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Phosphine Toxicity in *Caenorhabditis elegans*: Synergy and Cross-Resistance with Other Pest Control Treatments, Including Gamma Radiation

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Abstract

Hydrogen phosphide (PH_3), also known as phosphine, is an ideal fumigant to control insect pest infestation of stored grain, as it is inexpensive, easy to use and leaves little or no chemical residue. As no other general use fumigant is available, phosphine is used to protect 80% of the Australian grain harvest, with the remainder consisting primarily of animal feed and seed grain for planting. Heavy reliance on phosphine has resulted in the development of resistance among insect pests of stored products. In this project, the model organism, *Caenorhabditis elegans*, was utilized for exploring the mechanisms of phosphine toxicity and interaction with other treatments including gamma radiation as well as testing for synergistic actions between these treatments and phosphine.

By looking into the effect of oxygen, I found that hyperoxia synergistically enhances the toxicity of phosphine against wild type *C. elegans* at 15, 20 or 25 °C, but it only marginally increases the effectiveness of phosphine against phosphine-resistant animals at 20 °C. The sub-lethal concentration of phosphine (70 ppm) with 80% oxygen under 15, 20 and 25 °C gave 60%, 96%, and 99% mortality respectively, in the wild type nematodes. Interestingly, the nematodes of both strains consume significantly more oxygen at 20 °C comparing to the other temperatures. However, the wild type worms consume significantly more oxygen than *dld-(wr4)* at all three temperatures. The toxicity of arsenite, on the other hand, was negatively correlated with phosphine toxicity. The phosphine-resistant mutant exhibited sensitivity to arsenite, which was close to an arsenite-sensitive mutant. Combining 4 mM of arsenite ($\sim LC_{50}$) with 70 ppm phosphine resulted in elevated mortality of 89% in the phosphine-resistant mutant, whereas the combination was not lethal to wild type animals.

One method of grain disinfestation is gamma irradiation; a treatment that can co-exist with phosphine in the grain storage system. I tested the toxicity of two distinct forms of irradiation on *C. elegans*, UV and gamma irradiation. By utilizing mutant lines that are sensitive or resistant to either phosphine or radiation, I found hypersensitivity to phosphine of mutations originally selected for hypersensitivity to either UV or gamma radiation. The phosphine-resistant and the radiation-resistant mutants were each significantly more resistant to UV and ionizing radiation than wild type *C. elegans*. UV and gamma radiation-sensitive mutant exhibited hypersensitivity to phosphine, considerably higher than the wild type in most cases. Unexpectedly, a gamma and UV radiation-resistant mutant was also hypersensitive to phosphine.

The effect of pre-exposure to UV, ionizing radiation, and heat-shock was investigated, and I observed that these pre-treatments induced tolerance against phosphine in *C. elegans*. Heat-shock increased phosphine tolerance in the wild type strain by 3-fold, but no significant induction was observed in the phosphine-resistant mutant (*dld-1*(*wr4)*). On the other hand, mild exposure to UV and gamma radiation doubled phosphine resistance in the *dld-1*(*wr4*) mutant, but this effect was only observed with gamma radiation in the wild type strain.

The interaction between phosphine and the other treatments in my work demonstrates the involvement of phosphine toxicity with oxidative respiration, where temperature, oxygen, and arsenite have synergized phosphine. Also, the cross-resistance between phosphine and gamma radiation supports that oxidative damage is involved in the mode of action of phosphine. Finally, the observation that heat shock induces phosphine resistance in wild type, but not resistant animals provides a focus for future molecular studies.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

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Contributor	Statement of contribution
Alzahrani, Saad M.	Designed experiments (60%) Wrote the paper (100%) Statistical analysis of data (100%)
Ebert, Paul R.	Designed experiments (40%) edited paper (100%)

Contributions by others to the thesis

Saad Alzahrani is the sole responsible for all the works presented in this thesis.
Dr. Paul R. Ebert contributed in academic supervision including experiments design, interpretation of the data and editing papers for all studies presented in this thesis.
Dr. David I. Schlipalius contributed in part in academic supervision and editing chapters one and two.

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Keywords

phosphine, dihydrolipoamide dehydrogenase, synergism, phosphine-resistance, cross-resistance, ionizing radiation, UV radiation, preconditioning, heat shock, arsenite.

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List of abbreviations used in the thesis

AChE	Acetylcholinesterase
ANOVA	Analysis of variance
BCKDC	Branched-chain ketoacid dehydrogenase complex
°C	Celsius degree
C. elegans	*Caenorhabditis elegans*
CE1255	Radiation-resistant mutant of *C. elegans*
df	Degrees of freedom
DLD	Dihydrolipoamide dehydrogenase
dld-1	*C. elegans* gene that encodes dihydrolipoamide dehydrogenase
dld-1(wr4)	Phosphine-resistant mutant of *C. elegans*
DNA	Deoxyribonucleic acid
DW102	Ionizing radiation-sensitive mutant of *C. elegans*
DW103	Ionizing radiation-sensitive mutant of *C. elegans*
ED50	The median effective dose
ER	The endoplasmic reticulum
ETC	The electron transport chain
FAO	The Food and Agriculture Organization of the United Nations
FCCP	*p*-trifluoromethoxy carbonyl cyanide phenyl hydrazone
GCS	Glycine cleavage system
Gy	Gray
H_2O_2	Hydrogen peroxide
IPM	Integrated pest management
J cm^{-2}	Joule per square centimeter
KGDC	Alpha-ketoglutarate dehydrogenase complex
LC_{50}	The median lethal concentration
LC_{90}	Lethal concentration that gives 90% mortality
LD_{50}	The median lethal dose
LdP	Dose-Probit Line
LT_{90}	Lethal time that gives 90% mortality
mM	millimole
N2	Wild type isolate of *C. elegans*
NAD+	Oxidized nicotinamide adenine dinucleotide

NADH	Reduced nicotinamide adenine dinucleotide
NL147	Arsenite-sensitive mutant of *C. elegans*
O_2	Oxygen
O_2^-	Superoxide
OP50	*Escherischia coli*
PCP	Pentachlorophenol
PDC	Pyruvate dehydrogenase complex
PH_3	Phosphine
ppm	Parts per million
RF	Resistance factor
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
rph1, rph2	Phosphine-resistance genes
SD	Slandered deviation
SE	Standard Error
SEM	Standard error of means
SP483	UV-sensitive mutant of *C. elegans*
SP488	UV-sensitive mutant of *C. elegans*
Sy.x	Standard deviation of the residuals
TCA	The citric acid cycle
TPE	Time to population extend
UPR	Unfolded protein response
UV	Ultraviolet
X^2	Chi square

Chapter 1: Introduction

Grain is an essential food source for humans as well as feed for livestock with crops like wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), rice (*Oryza sativa* L.) and maize (*Zea mays* L.). Global population growth has driven increased demand for cereal crops and this, in turn, has led to increased challenges on grain production and storage practices. (Tilman et al., 2011). Storing grain in silos creates a suitable environment for pests such as insects, mites, and rodents (Sinha et al., 1995). According to the food and agriculture organization (FAO) of the United Nations, an estimation of 10% of the stored grain is lost annually due to pests' attacks on the stored grain. Loss from insects alone can range from 3-50% of stores, which depends on the storage period (Kumar, 2017). To counter this, pest management practices should be developed and followed to minimize or eliminate the infestation.

Chemical control is the preferred method for disinfesting stored grain pests due to its effectiveness and economic value (Boyer et al., 2012). Currently, fumigants are the most preferred and reliable chemicals for disinfestation of stored commodities, with hydrogen phosphide (PH_3), or phosphine, the most widely used gas by far for the protection of stored grain. The properties of phosphine that make it an ideal fumigant include its low cost, ease of application, ability to readily penetrate the grain bulk, and the lack of chemical residues. Moreover, it does not affect grain viability (Chaudhry, 1997).

Alternative fumigants exist but are limited in their use as they cause environmental damage, leave residues on grain or have limited efficacy. For example, methyl bromide causes depletion of the ozone layer in the stratosphere (Thomas, 1996), sulfuryl fluoride leaves residues on the grain and has limited efficacy against insect eggs (Derrick et al., 1990), whereas ethyl formate is unable to penetrate large grain bulks (Muthu et al., 1984). These limitations of alternative fumigants make phosphine the only fumigant approved for general use globally (Daft, 1987), which, when coupled with domestic and international market demand for insect-free grain has led to a heavy reliance on phosphine (Collins et al., 2003).

