, pupae and scale insects are alive or dead usually involves either checking morphological criteria or rearing them to observe development. These methods can be inaccurate, impractical and time consuming; thus, better methods are needed. To evaluate the potential for developing enzyme-based viability assays, we used electrophoretic gels to evaluate postmortem degradation of ten enzymes in *Musca domestica* L. (Diptera: Muscidae), four in *Bemisia flocculosa* Gill and Holder (Hemiptera: Aleyrodidae), and seven in *Listronotus bonariensis* (Kuschel) (Coleoptera: Curculionidae). Fresh insects displayed strong enzyme activity and distinct bands, but dead insects exhibited either no activity or weakened activity with reduced band resolution and increased migration of stained areas. Of ten enzymes investigated, seven showed clear indications of degradation just 1 day postmortem. Polyacrylamide gel electrophoresis of enzymes can be used to evaluate organism viability and has potential for estimating postmortem intervals. We also measured postmortem degradation rates of five *M. domestica* enzymes by assaying them in solution; these showed constant or gradually declining activity for 28 days postmortem, so live and dead specimens were less easily distinguished. By assaying enzymes in solution, it is possible to develop quick, easily

operated tests that can be used outside the laboratory for a variety of quarantine-related purposes.

Keywords Viability assessment · Phytosanitary inspection · Treatment efficacy · Postmortem interval · Biochemical assay

Introduction

Evaluating if organisms are alive or dead is the main method used to verify the efficacy of all phytosanitary treatments except irradiation (Hallman et al. 2010). However, it can be difficult to assess the viability of small arthropods, particularly those that do not move even when they are alive. Phytosanitary inspectors and pest managers often face this problem when dealing with sessile arthropods such as scale insects (Forney et al. 1991; Whiting et al. 1998; Witherell 1984) and immobile arthropod life stages such as eggs (Forney et al. 1991; Gadgil et al. 2000; Nagornaya 1981) and pupae (NZ Ministry of Agriculture and Forestry 2001; Whyte et al. 2006).

The viability of immobile arthropods is usually assessed by either maintaining them in a laboratory to observe development (Zettler et al. 2002), noting their response to physical stimulation (Yokoyama et al. 1992), or evaluating morphological characteristics such as turgidity (Barak et al. 2009; Witherell 1984) and color (Whiting et al. 1998). These methods can be impractical, time consuming, subjective or inaccurate so—based on the observation that many metabolites decline in concentration after death (Lockshin et al. 1977; Poloz and O'Day 2011)—researchers have developed alternative biochemical approaches.

Differences between live and dead insects have been found in concentrations of amylase (Nagornaya 1981)

All the authors belong to New Zealand Better Border Biosecurity Research Collaboration (<http://www.b3nz.org>).

C. B. Phillips (✉) · I. I. Iline · M. Novoselov · N. K. Richards
AgResearch, Lincoln, Private Bag 4749, Canterbury 8140,
New Zealand
e-mail: craig.phillips@agresearch.co.nz

and adenosine triphosphate (ATP) (Ebina et al. 2004; Ebina and Ohto 2007). We recently exploited this rapid postmortem degradation of ATP to develop a quick, easily used biochemical viability assay that works for a wide range of arthropods (Phillips et al. 2013). In the presence of ATP in a live arthropod, the assay solution changes color because of the reduction of tetrazolium to colored formazan, but no color change occurs if ATP is absent and the organism is dead (Phillips et al. 2013). After the assay, DNA can be extracted from the assay solution to make a taxonomic diagnosis (Richards et al. 2013).

Here, we describe postmortem degradation of a range of enzymes that could be useful for developing additional assays of either viability or other sample characteristics such as previous exposure to high temperature (Iline et al. 2013). We first evaluate postmortem enzyme degradation using polyacrylamide gel electrophoresis, then by assaying enzymes in solution. Our method for assaying enzymes in solution is particularly amenable to developing, quick easily used tests suited to use outside the laboratory for a range of applications (Iline et al. 2013; Novoselov et al. 2013; Phillips et al. 2013).

