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Resistance to phosphine in *Sitophilus oryzae* in Australia: A national analysis of trends and frequencies over time and geographical spread

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ABSTRACT

In Australia, along with many other parts of the world, fumigation with phosphine is a vital component in controlling stored grain insect pests. However, resistance is a factor that may limit the continued efficacy of this fumigant. While strong resistance to phosphine has been identified and characterised, very little information is available on the causes of its development and spread. Data obtained from a unique national resistance monitoring and management program were analysed, using Bayesian hurdle modelling, to determine which factors may be responsible. Fumigation in unsealed storages, combined with a high frequency of weak resistance, were found to be the main criteria that led to the development of strong resistance in *Sitophilus oryzae*. Independent development, rather than gene flow via migration, appears to be primarily responsible for the geographic incidence of strong resistance to phosphine in *S. oryzae*. This information can now be utilised to direct resources and education into those areas at high risk and to refine phosphine resistance management strategies.

1. Introduction

While strong resistance to phosphine has been identified and characterised in a number of stored grain insect species around the world (Collins et al., 2001; Lorini et al., 2007; Opit et al., 2012; Nayak et al., 2013; Nguyen et al., 2015), very little information is available on how it might spread or the factors that may be responsible for its development. It is important that this information is identified so that resistance to phosphine is managed and its sustainability as an efficacious fumigant is maintained.

Fumigation with phosphine is the primary method of controlling stored grain pests. Phosphine is popular because it has a number of attributes: it is cheap, permissible on most commodities, effective on all life stages of nearly all the major insect pests and it is considered to be residue-free, making it acceptable for most markets. It is also flexible and can be manipulated by adjusting the concentration and/or duration of the fumigation (Chaudry, 2000). In Australia, approximately 80% of grain is fumigated with phosphine (Collins et al., 2001). This includes grain for export and domestic markets, as well as much that is stored on farms for seed or later delivery to take advantage of market fluctuations.

A problem with the reliance on one chemical is the potential development of resistance. Resistance to phosphine has developed in several species (Chaudry, 2000; Collins et al., 2003; Lorini et al., 2007; Emery et al., 2011; Nayak et al., 2013) and occurs as two phenotypes: weak resistance (WR) and strong resistance (SR). Briefly, WR is controlled primarily by a single major gene, *rph1*, while SR is controlled by two major genes, *rph1* and *rph2* (Nguyen et al., 2015) that act synergistically to increase the level of resistance several times above that of susceptible or WR insects (Schlipalius et al., 2002). Therefore, rather than a continuum of resistance strength, as is often found, there is a distinct separation between WR and SR. Depending on the species, this can be resistance factors of between 9 and over 1000 (Nayak et al., 2013; Nguyen et al., 2015). Weak resistance appears to develop relatively easily in insect populations resulting in it being common and ubiquitous in many regions (White and Lambkin, 1990; Emery et al., 2003, Emery et al., 2011). However, it is readily controlled with current phosphine fumigation protocols registered in Australia.

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Strong resistance, on the other hand, is less common as it takes time to develop. This is because, to express this phenotype fully, insects must be homozygous for both resistance genes (Schlipalius et al., 2002; Jagadeesan et al., 2012). Development of SR to phosphine threatens continued control of insect populations. While phosphine concentrations and duration can be manipulated to levels sufficient to control these SR insects, in many cases current infrastructure may not be capable of containing these gas levels for the time period required. Therefore, alternative control measures must be used which, if available, are generally more expensive.

Strong resistance to phosphine has been detected in several important stored grain pest species in Australia, including *Sitophilus oryzae* (Emery et al., 2003, Emery et al., 2011). It was the need to be pro-active in developing strategies to manage these resistances that led to the initiation of a national phosphine resistance monitoring and management program in Australia in 1996 (Emery et al., 2003). This program is unique in the world and facilitates a quick response to the detection of SR populations in order to control them and prevent their spread. As part of the program, data including site and storage details, previous treatments and bioassay results for each population are recorded for each of the three grain growing regions (northern, southern and western) into the Australian Grain Insect Resistance Database (AGIRD) (Emery and Tassone, 1998). The information contained in AGIRD has been used to develop resistance management strategies (Collins, 2009) and forms the basis for this study.