The heavy reliance on phosphine has contributed to the selection of resistance against phosphine among insect pests of grain. Australia has had a national resistance-monitoring program for nearly three decades, which has detected highly phosphine-resistant insects from multiple species including the flat grain beetle *Cryptolestes ferrugineus*, lesser grain borer *Rhyzopertha dominica*, the psocid *Liposcelis bostrychophila*, red flour beetle *Tribolium*

castaneum and rice weevil *Sitophilus oryzae* (Emery et al., 2011). Phosphine resistance is also observed globally. In the 1970s the FAO conducted a large-scale global survey for insecticide resistance among insect pests of stored products, 10% of the collected insects around the world were resistant to phosphine (Champ & Dyte, 1976). Worldwide, reports on high level resistance to phosphine among stored product pests are common and widespread (Benhalima et al., 2004; Holloway et al., 2016; Koçak et al., 2015; Konemann et al., 2017; Pimentel et al., 2009; Rajan et al., 2017; Zuowei et al., 2004). This development of resistance is threatening the ability of the grain industry to maintain insect-free and residue-free grain, especially due to the lack of an alternative fumigant to replace phosphine, though methyl bromide is used as a quarantine treatment at port facilities and sulfuryl fluoride is used to a limited degree in rotation with phosphine.

Laboratory studies have revealed treatments that synergistically enhance phosphine toxicity, suggesting that this may be an effective strategy to more efficiently manage stored grain pests. Synergism can reduce the dose/concentration required to control pests (Ware, 1994), which may slow the development of pesticides resistance in the target insects.

Effective deployment of synergists will require an understanding of the mode of action of phosphine and how it synergistically interacts with other treatments. I used the free-living nematode *C. elegans* as a model organism due to the availability of suitable mutant strains. I employed wide-ranging chemical (arsenite and hyperoxia), environmental (temperature) and physical (ultraviolet (UV) and ionizing radiation) stresses to provide a rich understanding of interactions with phosphine toxicity and resistance.

Accordingly, the thesis will present in chapter two a review on phosphine resistance, mechanism of action and synergism. Chapter three will investigate phosphine synergists. Chapter four will explore the effects of radiation and cross-resistance between radiation and phosphine. Then chapter five will look at the preconditioning effect of stressors on phosphine resistance. Finally, chapter six will be a general discussion of the main findings of my research and will present models that succinctly summarize the conclusions derived from my results.

C. elegans in phosphine toxicity assays

In biological studies, *C. elegans* is favored as a model organism due to its fully characterized genetic background, which allowed the modification of that background for specific genetic characteristics (Consortium, 1998). This can significantly contribute to investigating the role of genes or biological pathways in phosphine resistance.

In 2003 Cheng et al. (Cheng et al., 2003) generated a phosphine resistant mutant of *C. elegans* that was subsequently found to be due to a variant of the dihydrolipoamide dehydrogenase gene (*dld-1*) (Schlipalius et al., 2012). The same gene was found to be responsible for high-level phosphine resistance in insects (Schlipalius et al., 2008; Schlipalius et al., 2002; Schlipalius et al., 2012). The shared resistance genetics between *C. elegans* and insects and the availability of the phosphine resistant *dld-1* gene variant in *C. elegans* has paved the way for investigating and studying phosphine resistance and the mechanisms of action in the *C. elegans* genetic model organism. In addition, phosphine, unlike contact pesticides that are selected based on specificity to the target organisms, is toxic to any obligate aerobically respiring species.

Moreover, the nematodes' small size, self-fertilization, and rapid reproduction make it easy to obtain large numbers of isogenic individuals for toxicology studies (Félix & Braendle, 2010). This makes laboratory manipulation of these animals far easier than the pest insects. Also, the ability of these animals to be grown on the surface of a solid medium enables the application of gases or dissolved chemicals simultaneously, which is much more difficult for the pest insects. These characteristics make *C. elegans* ideal for toxicity testing.

Chapter 2: Review of phosphine toxicology

Phosphine mechanisms of action

In a recent review of the mechanism of phosphine action, Nath et al. (Nath et al., 2011) summarized three proposed mechanisms. It should be noted that the proposed mechanisms are not mutually exclusive. Unlike most toxins, whose efficacy can be modelled as an inverse, linear relationship between time and concentration of exposure, the relationship between phosphine concentration and the duration of exposure is non-linear. Thus, phosphine is a fast acting toxin at very high concentrations, but a very slow acting toxin at low concentrations.

Oxidative stress. The first proposed mode of action is related to the ability of phosphine to initiate oxidative stress in aerobically respiring organisms. Reactive oxygen species (ROS) are primarily produced from enzymes of energy metabolism involved in electron transfer reactions. These molecules are highly reactive and damage biological macromolecules, eventually leading to cell death. A high rate of aerobic respiration is correlated with a high rate of oxygen consumption and elevated levels of ROS and is also associated with an increase in phosphine toxicity (Nath et al., 2011). Epigenetic suppression of mitochondrial electron transport chain (ETC) genes results in phosphine resistance, which is likely due to suppression of energy metabolism and possibly the generation of ROS (Zuryn et al., 2008). Furthermore, mitochondrial uncouplers, which increase the rate of electron flow through the ETC, were strongly synergistic with phosphine. These results confirm a positive relationship between phosphine toxicity and the rate of aerobic respiration inside the mitochondria (Valmas et al., 2008).

Despite the observed link between phosphine toxicity and aerobic respiration, *in vitro* inhibition of mitochondrial respiration by phosphine was not different between mitochondria that have been isolated from resistant versus susceptible insects (Price, 1980b). The author reported in another study that *in vivo* exposure to phosphine for a sublethal period triggered a response that resulted in reduce oxygen consumption. Notably, this event occurred only in the resistant animals (Price, 1980a). The ability of phosphine to disrupt mitochondrial function through inhibiting cytochrome *c* oxidase runs contrary to the other observations that respiration rate is positively correlated with toxicity. If the inhibition occurs *in vitro* and contributes to phosphine toxicity, it may be that the mechanism of action is to increase the rate of ROS generation despite an inhibition of the respiratory rate.

Metabolic crisis. An alternative proposal relates to suppression of energy metabolism, with phosphine toxicity due to energetic insufficiency resulting in a "metabolic crisis", leading to death (Nath et al., 2011). This interpretation is supported by a study conducted on rats. When the animals were treated with phosphine, glucose was synthesized in the liver, suggesting an increased rate of glycolysis in brain tissue. As a result of these observations, the authors (Dua et al., 2010) suggested a phosphine-mediated reduction of aerobic respiration created an energy crisis due to the difficulty of meeting energy needs via anaerobic respiration. The dramatic decrease in the levels of plasma glucose supports the emergence of a metabolic crisis.

Moreover, phosphine can inhibit cytochrome *c* oxidase (complex IV of the ETC) *in vitro*, which was initially proposed to be the site of action of phosphine *in vivo* as well. With the recent identification of phosphine resistance variants clustered around the active site of dihydrolipoamide dehydrogenase, this enzyme must also be considered a potential target of phosphine. As the enzymatic product of DLD is NADH, which feeds electrons into the ETC, many observations previously attributed to complex IV of the ETC in insects can equally well be attributed to mutation of the *dld-1* gene. Regardless of which is the actual target of phosphine, their roles in energy generation provide a possible mechanism to explain the phosphine-mediated inhibition of aerobic respiration (Kashi, 1981a, 1981b; Nakakita et al., 1974; Zuryn et al., 2008). In a histopathological study involving cytochrome *c* oxidase, exposing rats orally to phosphine significantly decreased the activity of mitochondrial complexes I, II and IV in liver tissue, while phosphine poisoning decreased the level of all cytochromes in the liver and brain of the treated animals.

Histological changes in the treated rats showed mitochondrial injury in the heart, liver and brain tissues, resulting in decreased energy output and an increase in oxidative stress. This acute exposure to phosphine in rats resulted in significant suppression in the activity of catalase, leading to an escalation of lipid peroxidation (Anand et al., 2012). This suggests that phosphine targets the mitochondria interfering with cellular respiration. To counter these insults in phosphine-poisoned patients, Duenas et al. reported an anti-ischemic metabolic agent Trimetazidine that can decrease the toxic effect of phosphine by preserving oxidative metabolism through improving glucose utilization by inhibiting fatty acid metabolism (Duenas et al., 1999).

Neurotoxicity. A third potential mechanism of phosphine toxicity is as a neurotoxin. Limited evidence suggests that, phosphine increases acetylcholine neurotransmission by inhibiting

acetylcholine esterase activity (AChE). The esterase activity is mandatory for attenuating acetylcholine signaling, so inhibition of the esterase results in elevated levels of synaptic acetylcholine results in excitotoxicity (Al-Hakkak et al., 1989; Al-Azzawi et al., 1990). Mirtta et al. (Mittra et al., 2001) concluded that phosphine causes inhibition of AChE, based on survival of rats exposed to a lethal dose of phosphine when they were treated with pralidoxime, a chemical inhibitor of anti-cholinesterase compounds. The animals treated with pralidoxime survived 2.5 times longer than control animals that were exposed to phosphine but were not treated with pralidoxime.

In stored product insects, phosphine suppressed the activity of AChE in fourth instar larvae of *Trogoderma granarium* up to 46% (Sher et al., 2004). In a phosphine resistant population of *T. granarium*, the rate of AChE activity was decreased after exposure to phosphine at the LC_{20} for 80 hours. After comparing five phosphine resistant strains of *T. granarium* with a susceptible one, the AChE activity was significantly higher in the phosphine resistant strains, which indicates that elevated activity of AChE is involved in phosphine resistance (Riaz et al., 2017).