Materials and methods

Experiments were conducted with *Musca domestica* L. (Diptera: Muscidae), *Bemisia flocculosa* Gill and Holder (Hemiptera: Aleyrodidae), and *Listronotus bonariensis* (Kuschel) (Coleoptera: Curculionidae). *Musca domestica* was used as a proxy for Diptera of quarantine significance, including other muscids such as *Atherigona* spp. and other families such as Tephritidae; we cultured it using established methods (Sawicki 1964; Shipp and Osborn 1967). *Bemisia flocculosa* also provided a realistic study system because it was discovered on a New Zealand native *Melicactus* spp. (Violaceae) and elicited a response by the New Zealand Ministry of Agriculture and Forestry (Wilson 2004); we sampled adults from *Melicactus* spp. at Christchurch Botanic Gardens. *Listronotus bonariensis* adults were vacuumed from pasture at Lincoln, Canterbury, as described by McNeill et al. (2002).

Adults of all three species were used for enzyme electrophoresis, but only *M. domestica* pupae were used to study enzymes in solution. In electrophoresis experiments, it was critical to accurately recognize live specimens; thus, we used adult insects so motility would confirm viability. Afterwards, we aimed to ascertain whether enzyme degradation became apparent as quickly in solution as it did on gels. Here, perfect recognition of live specimens was less critical, so we used pupae to more realistically simulate the needs of quarantine authorities.

Polyacrylamide gel electrophoresis

Table 1 shows the enzymes assessed for each of the three species and also lists their abbreviations and enzyme commission numbers; abbreviations are hereafter used in the text. We chose these enzymes because they had shown good activity in previous electrophoretic studies of *M. domestica* (Krafsur et al. 1992), *Bemisia tabaci* (Tovar et al. 2008), and *L. bonariensis* (unpublished data).

Enzymes in *M. domestica* (Table 1) were studied after different postmortem intervals by freezing live adults at -80°C for at least 1 day, then storing them, uncovered, in an incubator at 20°C for 1, 2, 7, 14, 21, or 28 days. Additional *M. domestica* adults were kept at -80°C as a source of fresh specimens; these were used for electrophoresis immediately upon removal from the freezer and are hereafter referred to as ‘0-day-postmortem samples’.

Enzymes in *B. flocculosa* (Table 1) were studied after different postmortem intervals by freezing live adults at -80°C for at least 1 day, then storing them, uncovered, in an incubator at 20°C for either 20, 24, 46, or 76 h. Additional 0-day-postmortem samples were kept at -80°C .

Listronotus bonariensis adults were maintained in cages as described by McNeill et al. (2002). Specimens that died while caged were recognized when they adopted body positions uncharacteristic of living specimens and became unresponsive when their antennae or legs were touched with forceps. Enzymes (Table 1) were studied in dead

Table 1 Enzyme activity assessed in dead specimens of *Musca domestica* (Md), *Bemisia flocculosa* (Bf) and *Listronotus bonariensis* (Lb)

Enzyme	Abbreviation	E. C. no.	Md	Bf	Lb
Esterase	EST	3.1.1.-	s(3)	–	s(6)
Fumarate hydratase	FUM	4.2.1.2	s(1)		
Glycerol-3-phosphate dehydrogenase	G3PD	1.1.1.8	s(1)		
Glucose-6-phosphate isomerase	GPI	5.3.1.9	s(1)	s(1)	s(1)
Glucose-6-phosphate dehydrogenase	G6PD	1.1.1.49	s(1)		
Hexokinase	HK	2.7.1.1	s(4)		s(1)
Isocitrate dehydrogenase	IDH	1.1.1.42	–		n
Malate dehydrogenase	MDH	1.1.1.37	s(2)	s(1)	s(1)
Malic enzyme	ME	1.1.1.40	s(2)	n	n
Phosphoglucumutase	PGM	5.4.2.2	s(2)		s(1)

Enzyme activity from the dead specimens was scored as (n)o activity, (s)ome activity, or not evaluated (blank). Bracketed numbers show the number of isozymes observed. All evaluated enzymes showed clear activity when live specimens were tested, except the two marked ‘–’.

specimens 1–2 days postmortem and in live insects by killing them during the extraction process. Using specimens that had died while caged provided an initial indication of whether enzyme degradation patterns could be influenced by cause of death. *Listronotus bonariensis* MDH was also studied using a wider range of postmortem intervals by freezing live adults at -80°C for at least 1 day, then storing them, uncovered, for either 1 or 2 weeks at 5°C . Additional 0-day-postmortem samples were kept at -80°C .