In this paper, we have analysed information amassed in AGIRD from 1996 to 2013 in order to develop some understanding of how SR in *Sitophilus oryzae* may have spread, as well as which factors are primary in its development. *Sitophilus oryzae* was chosen because of its importance as a pest of stored grain in Australia and the fact that SR in this species was not detected until 2003. Thus the initial development and subsequent geographic spread could be investigated in its entirety. The information will be used to refine existing resistance management strategies and focus resources and education to those regions and situations where it is most needed.

2. Material and methods

2.1. Sample collection

Australia's national phosphine monitoring program is conducted through three regional laboratories located across three grain growing regions: Wagga Wagga, New South Wales; Brisbane, Queensland; and Perth, Western Australia. Distances between the laboratories range from 1250 km (Brisbane/Wagga Wagga) to 4340 km (Brisbane/Perth). All laboratories follow a nationally agreed and statistically robust monitoring protocol to ensure integrity of data for comparison across the sites (Collins et al., 2003).

Insects were collected in two ways. Managers of central storages sent live insects that were either detected when grain was delivered to the site or, more frequently, immediately after a treatment such as fumigation or other insecticide treatment. The second method was by collectors sampling grain storage facilities, such as farms, grain merchants, millers and feedlots, either randomly or targeting sites where resistance was known to have occurred previously. In general, a similar number of sites were sampled each year across all states. However, not all regions within each state could be sampled every year due to the large distances required to be covered. Collected insects were sent to the nearest of the three laboratories, where they were cultured and their offspring, generally F1, tested for phosphine resistance. This ensured healthy specimens of a similar age, while reducing any effect of genetic drift or selection by long-term laboratory culturing. An insect "population sample" is defined as a culture or collection of insects sampled from a single storage at one time. This is because treatments, commodities, and/or environmental conditions differ between storages and over time, and, consequently, different pressures would be exerted on the insects. Therefore, multiple population samples could be collected from one site or storage over several years.

2.2. Testing for resistance - phosphine fumigation bioassays

Phosphine resistance tests were performed using the recommended FAO phosphine bioassay method (FAO, 1975), with modified discriminating doses of 0.04 mg/L and 0.25 mg/L to determine weak and strong resistance, respectively. Phosphine gas was generated from commercial formulations of aluminium phosphide in a 5% solution of sulphuric acid. Detailed methods of phosphine generation and measurement of concentration with a gas chromatograph are as described previously (Daglish et al., 2014a).

For each bioassay, batches of 50 randomly selected adult *Sitophilus* beetles were placed, without food, into plastic cups with perforated lids. These cups were then positioned in gas-tight desiccators and phosphine gas injected via a septum to achieve the required dose. Insect strains of known resistance status were included in the desiccators as a control to check that phosphine concentrations were maintained for the duration of the fumigation. For each bioassay, there were 2–4 replicate desiccators, plus a control desiccator in which no gas was injected. All desiccators were placed in a controlled temperature room (25–27 °C, 50–60% RH) and left for 20 h. On completion of the fumigation, the beetles were removed from the desiccators and placed on whole wheat (25–27 °C; 50–60% RH) for seven days to allow time to recover, after which mortality was assessed. Populations were assessed as susceptible if no test insect survived, weak resistant if any insect survived 0.04 mg/L but all died at 0.25 mg/L and strong resistant if there were any survivors at 0.25 mg/L. Survivors of the high dose were cultured and their offspring retested to confirm resistance diagnosis.

2.3. Australian Grain Insect Resistance Database (AGIRD)

The Australian Grain Insect Resistance Database was developed in 1996 by the Department of Agriculture and Food Western Australia to ensure all data from various stored grain databases and spreadsheets were held in a single repository (Collins et al., 2003; Emery et al., 2011). Data, obtained from the national resistance monitoring program, are entered for all sites, insect populations and bioassays by each of the three collaborating laboratories, and synchronised at regular intervals. The database currently holds information on over 40,000 insect populations collected from over 11,000 sites throughout Australia over a period of 30 years. For this study, analysis was based on a total of 2414 and 2137 bioassay tests for diagnosing strong and weak resistance, respectively, to phosphine in *S. oryzae* from 1996 to 2014.

2.4. Statistical analysis

Analysis was a multi-step process. Initially descriptive statistics were applied to the randomly collected data using the Chi-Square Test, followed by simple linear and smoothed (generalised addition models (GAMs)) trend models. All proportions of strong resistance were calculated to take into account the different weightings afforded by the different number of populations collected for each criterion.

The next step in the analysis was to use the Bayesian hurdle modelling approach to further investigate trends and significant