Phosphine resistance

Biochemical and genetic studies were conducted to investigate the mechanisms of phosphine resistance deeply. In 2002 Collins et al. (Collins et al., 2002) showed that the resistance to phosphine in the lesser grain borer *R. dominica* is genetically inherited and is the product of more than one gene. These genes were incompletely recessive. Of the two resistant strains that they tested, one was weakly resistant, and the other was strongly resistant to phosphine. Subsequently, two genes responsible for resistance were identified by Schlipalius et al. (Schlipalius et al., 2002). One gene, *rph2*, provides up to 12-fold phosphine resistance while the other, *rph1*, provides resistance up to 50-fold. The two loci that provide resistance to phosphine act synergistically when both are homozygous for the resistance alleles, resulting in >250-fold phosphine resistance when compared with completely susceptible insects. The same two genes are the primary contributors to high level resistance in *T. castaneum*, *S. oryzae* and *C. ferrugineus* (Chen et al., 2015; Jagadeesan et al., 2012; Koçak et al., 2015; Oppert et al., 2015). Similarly, in the model organism *C. elegans*, a mutant line carrying a phosphine resistance variant of the *dld-1* gene (orthologous to *rph2*) can resist phosphine toxicity nine times greater than the wild type which is fully susceptible to phosphine (Cheng et al., 2003).

Nematodes facilitated the biochemical studies for phosphine resistance. Phosphine was found to increase the levels of free iron in exposed animals, contributing to lipid peroxidation, broader cellular damage and eventually cell death. Suppression of the iron sequestering ferritin-2 gene in *C. elegans* increased phosphine sensitivity (Cha'on et al., 2007). Also, mitochondrial respiratory genes were found to be directly involved in phosphine resistance (Zuryn et al., 2008). When Zuryn et al. silenced twenty-one mitochondrial respiratory chain genes in wild type *C. elegans* using RNAi (RNA interference/Post-Transcriptional Gene Silencing), several of the genes inhibited the respiration rate in a way that led to elevated phosphine resistance by 10-fold compared to the controls. Valmas et al. (Valmas et al., 2008) created a situation of accelerated ETC activity by co-exposing wild type and phosphine resistant mutants of *C. elegans* to non-lethal doses of mitochondrial uncouplers (FCCP and PCP) plus a nonlethal concentration of phosphine. The combination caused complete mortality in both strains compared to complete survival when exposed to either compound individually.

Phosphine resistant insects take up smaller amounts of phosphine compared to their susceptible counterparts (Nakakita & Kuroda, 1986). These claims have been supported by Pratt (Pratt, 2003), who showed that the retention of phosphine is associated with the oxidation of the phosphine molecule in the cell. There is a correlation between phosphine oxidation and the toxicity of the molecule, but the nature of this relationship is not understood. The oxidation of phosphine is also associated with an increase in ROS leading to mortality in the exposed organism.

Resistance to phosphine in stored-products pests was reasoned to be due to a number of physical and biological mechanisms. Early studies suggested that a narcosis effect observed at higher doses was a protective mechanism against phosphine in resistance insects (Chaudhry, 1997; Nakakita et al., 1974; Winks, 1985; Winks, 1974). Later, that claim was discounted as a cause of phosphine resistance when Winks and Waterford (Winks & Waterford, 1986) conducted a phosphine toxicity assay on a resistant strain of *T. castaneum*. They found that the concentration where the strain exhibits a narcotic effect, was ten times higher in resistant animals than in susceptible ones. Therefore, narcosis is not involved in phosphine resistance. In a previous study, nitrogen-induced narcosis could not protect *S. oryzae* and *S. granarius* from phosphine toxication. That led Kashi (Kashi, 1981a) to suggest further studies in the involvement of narcosis in phosphine resistance. In fact, animals exposed to phosphine will exhibit hyperactivity followed by twitching (Chaudhry, 1997; Nath et al., 2011), those effects

precede the narcotic effect of phosphine in exposed animals. These characteristics of phosphine exposure suggest activity as a neurotoxin.

Phosphine interaction with other treatments

To enhance phosphine potency, or to understand the precise mode of action, the interaction between phosphine and other treatments has been a focus of study. Oxygen was found to be directly involved in phosphine poisoning. In fact, phosphine and other fumigants have a direct relationship with metabolism (Cotton, 1932). Mortality-induced by seven fumigants including phosphine were increased in two species of insects when the insects were exposed to oxygen during the fumigation. However, the oxygen-enhanced toxicity of phosphine was significantly greater than the oxygen-enhanced toxicity of six other fumigants (hydrogen cyanide, acrylonitrile, methyl bromide, ethylene dibromide, ethylene oxide and chloropicrin) (Bond, 1963). Under anoxic conditions (0% oxygen, 100% nitrogen), the wheat weevil (*S. granarius*) was protected against phosphine, with more than 22 mg L^{-1} required to achieve the LC_{50} compare to 1 mg L^{-1} in the presence of oxygen (Bond et al., 1967). A high oxygen atmosphere was even able to enhance phosphine toxicity when applied up to thirty hours after phosphine fumigation (Bond & Monro, 1967).

Phosphine toxicity and oxygen are closely interrelated. Under anoxic conditions, phosphine is not toxic to insects, while increasing oxygen concentration in the fumigation chambers increased the toxicity of phosphine (Kashi, 1981a). Three species of stored product pests *T. castaneum, T. confusum* and *R. dominica* were able to tolerate 10 mg L^{-1} of phosphine fumigation for 12 hours in anoxia (~98% survival). On the other hand, 2 mg L^{-1} of phosphine was lethal causing 100% mortality when the oxygen concentration increased during the fumigation (Kashi, 1981b). The non-lethal concentration of phosphine of 0.1 mg L^{-1} to the wild type *C. elegans* caused 100% mortality when the fumigation was combined with 80% oxygen (Cheng et al., 2003). Also, in postharvest pest insects of horticulture, hyperoxia during fumigation significantly reduced the time and concentration of phosphine required to achieve complete pest control (Liu, 2011, 2012).

Arsine and arsenite interact directly with the lipoic acid cofactor of the four enzyme complexes that contain the phosphine resistance factor, DLD (Bergquist et al., 2009; Hughes, 2002). Sensitivity to arsine was found to be negatively correlated with phosphine resistance. For

example, 25 ppm phosphine causes 100% mortality of a phosphine susceptible strain of *R. dominica* no mortality of a phosphine resistant strain. The same concentration of arsine was 50% more lethal to resistant strains than to phosphine susceptible ones (Chaudhry & Price, 1991). Phosphine resistant animals of *C. elegans* were significantly more sensitive to arsine as well as arsenite than the wild type (Schlipalius et al., 2012).

Synergizing phosphine by other treatments was not limited to chemicals as temperature also plays a vital role in the efficiency of the fumigation. A combination of high temperature (32-37 °C) and 4-6% of carbon dioxide decreased phosphine concentration that is required to achieve 100% mortality in mills, from 850-1500 ppm (the conventional concentration range) to 65-165 ppm (Mueller, 1994). Also, elevated temperature significantly reduced the time to population extinction (TPE) for the phosphine resistant psocid, *L. bostrychophila*. The TPE was 11 days with 1 mg L^{-1} of phosphine at 15 °C, and it became only two days when the fumigation temperature increased to 35 °C (Nayak & Collins, 2008).

Temperature directly affects the respiration rate in the stored product insects. High temperature accelerates the respiratory rate in cold-blooded animals including insects (Cossins, 2012; Keister & Buck, 1964), so when combined with phosphine, the exposed animal will uptake and respond to phosphine more rapidly. The reverse is true at low temperatures (Chaudhry et al., 2004). High temperature (37-40 °C) is stressful to many insects in the standard atmosphere (Burks Charles S., 2012), thus, combining it with other stressors will exaggerate the harmful effects. Mbata and Philips (Mbata & Phillips, 2001) had intensified the injurious effect of low pressure to stored product insects when they conducted their experiment at high temperature. Under normal conditions, the lethal time LT_{90} for *R. dominica* larvae in low pressure was 64 hours at 25 °C, while at high temperature 40 °C the LT_{90} decreased to 5 hours. They assumed in their discussion that high temperature increases the respiration and metabolic rates resulting in rapid mortality among the exposed insects (Mbata & Phillips, 2001).

Managing phosphine resistance can also be achieved through applying the integrated pest management (IPM). One of which is gamma radiation. Irradiation with gamma rays has gained an excellent reputation in stored-product pest management (Aldryhim & Adam, 1999; Arthur, 2004; Aye et al., 2008; Ayvaz & Tunçbilek, 2006; Beetle & du Val, 2002; Follett et al., 2013; Ignatowicz, 2004; Kirkpatrick et al., 1973; Zolfagharieh, 2004). For instance, treating infested wheat grain with immature stages of *R. dominica* with 250 Gy of gamma-ray reduced the rate

of adult emergence by 54% compared with the untreated insects (Kirkpatrick et al., 1973). Also, irradiating eggs of *S. granaries* with 30-500 Gy inhibited development, preventing adult emergence (Aldryhim & Adam, 1999). Ninety-nine percent of *T. confusum* adults were killed at 30 days from the time of exposure to 200 Gy of gamma radiation (Beetle & du Val, 2002).