Individual *M. domestica* were macerated in cold extraction buffer (0.1 M Tris–HCl, 0.1 % DTT, 0.1 % 2-mercaptoethanol, 12 % sucrose, pH 7.2) in a porcelain mortar chilled to 4°C , then the macerate was transferred to a 1.7-ml Eppendorf tube. Fifteen μl of extraction buffer was used per mg of fly tissue. The macerates were centrifuged (Eppendorf centrifuge 5810R) for 10 min at 10,000 rpm at 4°C , then stored at 4°C pending electrophoresis, which was always conducted within 2 h of initiating the protein extraction process.

Individual adults of *B. flocculosa* and *L. bonariensis* were macerated in 5–30 μl and 80–100 μl of cold extraction buffer, respectively (0.05 M Tris–HCl pH 7.2–7.4, 0.1 % DTT, 0.1 % 2-mercaptoethanol, 15 % sucrose, 0.01 % NADP, 0.01 % NAD; or 0.1 M tris–phosphate pH 7.5, 15 % sucrose, 0.1 % Triton X100) in 1.5-ml Eppendorf tubes, centrifuged at 14,000–16,000 rpm for 5 min at 4°C , and stored at 4°C pending electrophoresis.

The Ornstein-Davis discontinuous buffer system (Rothe 1994) was used for separating enzymes in 5–9 % vertical polyacrylamide gels in a Bio-Rad (California) Protean II xi cell. Supernatant volumes of 3–3.5 μl provided the best results for GPI and MDH, and 5–6 μl was better for other enzymes. After loading samples, gels were run at 80 V for 30–40 min, then for 1–2.5 h (depending on percent acrylamide) at 220–280 V at $4-6^{\circ}\text{C}$. Voltages greater than 300 V, or run times over 3 h, overheated the gels and distorted the bands.

Activity stains for all enzymes (Manchenko 2003; Murphy et al. 1996) except EST were modified by replacing NBT with MTT (0.1 mg/ml) and using PMS at 0.02 mg/ml. EST activity was visualized with α and β naphthyl acetate. MDH and ME were initially assayed separately to determine their electrophoretic mobility, then studied on the same gels using activity stain containing a mixture of NAD and NADP cofactors as well as MgCl_2 . Addition of G6PD to the separation gel improved activity staining for PGM, but not HK. Addition of NADP to the upper electrode buffer and the upper gel did not improve activity staining for G6PD, IDH, and ME.

Each gel was photographed several times as staining progressed, and the optimal gel staining duration for each

enzyme was identified when the color and resolution of isozyme bands from control specimens, included in each electrophoretic run, were maximized. Bands representing different isozymes and proteins were designated sequential numbers starting from the top of the gel.

For *M. domestica* and *L. bonariensis*, gels were digitally photographed (Canon Coolpix 4500) using a standardized procedure, and the pictures were opened with image analysis software (ImageJ version 1.29, <http://rsbweb.nih.gov/ij>) for processing. The background of each picture was subtracted (rolling ball radius of 300 pixels), gel dimensions were calibrated to mm, and isozyme band optical density, width (distance between the band's anodal and cathodal edges), and mobility (distance from the origin to the cathodal side of the band) were measured. Band width and mobility were used to gauge band resolution. Activity staining for *B. flocculosa* enzymes was too faint to obtain clear photographs, so band mobility was measured manually, and band intensity and resolution were recorded in writing.

Activity staining of enzymes in solution

Musca domestica that had pupated 1 day beforehand were frozen at -80°C to obtain samples with postmortem intervals of 1, 2, 7, 14, 21, and 28 days. Additional 0-day-postmortem samples were kept at -80°C .

EST, G3PD, G6PD, GPI, and MDH activity was visualized using published methods (Manchenko 2003) with the following modifications. For all enzymes except EST, Triton X-100 was added at a final concentration of 0.1 % to slow sedimentation of MTT formazan, and PMS was replaced with mPMS (5 $\mu\text{g}/\text{ml}$) to reduce light sensitivity. Five pupae from each postmortem interval were studied separately for each enzyme. For G3PD, five additional fresh *M. domestica* adults were studied using the same method as for *M. domestica* pupae because activity of this enzyme is known to vary between life stages (Colgan 1992).