Moreover, gamma irradiation was sufficient for the disinfestation of *Oryzaephilus surinamensis* in dates. The optimum dose for controlling all developmental stages of the insect was 700 Gy of gamma radiation. Furthermore, only 85 Gy was enough for reproductive sterilization of this pest (Zolfagharieh, 2004). Exposing eggs of *Ephestia kuehniella* to 400 Gy reduced the hatchability to 27%, and no adult emerged from the hatched eggs (Ayvaz & Tunçbilek, 2006). The inhibitory effect of gamma radiation was observable on *Plodia interpunctella*; immature stages, which failed to develop when irradiated with 500 Gy (Aye et al., 2008).

In addition to control pests, many countries utilize gamma irradiation as a quarantine treatment to disinfest commodities in the stored-products industries (Follett, 2009; Follett et al., 2013; Hallman, 2013). In these countries, gamma radiation usually co-exists with phosphine in stored-products pest management. This co-existence created the necessity of investigating the interaction between the two treatments. The interaction between phosphine and gamma radiation was not observed when two strains of *T. castaneum* were tested, one susceptible and the other resistant to phosphine (Saxena & Bhatia, 1981).

On the other hand, Mehta et al. (Mehta et al., 2004) reported that exposing *T. castaneum* insects to gamma radiation altered their susceptibility to fumigants. If the irradiation preceded the fumigation, the insects became more tolerant, but if the insects were first treated with the fumigant, then irradiated with gamma rays, their radiosensitivity remained the same. Cross-resistance between gamma radiation and phosphine was observed in *R. dominica*. The phosphine resistant strain showed a significant increase in resistance to gamma irradiation compared with a phosphine susceptible strain (Hasan et al., 2006).

On an experimental scale, Ultraviolet radiation has been reported as an approach for stored product pest control and as a hygiene treatment (Bruce & Lum, 1978; Collins & Kitchingman, 2010; Faruki et al., 2005; Ghanem & Shamma, 2007). UV radiation can stop the development process of the khapra beetle *T. granarium* at different stages. A hundred percent mortality was achieved after irradiating the eggs with 56.52 J cm^{-2} of UV light. The radiation caused damage to the eggs' chorions resulting in a leakage of the inner contents. Other premature stages of this

pest were sensitive to UV, and the same dose produces 98.3% and 91.7% mortality in larvae and pupae respectively (Ghanem & Shamma, 2007). In another stored product pest the lesser mealworm *Alphitobius diaperinus*, the LT_{50} for the second instar larvae at 15 W from a germicidal lamp was 24 minutes. Also, UV inhibited pupation and adult emergence in this pest. Fecundity was also affected by UV radiation, where the fecundity percentage of *A. diaperinus* females decreased from 78% to 23.5% after four minutes exposure (Faruki et al., 2005). In the same manner, a two-hour exposure to 9 mW cm^{-2} at a wavelength of 254 nm of UV light caused 21.5% and 53.6% reduction respectively in the mean number of progeny of *O. surinamensis* and *T. castaneum*. Only 12 seconds of the same intensity of UV light was enough to reduce the average number of progeny of two stored product mites, *Acarus siro* and *Tyrophagus putrescentiae* by 64.6% and 92.2% respectively (Collins & Kitchingman, 2010). However, the interaction between UV and phosphine has not been looked at, probably due to the inability to utilize UV in grain protection. UV light cannot penetrate the grain, which makes grain pests protected from its toxic action by the grain.

Besides DNA-damage radiation injures biological systems by generating ROS. Therefore, antioxidant enzymes play an essential role in cellular defenses against radiation-induced damage (Riley, 1994). Even exogenous antioxidants such as catalase and superoxide dismutase have significantly protected cells from the damaging effect of UV radiation in the exposed organism (Santos et al., 2012). The oxidative stress caused by ionizing radiation can also be reduced by the antioxidant Resveratrol which decreased the injurious effect of ionizing radiation (Ye et al., 2010). The relationship between oxidative stress and radiation is observable since the levels of ROS in living cells and radioresistance are negatively correlated (Diehn et al., 2009).

Since phosphine has been identified as a redox active toxin that generates significant oxidative stress (Chaudhry, 1997; Nath et al., 2011), there is overlap between the biological pathways of the toxic action of the treatments. Also, gamma irradiation, as mentioned above, can co-exist with phosphine in the grain industry for controlling pest infestation which creates the imperative to investigate the interaction between them.

Chapter 3: Phosphine synergism: Oxygen and Arsenite Synergize Phosphine Toxicity by Distinct Mechanisms[1]

Abstract

Phosphine is the only fumigant approved globally for general use to control insect pests in stored grain. Due to the emergence of resistance among insect pests and the lack of suitable alternative fumigants, we are investigating ways to synergistically enhance phosphine toxicity, by studying the mechanism of action of known synergists, such as oxygen, temperature, and arsenite. Under normoxia, exposure of the model organism *C. elegans* for 24 hours at 20 °C to 70 ppm phosphine resulted in 10% mortality, but nearly 100% mortality if the oxygen concentration was increased to 80%. In wild type *C. elegans*, toxicity of phosphine was negatively affected by a decrease in temperature to 15 °C and positively affected by an increase in temperature to 25 °C. The *dld-1(wr4*) strain of *C. elegans* is resistant to phosphine due to a mutation in the dihydrolipoamide dehydrogenase gene. It also exhibits increased mortality that is dependent on hyperoxia, when exposed to 70 ppm phosphine at 20 °C. As with the wild type strain, mortality decreased when exposure was carried out at 15 °C. At 25 °C, however, the strain was completely resistant to the phosphine exposure at all oxygen concentrations. Arsenite is also a synergist of phosphine toxicity, but only in the *dld-1(wr4*) mutant strain. Thus, exposure to 4 mM arsenite resulted in 50% mortality, which increased to 89% mortality when 70 ppm phosphine and 4 mM arsenite were combined. In stark contrast, 70 ppm phosphine rendered 4 mM arsenite non-toxic to wild type *C. elegans*. These results reveal two synergists with distinct modes of action, one of which targets individuals that carry a phosphine resistance allele in the dihydrolipoamide dehydrogenase gene.

Keywords: *Oxygen synergism, Phosphine, Temperature, Respiration rate, Oxygen, Oxygen consumption, Arsenite, Arsenite synergism, C. elegans*

[1] Submitted for publication to the Journal of Toxicological Sciences (https://academic.oup.com/toxsci)

Introduction

Controlling insect infestations in the grain industry is essential for global food security. Fumigants are favored for pest control because contact pesticides leave residues on the grain, a problem that is greatly exacerbated when insects become resistant, requiring the application of greater amounts of pesticide to control pests. Phosphine is the only fumigant available for general use. While alternative fumigants exist, their use is restricted due to environmental damage, an inability to penetrate large bulks of grain, unacceptable residues or high cost. Phosphine (PH_3) is an ideal gas for fumigation to control insect pest infestation in stored commodities, as it is inexpensive, easy to use and does not leave harmful chemical residues (Chaudhry, 1997). If synergists can be found that enhance the efficacy of phosphine, it may be possible to achieve the benefits of fumigation with phosphine without the disadvantages associated with the alternative fumigants.

Three mechanisms of phosphine toxicity have been proposed as reviewed by Nath et al. (Nath et al., 2011). The mechanism most relevant to this work is that phosphine can initiate oxidative stress in the exposed organism. Under normal conditions, a small amount of reactive oxygen species (ROS) are typically produced from the mitochondrial electron transport chain (ETC) or other metabolic enzymes as a byproduct of electron transfer reactions. Elevated levels of ROS are harmful to biological macromolecules and can ultimately lead to cell death. A high rate of aerobic metabolism, which is characterized by a high rate of oxygen consumption as well as higher levels of ROS, also increases phosphine toxicity. Epigenetic suppression of ETC genes results in phosphine resistance, which is likely due to suppression of energy metabolism (Zuryn et al., 2008).

Therefore, it is evident that oxygen is an essential component of the toxicity of phosphine. In fact, phosphine is not toxic to insects if oxygen levels are lower than about 2% (Kashi, 1981a). Thus, there is a positive correlation between aerobic metabolism and the phosphine poisoning process, since toxicity increases as the rate of aerobic respiration increases (Bond, 1963). Interestingly, insect mortality also increases when they are exposed to elevated levels of oxygen after fumigation with phosphine has been terminated. Oxygen has been tested for its ability to enhance the toxicity of a variety of fumigants and has been found to be most active in combination with phosphine (Bond et al., 1967).

Robust synergism was also observed between oxygen and phosphine in the model organism *C. elegans* (Cheng et al., 2003), in which a concentration of phosphine that was non-lethal in normal air produced 100% mortality of wild type nematodes under hyperoxic conditions. This is a similar to the response subsequently observed in postharvest insect pests, in which fumigation with phosphine under hyperoxia was significantly more effective than fumigations in regular air in all four species that were tested (Liu, 2011). Consequently, the author proposed that oxygen should increase phosphine toxicity against a broad range of insect pests. In support of this, the same author found that 60% oxygen could reduce the phosphine concentration and fumigation time required for the complete control of the aphid, *Nasonovia ribisnigri* (Liu, 2012). Caution is warranted, however, as a phosphine resistance mutation in the *dld-1* gene (*rph2* in insects) results in resistance to the synergistic effect of oxygen plus phosphine (Cheng et al., 2003; Schlipalius et al., 2012).

The relationship between phosphine resistance and arsine hypersensitivity was initially observed in grain pests (Chaudhry & Price, 1991) and was later found to result from resistance variants in the *dld-1* gene (also referred to as the *rph2* locus in insects) and to extend to arsenite hypersensitivity in both insects and *C. elegans* as well (Schlipalius et al., 2012). The observed response to arsenite is not surprising as the redox forms of arsenic are readily interchangeable intracellularly, with arsenite as the most common form. Arsenite can interfere with a range of metabolic enzymes, notably the lipoamide cofactor that transfers electrons to the DLD enzyme itself - the phosphine resistance factor (Schlipalius et al., 2012).

In this work, we aimed to investigate the synergistic action of oxygen and arsenite on phosphine toxicity using mutants of the model organism *C. elegans*. The results obtained from this work should contribute to the understanding of the precise mode of action of phosphine. Additionally, it holds the promise that strategies can be devised to enhance the toxicity of phosphine in a way that specifically targets resistant insects. This would constitute a great advance in the use of phosphine to control pest insects of stored products.

Materials and Methods

Nematode strains

The *C. elegans* strains used in this study were wild type, N2, phosphine resistant, *dld-1*(*wr4*) (Cheng et al., 2003), and arsenite hypersensitive, NL147 (Broeks et al., 1996). The nematodes were maintained at 20 °C according to standard protocols for maintaining *C. elegans* (Stiernagle, 1999).

Chemicals

Phosphine gas was generated from aluminum phosphide tablets (570g/kg aluminum phosphide, BEQUISA Co. (GASTION), Brazil). The generation of the gas was carried out according to (Valmas & Ebert, 2006). Oxygen was supplied from a medical oxygen cylinder (High purity oxygen compressed, U.N. No. 1072, BOC®). The arsenite solution was prepared from sodium arsenite (Sigma-Aldrich; CAS 7784-46-5; VMT code A4129) and was added to the NGM agar immediately prior to pouring the plates as described in (Schlipalius et al., 2012).

Phosphine and oxygen exposure

Phosphine exposure was performed as previously described in (Cheng et al., 2003; Valmas & Ebert, 2006; Valmas et al., 2008; Zuryn et al., 2008). Briefly, the nematodes were age-synchronized by harvesting eggs from gravid adults using alkaline sodium hypochlorite as described by stiernagle (Stiernagle, 1999). Eggs were maintained with gentle agitation in M9 buffer for 18-20 hours to allow them to hatch. They enter L_1 diapause in the absence of food and begin synchronized growth when transferred to fresh NGM agar plates (0.3 % NaCl, 0.25 % peptone, 5 mg/ml cholesterol, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1.7 % agar) seeded with a lawn of OP50 bacteria (*Escherichia coli*) as a food source. The nematodes were allowed to grow at 20 °C for 48 hours, at which time they had reached a late L_4-early adult stage of development.

The plates were placed in a double ported, air-tight chamber. Oxygen was bubbled through water to humidify it and was passed through the chamber until the desired concentration was achieved. Oxygen concentration was determined by a Witrox 1 Fibox oxygen meter (Loligo Systems, #OX11800) and PreSens O_2 sensor spots (Loligo Systems, #OX11050). Oxygen concentrations used in this experiment were 20.9% (normal air), 40%, 60% and 80%. Seventy ppm phosphine was injected into the desiccators once the desired oxygen concentration was

reached and the chambers were sealed. After fumigation for 24 hours, the nematodes were transferred to fresh air to recover for 48 hours. Fumigations were carried out at each of three temperatures, 15, 20 and 25 °C.

Oxygen consumption rate

The rate of respiration for each of the two strains at 15, 20 and 25 °C was determined as the oxygen consumption rate as described in (Zuryn et al., 2010). Nematodes were grown on a lawn of *E. coli* bacteria of strain OP50 to a late L_4/early adult stage of development. The nematodes were then washed with M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl in 1 L sterilize H_2O) to remove the bacteria. ~300 washed nematodes in fully aerated M9 buffer were placed in a 350 µl water-jacketed respirometer cell (SI782) maintained at 15, 20 or 25 °C. An oxygen meter (MT200A) was utilized to measure the oxygen consumption in a five minute period.

Arsenite toxicity assay

A lawn of OP50 bacteria was grown for 24 hours at 30 °C on NGM agar plates supplemented with 3, 3.5, 4, 4.5, 5, 6 or 7 mM arsenite (Schlipalius et al., 2012). Nematodes were grown on OP50 bacteria on normal NGM plates at 20 °C for 48 hours until they reached late L_4/early adulthood. The nematodes were then washed from the plates with M9 buffer and transferred to the arsenite containing plates. One set of worms was exposed to arsenite alone, whereas the other was exposed to arsenite in combination with 70 ppm phosphine for an initial 24 hours. The phosphine response of the arsenite sensitive strain was also determined by exposure to phosphine by itself for 24 hours at concentrations of 0, 100, 200, 400, 800, 1600, 3200 and 6400 ppm. The worms were then removed from the phosphine, and all plates were incubated at 20 °C for an additional 48 hours prior to scoring mortality.

Statistical analysis

Each experiment was independently replicated three times. Mortality scoring was conducted using the Automated WormScan procedure (Mathew et al., 2012; Puckering et al., 2017), i.e., individuals that do not move in response to a light stimulus for a period of ten minutes were considered dead. For mortality rates, the average rates with standard error were calculated using Excel 2016. Mortality analysis for calculating the median lethal concentration (LC_{50}) was

carried out by subjecting the mortality percentages to best-fit concentration response curves. We use this analysis to facilitate biological interpretation of the data. A more standard probit analysis of the mortality data can be found in appendix I. LC_{50} values were compared with One-way ANOVA followed by Dunnet's multiple comparison to identify significant differences between the strains' responses for each treatment. Finally, two-way ANOVA followed by Sidak's multiple comparisons test to determine significant differences in oxygen consumption at each temperature between the wild type and the phosphine resistant nematodes. All statistical analyses were performed using GraphPad Prism 7.03.

Results

We wished to identify potential phosphine synergists and compare their modes of action. The approach was to expose the nematodes to a minimal concentration of phosphine (70 ppm) at various temperatures in combination with oxygen. In addition, the same phosphine concentration was combined with a range of arsenite concentrations.

Phosphine and oxygen exposure at various temperatures

This experiment was designed to test the interaction between oxygen concentration and temperature in combination with phosphine. We employed a single concentration of phosphine, 70 ppm, that causes approximately 10% mortality of both the wild type strain, N2 and the phosphine resistant mutant, *dld-1*(*wr4*) at 20 °C under normoxia (Figure 3.1B). Oxygen concentrations included normoxia (21% O_2), 40%, 60% and 80%, none of which cause any mortality in the absence of phosphine. Likewise, the temperatures of 15 °C, 20 °C and 25 °C that were used in the experiment do not on their own affect survival over the timespan of the experiment. While increase in temperature has the biological effect, of increasing the rate of development (Byerly et al., 1976).

We found temperature dependent differences in how each of the two strains responded to phosphine plus oxygen. For the wild type nematodes, the effect of increasing the temperature during exposure consistently increased sensitivity to the phosphine plus hyperoxia combination. Thus, at 15 °C, mortality up to 60% oxygen was equivalent to the phosphine plus normoxia control. At 80% oxygen, however, mortality increased to 46% (Figure 3.1A). At 20 °C,

the mortality from 70 ppm phosphine was 9% in normal air (21% oxygen), but this rose to 21%, 67%, and 95% respectively under 40%, 60% and 80% oxygen (Figure 3.1B). At 25 °C, mortality under 40%, 60% and 80% oxygen was 38%, 99%, and 99.6% respectively (Figure 3.1C). This increase in the toxicity of phosphine as the temperature was increased from 15 °C to 20 °C and 25 °C is consistent with an increased rate of chemical and biochemical reactions at elevated temperatures. Under normoxia, however, the wild type strain had equivalent mortality at 15 °C and 20 °C, but no mortality at 25 °C.