Each insect was macerated in 50 μl distilled water per mg of insect in a 1.7-ml Eppendorf tube. Ten μl of supernatant was mixed with 10 μl of stain in a 0.2-ml tube, incubated at room temperature for 10 min, then digitally photographed using a standardized procedure (Phillips et al. 2013). The pictures were opened in ImageJ (version 1.29) for processing, and the optical density of the solution was recorded. To calibrate the measurements against optical densities obtained when no enzyme reaction occurred, *M. domestica* macerate was combined with stains without enzyme substrate, then processed as previously described.

Data presentation

Graphs were prepared using R (R Core Team 2012) and the R package ‘ggplot2’ (Wickham 2009).

Results

Musca domestica polyacrylamide gel electrophoresis

At 0 day postmortem, activity staining was evident for nine of ten *M. domestica* enzymes, and a total of 17 isozymes with differing mobilities were observed (Table 1). Figure 1

shows data for isozyme band optical density, width, and mobility for all enzymes except FUM and IDH. Figure 2 shows variation in banding patterns for *M. domestica* MDH2, GPI, G3PD, and G6PD over the range of post-mortem intervals (corresponding quantitative data for these enzymes are given in Fig. 1). With the exception of EST2, enzyme band optical densities either remained relatively constant or declined, with postmortem interval (Fig. 1). As the postmortem interval increased, isozymes that lost activity relatively quickly (e.g., EST1, G3PD, HK1-4, and PGM1-2; Figs. 1, 2) initially tended to show minor increases in band mobility and width, which then declined as optical density dropped below about 0.1–0.2, reaching

Fig. 1 Electrophoretic isozyme band optical density, width in mm, and mobility in mm, by postmortem interval in days for eight *Musca domestica* enzymes (EST, G3PD, G6PD, GPI, HK, MDH, ME, and PGM) and *Listronotus bonariensis* MDH ('Lb-MDH'). Five *M.*

domestica tested per enzyme and postmortem interval and 3–7 *L. bonariensis* tested per postmortem interval. Vertical bars show 95 % confidence interval

Fig. 2 Polyacrylamide electrophoretic gels stained for *Musca domestica* MDH2, GPI, G3PD, and G6PD, and *Listronotus bonariensis* MDH, following different postmortem intervals (days)

zero once bands were no longer visible (Fig. 1). Exceptions were G6PD bands, which showed rapid declines in optical density, but little change in width (Figs. 1, 2). G6PD bands also showed greater variation in stain intensity between samples within treatments than other enzymes, which sometimes made it difficult to distinguish between 0-, 1-, and 2-day-postmortem samples (e.g., Fig. 2; fourth sample from the left on day 0 c.f. fifth sample from the left on day 1 c.f. second sample from the left on day 2).

Isozymes that maintained postmortem activity throughout the 28 days showed either gradually increasing band widths and mobilities (e.g., EST3, GPI and MDH1-2; Figs. 1, 2) or fairly constant values (e.g., ME; Fig. 1). EST2 was unusual because activity was not apparent in control specimens, but first appeared 1 day after death, then became relatively strong for the duration of the experiment (Fig. 1).

All *M. domestica* isozymes were evident in all specimens except HK1, which was only observed in two of the five specimens tested on each of days 0, 1, and 2. Due to its low frequency in the samples, any absences on days 7–28 were recorded as missing values, rather than as zeros, for band density, width, and mobility (Fig. 1). FUM was studied in flies with postmortem intervals of 0 and 2 days, and no differences in banding patterns were observed. FUM activity was relatively strong, with optical densities in the range 0.3–0.35 for the ten specimens examined (c.f. the other enzymes shown in Fig. 1).

Figure 2 shows how *M. domestica* enzyme degradation was apparent 1 day after death because of increased band width and mobility of MDH2, GPI, and G3PD, and also because of reduced activity staining for MDH2, G3PD and G6PD. These changes are also apparent in the measurements shown in Fig. 1, though the increases in band width and mobility through the 0–28-day range for MDH2 and GPI are less obvious in Fig. 1 than in Fig. 2.

Bemisia flocculosa polyacrylamide gel electrophoresis

In 0-day-postmortem samples, GPI, MDH, and ME exhibited activity, but EST did not (Table 1). In dead

Table 2 Description of *Bemisia flocculosa* GPI isozyme bands in individual insects after different postmortem intervals

Isozyme	Hours since death	Band description	Distance from cathode (mm)	No. of specimens
<i>Bemisia flocculosa</i> GPI	0	1–3 sharp bands, very high activity	8, 10 and 12 ^a	158
	20	Bands still separated, but less well resolved	16–18 ^b	8
	24	Bands no longer separated, poor resolution	19.5–20.5 ^b	8
	46	Bands no longer separated, poor resolution	21.5 ^b	8
	76	Bands no longer separated, poor resolution	23 ^b	4

^a Due to the dimeric structure of GPI, one band was observed at either 8 or 12 mm in homozygotes (AA or BB), while three bands were observed in heterozygotes (AB)

^b Maximum mobility of merged GPI bands

specimens, GPI and MDH exhibited traces of activity, but EST and ME did not (Table 1).

The intensity, pattern, and resolution of GPI bands obtained from individual *B. flocculosa* that had been dead for varying periods are summarized in Table 2. GPI activity was observed in specimens that had been dead for ≤76 h. Variation in enzyme banding with postmortem interval was characterized by declining band resolution, declining activity, and increasing mobility of stained areas towards the anode (Table 2).

Listronotus bonariensis polyacrylamide gel electrophoresis

For all seven enzymes, clear bands were observed both in 0-day-postmortem samples and in specimens that had been

Fig. 3 Optical densities of five *Musca domestica* enzymes (in solution) by postmortem interval, with five insects tested per sample time and enzyme. Vertical bars show 95 % confidence interval

killed during the extraction process (Table 1). However, weevils that had died in their cages 1–2 days earlier showed no activity at IDH and ME, and only exhibited traces of activity without clear bands at EST, GPI, HK, MDH and PGM (Table 1).

The optical density, width, and mobility of MDH bands obtained from individual *L. bonariensis* adults that had been dead for 0, 7, and 14 days are given in Fig. 1 ('Lb_MDH'), and photographs are shown in Fig. 2. MDH activity was observed in specimens that had been dead for ≤ 336 h (14 days). Variation in enzyme banding with postmortem interval was characterized by declining stain intensity, and increasing band width and mobility (Figs. 1, 2).

Activity staining of *Musca domestica* enzymes in solution

When enzyme activity stains without enzyme substrates were combined with *M. domestica* macerates, the resulting solutions, in which no enzyme reaction could occur all had optical densities of approximately 0.1. Before analysis, therefore, the contribution of the macerated insect to optical density, rather than the contribution of enzyme activity staining, was eliminated by subtracting 0.1 from each optical density measurement.

At 0-day postmortem, activity staining was strong for GPI, moderate for EST, weak for MDH and G6PD, and negligible for G3PD (Fig. 3). However, strong G3PD activity occurred at 0 day postmortem when adults were

used rather than pupae (Fig. 3). The activity of most enzymes remained relatively constant throughout the 0–28-day range of postmortem intervals, but EST and G6PD activity declined with postmortem interval (Fig. 3). The relatively gradual decline in EST activity mostly occurred between days 2 and 21, though EST activity was still clearly evident after 28 days. In contrast, G6PD activity declined rapidly between days 0 and 2, but a slower subsequent rate of decline meant faint residual activity was observed until day 21 (Fig. 3).

Discussion

When studied by gel electrophoresis, ten *M. domestica* isozymes provided reliable indications of viability because they either quickly lost activity after death (e.g., EST1, G3PD, HK1–4 and PGM1), or exhibited changes in band width and mobility (e.g., GPI, MDH1–2). Using any one of these isozymes, it was straightforward to distinguish between 0-day-postmortem and 1-day-postmortem *M. domestica*. Of these, EST1, G3PD, GPI, and MDH2 were particularly useful because they exhibited high activity at 0-day postmortem, which suggests they will provide good sensitivity when testing small insects, along with rapid postmortem changes in activity and/or band resolution. GPI and MDH also showed good potential with both *B. flocculosa* and *L. bonariensis*, as did EST with *L. bonariensis*.

Musca domestica isozymes that exhibited persistent postmortem activity, combined with relatively constant postmortem isozyme band widths and mobilities, such as ME and EST2, did not provide clear indications of insect viability. However, ME clearly indicated the viability of *B. flocculosa* and *L. bonariensis*, thus showing the suitability of particular enzymes for inferring viability can vary between species. G6PD had intermediate potential for evaluating viability, with rapid postmortem declines in activity being partly obscured by relatively high within-treatment variation and only minor postmortem changes in band mobility.