The phosphine resistant strain showed greater resistance to phosphine at each oxygen level and temperature condition at which mortality was observed for the wild type strain. Conditions that did not cause mortality of the wild type strain likewise did not affect the phosphine resistant mutant strain. As with the wild type animals, resistant nematodes were more sensitive to phosphine plus hyperoxia at 20 °C than at 15 °C. Thus, at 15 °C, there was no increase in mortality over that of the normoxia control up to 60% oxygen. Even at 80% oxygen mortality only reached 10%. At 20 °C, mortality rates of the mutant were 7%, 8%, 37%, and 38% respectively, under normal air, 40%, 60% and 80% oxygen (Figure 3.1B). Under normoxia, mortality of the mutant strain was only observed at 20 °C (Figure 3.1A-C).

At 25 °C, the responses of the two strains diverged sharply (Figure 3.1C). Whereas the wild type strain continued with a predictable increase in mortality with increasing temperature, the phosphine resistant mutant became entirely insensitive to phosphine. Thus, it appears that the mild stress associated with an increase in temperature to 25 °C uniquely affected the mutant strain, by either triggering a resistance mechanism or by eliminating a mechanism of phosphine toxicity.

<u>Oxygen consumption rate</u>

We previously observed that at 25 °C, hyperoxia increased the toxicity of phosphine in the wild type strain, but enhanced the phosphine resistance of *dld-1*(*wr4*). We initially assumed that respiration rate would increase with temperature, which we had anticipated would increase sensitivity to phosphine – not result in resistance. To clarify this situation, we compared respiration rates between the two strains of *C. elegans* at each of the three temperatures. Both strains consumed oxygen most rapidly at the optimal growth temperature of 20 °C. The rate was lower for both strains under the mild temperature stress of 15 °C and 25 °C (Figure 3.2). The same pattern of maximal respiration under the non-stress condition of 20 °C was observed

in each strain. Though at each temperature, the respiration rate was significantly lower in the *dld-1(wr4)* mutant than in the wild type strain. The mean oxygen consumption rates of the mutant were 39%, 57% and 55% of the rates observed for the wild type strain at 15 °C, 20 °C, and 25 °C respectively.

Arsenite plus phosphine toxicity

Resistance variants of the *dld-1* gene cause a secondary phenotype of hypersensitivity to arsenite in both insects and *C. elegans* (Schlipalius et al., 2012). We used three strains of *C. elegans* to explore the relationship between arsenite and phosphine toxicity – the wild type strain, N2, and two mutants. The first mutant is *dld-1*(*wr4*), which was initially selected for phosphine resistance and later was found to be sensitive to arsenite as well (Cheng et al., 2003; Schlipalius et al., 2012). The second mutant, NL147, has a defective efflux pump that renders it hypersensitive to arsenite (Broeks et al., 1996; Leslie et al., 2001). We first exposed each strain to a range of concentrations of either arsenite or phosphine individually. The LC_{50} values for phosphine exposure were 314.8, 1019.0 and 178.6 ppm for the wild type, *dld-1(wr4)* and NL147 strains (Table 3.1), confirming the strong phosphine resistance of *dld-1*(*wr4*) and revealing a significant hypersensitivity toward phosphine of NL147 (Figure 3.3A). On closer inspection, the resistance is restricted to low dose phosphine exposure, suggesting that the arsenite pump, while providing cross-protection against phosphine, is only capable of expelling low levels of the gas.

When the strains were exposed to arsenite, both mutants were more sensitive than the wild type, with LC_{50} values for the wild type, *dld-1(wr4)* and NL147 strains of 5.1, 4.5 and 4.0 mM (Figure 3.4A, Table 3.1). It was apparent that the defect in the efflux pump of strain NL147 rendered it sensitive to arsenite relative to the wild type N2 strain that was significantly more tolerant. The pattern of susceptibility suggests that the efflux pump provides no protection at low doses of arsenite, but rather, provide protection at doses above 3.5 mM. The *dld-1*(*wr4*) strain is sensitive to arsenite, indicating a particular vulnerability due to the mutation in the dihydrolipoamide dehydrogenase gene. The level of susceptibility is less severe than that in NL147. indicating that a functioning pump is capable of overcoming the hypersensitivity phenotype due to the *dld-1(wr4)* mutation.

We then exposed each strain to a range of arsenite concentrations together with 70 ppm phosphine, a level of phosphine exposure that at 20 °C results in less than 10% mortality on its

own (Figure 3.4B). The mortality curve of NL147 is identical to that resulting from exposure to arsenite alone. In contrast, the functional pump in the other two strains seemed to be activated by exposure to the low concentration of phosphine. This was apparent by the induced resistance to low concentrations of arsenite, against which the pump was not effective when phosphine was not present (Figure 3.4A). The shape of the mortality curve of the wild type strain indicated that the pump lost its effectiveness at arsenite concentrations above 4mM. The steep slope of the response curve of the wild type strain was identical to that of the *dld-1(wr4)* mutant strain, indicating that there was no interaction between the pump and the DLD protein. This interpretation is supported by the observation that the relative sensitivity of the *dld-1(wr4)* mutant relative to the wild type strain (Figure 3.4B).

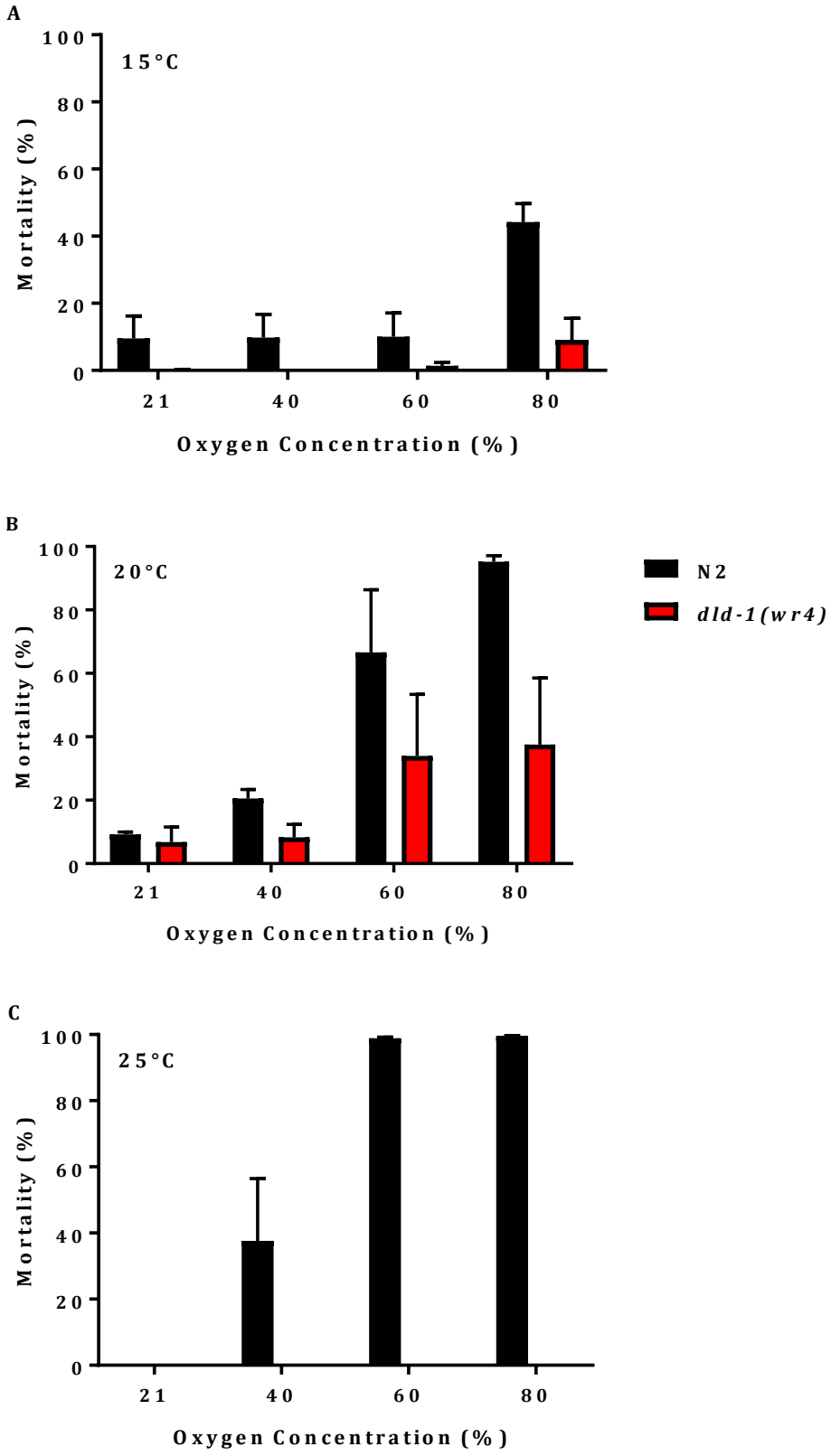

Figure 3.1: Phosphine-induced mortality after exposing to hyperoxic phosphine fumigation with 70 ppm at (A) 15 °C. (B) 20 °C. (C) 25 °C. The oxygenated phosphine fumigation was carried out for 24 hours, after which the animals were allowed a 48 hours recovery followed by mortality scoring. For all panels, the black bars represent the wild type strain, N2, and the red bars represent the phosphine-resistant *dld-1*(*wr4*) mutant, error bars represent standard error.