Enzymes in *L. bonariensis* that died while caged showed degradation patterns consistent with those observed in *M. domestica* and *B. flocculosa* that were killed by freezing. This suggests postmortem enzyme degradation—indicated on gels by declining activity, reduced band resolution, and increased band mobility—should occur irrespective of the cause of death. However, enzyme degradation rates can vary with cause of death. For example, some enzymes are quickly inactivated by high temperature (Iline et al. 2013).

In addition to the electrophoresis experiments described here, we have made ad hoc comparisons of enzyme banding patterns between live and dead specimens of various species of Tetranychidae (Acari), Desidae, and

Theridiidae (Araneae), Tenebrionidae (Coleoptera), Drosophilidae and Simuliidae (Diptera), Cicadellidae and Diaspididae (Hemiptera), Braconidae (Hymenoptera), and Gelechiidae (Lepidoptera). Specimens had either died while caged, or were killed by freezing at -80°C or heating at 90°C , and postmortem storage (range 1–23 days) was at 5 or 20°C . The main enzymes tested were EST, GPI, MDH and ME, and clear differences between live and dead specimens of the same species were observed in all cases ($n > 300$ specimens). GPI and MDH always showed at least some activity in dead specimens, EST usually did, while ME often showed no activity. Tests of an additional eight enzymes, not reported here, have also shown clear activity in fresh specimens and weak or no activity in dead specimens.

The results from activity staining of *M. domestica* GPI, G6PD and MDH in solution were generally consistent with those obtained from electrophoresis, with GPI and MDH showing persistent postmortem activity and EST and G6PD showing postmortem declines in activity. An exception occurred with G3PD, which showed high activity in adults at 0 days postmortem, but not in pupae at 0 days postmortem; possible reasons are discussed below. Although *M. domestica* EST and G6PD showed postmortem activity declines, residual activity remained for at least 21 days postmortem, so inferring viability before this time would require quantitative analysis of the staining reaction rather than a qualitative interpretation of the presence or absence of a reaction.

Variation in G3PD activity between control adults and control pupae in solution was almost certainly due to differences in wing muscle development and flight capability between adult and pupal stages of *M. domestica*. G3PD is critical to glucose combustion during insect flight, and its activity is known to vary widely with flight capability both within and between species (Colgan 1992), so this enzyme could be unsuitable for broad application in insect viability assessment, but might be useful for other test purposes.

We expect many enzymes except G3PD to behave similarly in all life stages of a species. For example, in preliminary electrophoresis experiments, we obtained the same isozyme banding patterns for GPI and MDH from *M. domestica* pupae and adults. Nevertheless, the result from G3PD indicates research to develop an enzyme-based viability test for a particular set of species should focus on the life stages that are most problematic for quarantine authorities. Our use of *M. domestica* adults for gel electrophoresis and pupae for activity staining of enzymes in solution was sufficient to demonstrate the concept of enzyme-based viability testing, but not to develop a test that is ready for routine use.

The increasing activity of *M. domestica* EST2 on electrophoretic gels for 2 days after death was atypical among

the isozymes we evaluated, though postmortem increases in the activities of various enzymes have previously been recorded in samples from humans (Gupta et al. 1983) and noble scallops (Wongso and Yamanaka 1998). It was probably associated with changes in tissue pH during autolysis (Marquez-Rios et al. 2007; Wongso and Yamanaka 1998) and release of enzymes from necrotic tissue (Gos and Raszeja 1993). If used alone, EST2 could therefore lead to erroneous conclusions about *M. domestica* viability.

The unusual results for G3PD and EST2 indicate that careful validation would be needed to develop reliable enzyme electrophoretic viability assessment methods for particular taxa and suggest that conclusions should ideally be based on two or more enzymes.

Postmortem changes in DNA band resolution on electrophoretic gels have been investigated for estimating postmortem intervals in forensic investigations (Alaeddini et al. 2010), and our results indicate that postmortem changes in enzyme band resolution could also be helpful. To date, forensic research on enzymes has focused on postmortem changes in activity and concentration (Gos and Raszeja 1993; Gupta et al. 1983; Poloz and O'Day 2011) rather than on band resolution. We suggest the best enzymes for estimating postmortem intervals would be those that give strong activity and sharp bands with minimal intraspecific isozyme and allozyme variation.

Our results show that gel electrophoresis has excellent potential for assessing viability. GPI and MDH performed well for the purpose of assessing viability in all of the species tested in both experiments and ad hoc tests, and they are our first recommendations for future methodological development. The next main hurdle will either be to develop ways of reducing persistent postmortem activity of enzymes in solution or to streamline and simplify the electrophoretic procedure so it is more suitable for adoption by pest managers. Our method for producing histochemical reactions in microtubes from crude macerates of small samples holds promise for developing a variety of quick, easily used assays for use outside of the laboratory (e.g., Iline et al. 2013; Novoselov et al. 2013; Phillips et al. 2013).

Acknowledgments We thank P. Holder (Lincoln University, New Zealand) for assistance with a preliminary experiment using whiteflies and M. O'Callaghan (AgResearch, Lincoln, New Zealand) and two anonymous referees for reviewing earlier versions of our manuscript. The research was funded by AgResearch through the Better Border Biosecurity (B3) research collaboration (<http://www.b3nz.org>).

Open Access This article is distributed under the terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided the original author(s) and the source are credited.

References

- Alaeddini R, Walsh SJ, Abbas A (2010) Forensic implications of genetic analyses from degraded DNA—a review. *Forensic Sci Int Gen* 4:148–157
- Barak AV, Yang W, Yu D, Jiao Y, Kang L, Chen Z, Ling X, Zhan G (2009) Methyl bromide as a quarantine treatment for *Chlorophorus annularis* (Coleoptera: Cerambycidae) in raw bamboo poles. *J Econ Entomol* 102:913–920
- Colgan DJ (1992) Glycerol-3-phosphate dehydrogenase isozyme variation in insects. *Biol J Linn Soc* 47:37–47
- Ebina T, Ohto K (2007) ATP assay for determining the viability of the two-spotted spider mite (*Tetranychus urticae* Koch) and the European red mite (*Panonychus ulmi* (Koch)) (Acari: Tetranychidae) during diapause. *Appl Entomol Zool* 42:291–296
- Ebina T, Kaneda M, Ohmura K (2004) ATP assay for determining egg viability of the Fuller rose beetle, *Pantomorus cervinus* (Boheman) (Coleoptera: Curculionidae). *Res B Plant Prot Serv* 40:69–73
- Forney CF, Aung LH, Brandl DG, Soderstrom EL, Moss JI (1991) Reduction of adenosine triphosphate in eggs of Fuller Rose Beetle (Coleoptera: Curculionidae) induced by lethal temperature and methyl bromide. *J Econ Entomol* 84:198–201
- Gadgil PD, Bulman LS, Crabtree R, Watson RN, O'Neil JC, Glassey KL (2000) Significance to New Zealand forestry of contaminants on the external surfaces of shipping containers. *New Zeal J For Sci* 30:341–358
- Gos T, Raszeja S (1993) Postmortem activity of lactate and malate dehydrogenase in human liver in relation to time after death. *Int J Legal Med* 106:25–29
- Gupta H, Dixit PC, Chandra J (1983) Relationship of phosphoglucose mutase in postmortem blood with time since and cause of death. *Indian J Med Res* 77:154–162
- Hallman GJ, Levang-Brilz NM, Zettler JL, Winborne IC (2010) Factors affecting ionizing radiation phytosanitary treatments, and implications for research and generic treatments. *J Econ Entomol* 103:1950–1963
- Iline II, Novoselov M, Phillips CB (2013) Proof of concept for a biochemical test that differentiates between heat-treated and non-heat-treated food products. *New Zeal Plant Protect* 66:34–39
- Krafsur ES, Helm JM, Black WC IV (1992) Genetic diversity at electrophoretic loci in the house fly, *Musca domestica* L. *Biochem Gen* 30:317–328
- Lockshin RA, Schlichtig R, Beaulaton J (1977) Loss of enzymes in dying cells. *J Insect Physiol* 23:1117–1120
- Manchenko GP (2003) Handbook of detection of enzymes on electrophoretic gels, 2nd edn. CRC Press, Florida