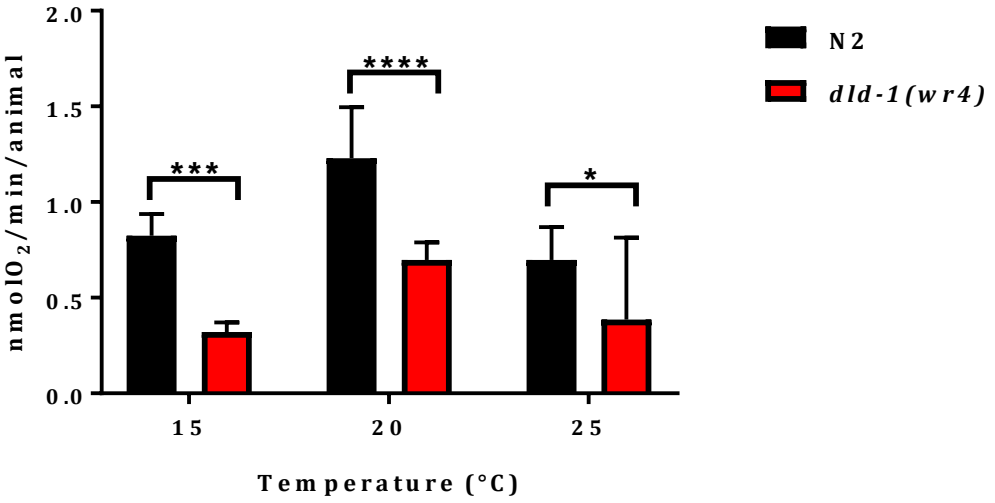

Figure 3.2: Oxygen consumption rates in *C. elegans* strains at various temperatures. Temperature affects oxygen consumption rate in *C. elegans*; the highest rate was at 20 °C while altering that temperature resulted in decreased oxygen consumption. The wild type nematodes (sensitive to phosphine) have significantly higher rate of oxygen consumption than the *dld-1(wr4)* mutants (phosphine resistant) regardless of the temperature change. Subjecting the respiration means to two-way ANOVA followed by Sidak's multiple comparisons test to compare the two strains. The two strains are significantly different at $^{****}p < 0.001$, $^{***}p < 0.01$ and $^{*}p < 0.05$, error bars represent standard error.

Table 3.1: Best-fit values from concentration response curves in *C. elegans* strains after exposing to a range of concentrations from phosphine and arsenite. One way ANOVA followed by Dunnett's multiple comparison test was performed to identify significant differences in LC_{50} values due to exposure of each treatment between N2 (wild type) and *dld-1(wr4)* (phosphine-resistant) and NL147 (arsenite-sensitive).

Treatment	Strain	LC_{50} ± SE	Slope ± SE	R	Sy.x†
Phosphine (ppm)	N2 (wild type)	314.8±1.6	4.59±0.71	0.999	0.37
	dld-1(wr4) (phosphine-resistant)	1019±51.9****	2.29±0.22	0.998	1.99
	NL147 (arsenite-sensitive)	178.6±10.5****	1.92±0.20	0.998	2.38
Arsenite (mM)	N2 (wild type)	5.1±1.2	3.81±1.94	0.952	9.5
	dld-1(wr4) (phosphine-resistant)	4.5±0.6	5.44±2.56	0.964	9.02
	NL147 (arsenite-sensitive)	4.0±0.2*	6.33±3.55	0.947	12.73
Arsenite+70 ppm Phosphine (mM)	N2 (wild type)	4.5±0.05	25.01±7.96	0.991	5.55
	dld-1(wr4) (phosphine-resistant)	3.7±0.03****	31.68±4.13	0.998	3.02
	NL147 (arsenite-sensitive)	3.8±0.23****	5.87±1.72	0.982	7.08

*$p < 0.05$, ****$p < 0.0001$, ppm parts per million, mM millimole
† Standard deviation of the residuals

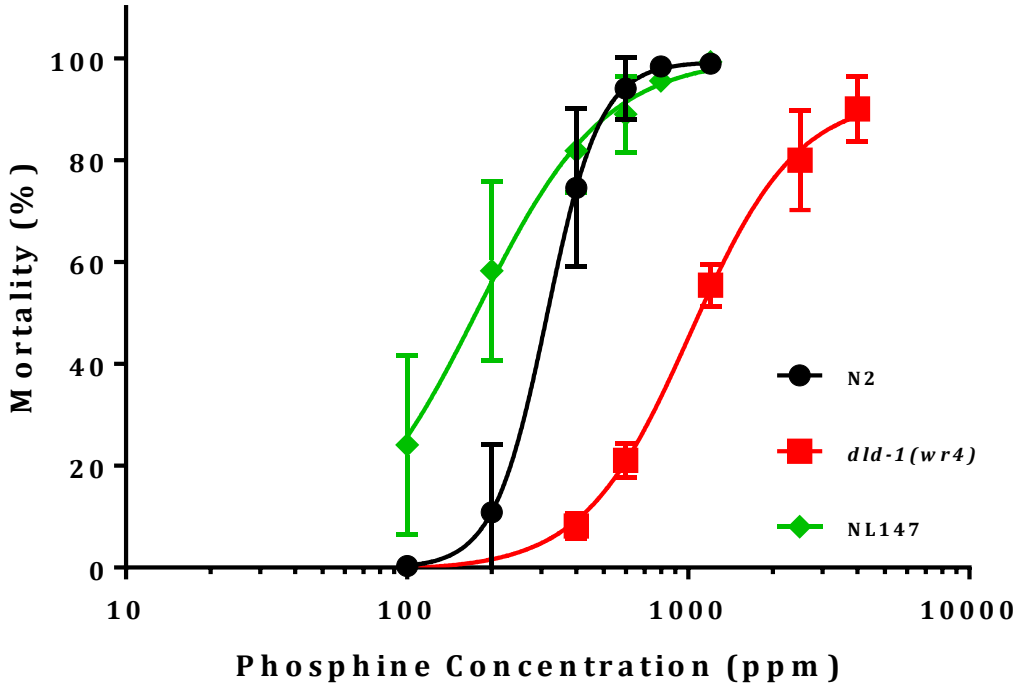

Figure 3.3: Phosphine-induced mortality for the three strains, N2 (wild type), *dld-1(wr4)* (phosphine-resistant) and NL147 (arsenite-sensitive), **after exposure to a range of phosphine concentrations for 24 hours.** Forty-eight hours after the exposure, mortality was scored for each strain. Mortality lines are based on average mortality from three replications; error bars represent standard error.