- Marquez-Rios E, Moran-Palacio EF, Lugo-Sanchez ME, Ocano-Higuera VM, Pacheco-Aguilar R (2007) Postmortem biochemical behavior of giant squid (*Dosidicus gigas*) Mantle Muscle stored in ice and its relation with quality parameters. *J Food Sci* 72:356–362
- McNeill MR, Goldson SL, Proffitt JR, Phillips CB, Addison PJ (2002) A description of the commercial rearing and distribution of *Microctonus hyperodae* (Hymenoptera: Braconidae) for biological control of *Listronotus bonariensis* (Kuschel) (Coleoptera: Curculionidae). *Biol Cont* 24:167–175
- Murphy RW, Sites JW, Buth DG, Haufler CH (1996) Proteins: isozyme electrophoresis. In: Hillis DM, Moritz C, Mable BK (eds) *Molecular systematics*, 2nd edn. Sinauer Associates, Massachusetts, pp 51–120
- Nagornaya IM (1981) A rapid method for determining the mortality of comstock mealybug eggs *Pseudococcus-Comstocki* after fumigation. *Vestn Zool* 3:78–82
- Novoselov MA, Iline II, Phillips CB (2013) How fresh is that frass? *New Zeal Plant Protect* 66:376
- NZ Ministry of Agriculture and Forestry (2001) Standard BMG-STD-PESTI: Requirements for the diagnosis and reporting of organisms intercepted at the border, or within transitional facilities. <http://www.biosecurity.govt.nz/files/regs/stds/bmg-std-pesti.pdf>
- Phillips CB, Iline II, Richards NK, Novoselov M, McNeill MR (2013) Development and validation of a quick, easily used biochemical assay for evaluating the viability of small immobile arthropods. *J Econ Entomol* 106(5):2006–2019. doi:10.1603/EC13028
- Poloz YO, O'Day DH (2011) The use of protein markers for the estimation of postmortem interval. *Forensic Pathol Rev* 6:277–294
- R Core Team (2012) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0. <http://www.R-project.org/>
- Richards NK, Winder L, Iline II, Novoselov M, McNeill MR, Phillips CB (2013) A biochemical viability assay is compatible with molecular methods for species identification. *New Zeal Plant Protect* 66:29–33
- Rothe GM (1994) *Electrophoresis of enzymes: Laboratory methods*. Springer, Berlin
- Sawicki RM (1964) Some general considerations on housefly rearing techniques. *B World Health Organ* 31:535–537
- Shipp E, Osborn AW (1967) The effect of protein sources and of the frequency of egg collection on egg production by the housefly (*Musca domestica* L.). *B World Health Organ* 37:331–335
- Tovar L, Díaz A, Arnal E, Ramis C (2008) Isoenzymatic diversity of *Bemisia tabaci* Gennadius 1889 (Hemiptera: Aleyrodidae) on sesame plantations (*Sesamum indicum* L) in Venezuela. *Entomotropica* 20:249–263
- Whiting DC, Hoy LE, Connolly PG, McDonald RM (1998) Effects of high-pressure water jets on armoured scale insects and other contaminants of harvested kiwifruit. *New Zeal Plant Protect* 51:211–215
- Whyte C, Knight G, Mavengere T, Eccersall S, Wedde S, Tohovaka S, Chimside J, Allison V, Song V, McLaggan J (2006) Monitoring research and pathway review: sea containers. MAF Biosecurity New Zealand, Wellington, p. 82
- Wickham H (2009) *ggplot2: elegant graphics for data analysis*. Springer, New York
- Wilson J (2004) Report on the detection of an undescribed whitefly (*Bemisia* sp.) (Hemiptera: Aleyrodidae) on *Melicytus* sp. (Violales: Violaceae) at the Christchurch Botanical Gardens. New Zealand Ministry of Agriculture and Forestry, Wellington, 15 p
- Witherell PC (1984) Methyl bromide fumigation as a quarantine treatment for latania scale, *Hemiberlesis lataniae* (Homoptera: Diaspididae). *Fla Entomol* 67:254–262
- Wongso S, Yamanaka H (1998) Postmortem changes in contents of glycolytic intermediates in the adductor muscle of noble scallop during storage. *Fisheries Sci* 64:125–130
- Yokoyama VY, Miller GT, Hartsell PL (1992) Pest-free period and methyl bromide fumigation for control of walnut husk fly Diptera Tephritidae in stone fruits exported to New Zealand. *J Econ Entomol* 85:150–156
- Zettler JL, Follett PA, Gill RF (2002) Susceptibility of *Maconellia coccis hirsutus* (Homoptera: Pseudococcidae) to methyl bromide. *J Econ Entomol* 95:1169–1173