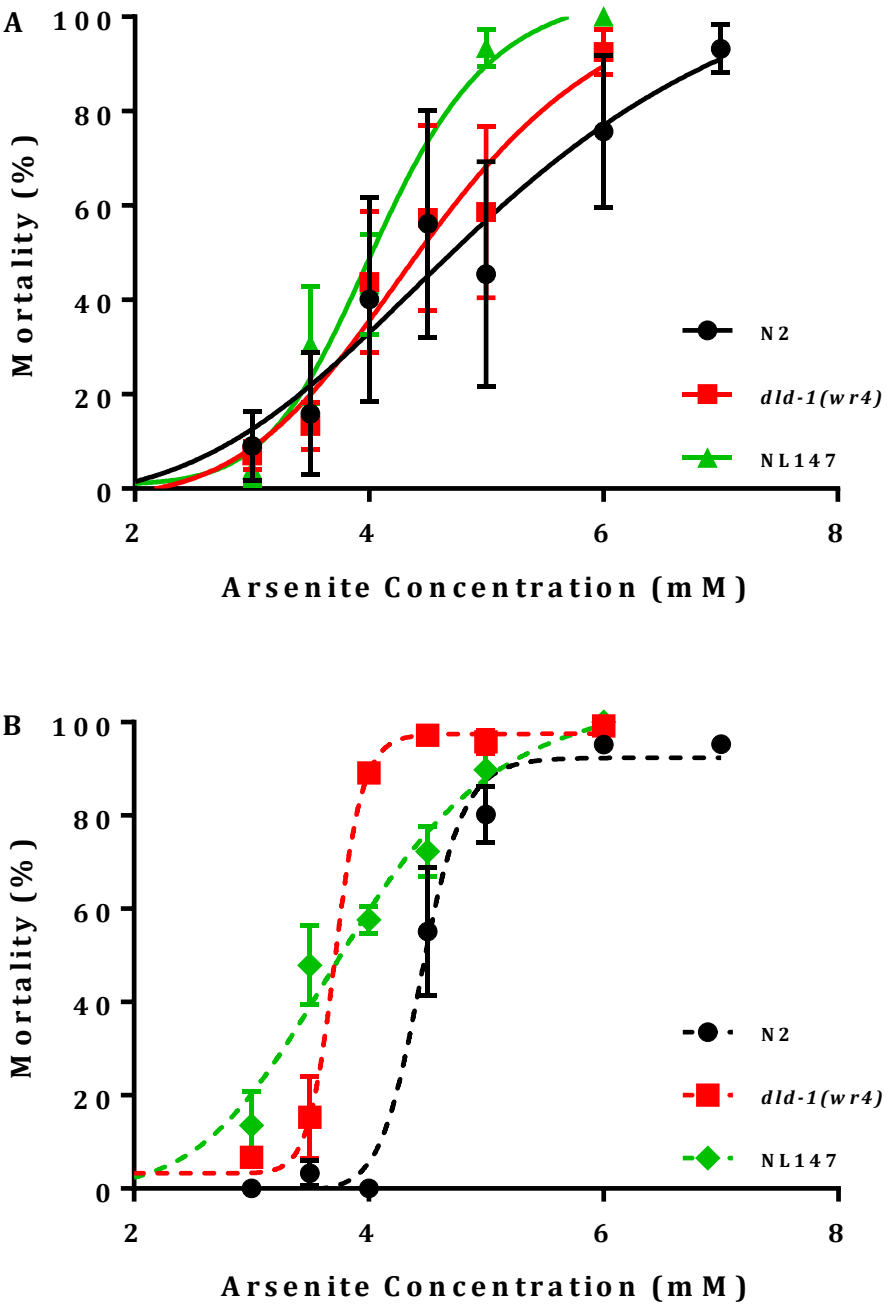

Figure 3.4: Arsenite-induced mortality of the three nematode strains, N2 (wild type), *dld-1(wr4)* (phosphine-resistant) and NL147 (arsenite-sensitive), **from exposure to a range of arsenite concentrations, without (solid) or with 70 ppm phosphine (dotted**). Mortality lines are based on average mortality from three replications. (A) Solid lines represent arsenite; (B) dotted lines represent the mixture of arsenite and 70 ppm phosphine, error bars represent standard error.

Discussion

This work builds on our discovery that the enzyme dihydrolipoamide dehydrogenase (DLD), a key contributor to core energy metabolism, is a phosphine resistance factor (Schlipalius et al., 2012). The involvement of energy metabolism in phosphine toxicity/resistance was anticipated as phosphine toxicity is dependent on oxidative respiration (Bolter & Chefurka, 1990; Nakakita et al., 1974; Pratt, 2003; Price & Dance, 1983; Price et al., 1982; Quistad et al., 2000). Furthermore, insects and nematodes that are resistant to phosphine are hypersensitive to arsine gas and arsenite (Chaudhry & Price, 1991; Schlipalius et al., 2012), which are able to disrupt energy metabolism through a covalent interaction with dihydrolipoamide a substrate of the DLD enzyme and a cofactor of DLD-containing enzyme complexes. In this chapter, we explore the interaction of the phosphine resistance allele *dld-1*(*wr4*) with the synergism between phosphine and oxygen as well as a newly discovered synergism between phosphine and arsenite.

Elevated oxygen levels can accelerate the respiration rate in aerobic organisms (Freeman & Crapo, 1981) and hyperoxia is known to synergistically enhance phosphine toxicity (Cheng et al., 2003; Hobbs & Bond, 1989). Interestingly, the increase in the toxicity of phosphine under hyperoxia was not observed in resistant animals, which have a constitutively suppressed rate of respiration (Cheng et al., 2003; Zuryn et al., 2008). The role of active respiration in the toxicity of phosphine is corroborated by the fact that *C. elegans* is hypersensitive to phosphine when the nematodes are simultaneously exposed to respiratory accelerators, i.e., mitochondrial uncouplers (Valmas et al., 2008).

The positive relationship between aerobic respiration and phosphine toxicity is indicated by shifting the effect of 70 ppm phosphine from being sublethal in the wild type nematodes to highly toxic with increased mortality. The same but less dramatic shift occurred in the phosphine resistant animals at 20 °C, making the non-lethal 70 ppm slightly lethal (Figure 3.1). Our results suggest that this shift in phosphine toxicity was significantly influenced by oxygen, and this agrees with Kashi's conclusion (Kashi, 1981a) that phosphine is ineffective toward stored grain pests maintained at <2% oxygen.

The increased mortality from 70 ppm phosphine in both strains was exhibited at 20 °C. Our data demonstrate that at that temperature the nematodes consumed more oxygen than any other temperature (Figure 3.2), which is consistent with the association of phosphine toxicity

with aerobic respiration, as measured by oxygen consumption. These findings support the linkage of phosphine uptake with its oxidation to its toxic derivatives within the exposed organism (Pratt, 2003), accelerating phosphine toxic action.

Now that the gene for strong resistance to phosphine is known to be dihydrolipoamide dehydrogenase (*dld-1*) (Schlipalius et al., 2012), a probable link between phosphine toxicity and respiration has emerged. The DLD enzyme is a subunit of the pyruvate dehydrogenase complex (PDC). PDC links glycolysis (anaerobic respiration) to the tricarboxylic acid (TCA) cycle, which is coupled to aerobic respiration (Sugden & Holness, 2003). PDC acts as a metabolic rheostat that controls the flow of metabolites from glycolysis to the TCA cycle. This property allows it to mediate the transition from active to suppressed aerobic respiration in mammals that are capable of hibernation (Andrews, 2007). The alpha ketoglutarate dehydrogenase complex (KGDC) is an integral component of the TCA that also contains DLD as a subunit. Variants of DLD may directly alter TCA cycle activity through KGDC. As DLD produces NADH from NAD^+, it is intimately coupled to the mitochondrial electron transport chain (ETC) that uses NADH as substrate. The mitochondrial ETC is also the site of action of respiratory uncouplers that are known to affect phosphine toxicity and is also the primary site of oxygen consumption. Thus, our data fit DLD seamlessly into the narrative of how phosphine works and how animals might become resistant to its toxicity (Chen et al., 2015; Koçak et al., 2015; Oppert et al., 2015; Park et al., 2008; Schlipalius et al., 2008; Schlipalius et al., 2012; Zuryn et al., 2008).

Because aerobic respiration can generate reactive oxygen species (ROS) as a byproduct, the effect of hyperoxia and respiration rate on phosphine toxicity may actually be a function of the generation of ROS (Freeman & Crapo, 1981). ROS is not simply a causative agent of cellular oxidative stress, however, but also serves a role in metabolic regulation. Thus, excessive ROS levels can trigger the inhibition of PDC (Tabatabaie et al., 1996), as can exposure to high levels of oxygen, possibly via ROS signaling (Turrens, 2003). Consistent with these observations, exposure to phosphine triggers suppression of oxidative respiration in wild type *C. elegans* (Zuryn et al., 2008). The situation is different in the phosphine resistant animals, however, as their metabolic rate is constitutively suppressed as determined by a reduction in oxygen consumption across all temperatures that we tested. The constitutively suppressed rate of respiration may act as a protection mechanism against the production of ROS. This may explain why hyperoxia fails to synergistically enhance phosphine toxicity in the phosphine resistant strain, as suppressed aerobic respiration could prevent the generation of ROS despite elevated

levels of oxygen. The situation in insects is similar to that which we observed in *C. elegans*, where the respiratory rate of phosphine resistant strains of *T. castaneum* is lower than in phosphine susceptible strains, resulting in a negative correlation between respiration rate and resistance to phosphine (Pimentel et al., 2007).

Another interesting observation is that resistance to phosphine is associated with sensitivity to arsine gas as well as to arsenite, a partially oxidized derivative of arsine. Resistance mutations at the *dld-1* locus result in sensitivity to these arsenicals. The lipoamide cofactor of the DLD enzyme is a primary target of arsenite, which can covalently modify the cofactor, thereby inactivating DLD and inhibiting the activity of the enzyme complexes to which it contributes (Schlipalius et al., 2012). Moreover, trivalent arsenicals, such as arsenite, were found to be the only form of arsenicals that can inhibit both PDC and KGDC (Bergquist et al., 2009).

The hypersensitivity of the phosphine resistant *dld-1(wr4)* strain of *C. elegans* to synergism between arsenite and phosphine is consistent with the mutation causing a decrease in DLD enzyme activity due to the mutation. Because the role of DLD in the enzyme complex is to re-oxidize the lipoamide cofactor, a decrease in DLD enzyme activity will result in the lipoamide remaining in the reduced state for an extended period of time. The reduced form of lipoamide is required for it to react with arsenite (Bergquist et al., 2009). This model provides a plausible explanation for how mutation of the *dld-1* gene can cause phosphine resistance by suppressing energy metabolism in a way that increases the reactivity of lipoamide toward arsenite.

The toxicity assay revealed that an efflux pump is able to suppress the toxic effect of arsenite., but only up to 4 mM. This was revealed by the observation that the NL147 strain that harbors a mutation in *mrp-1* was more sensitive to low dose arsenite than either wild type animals or the *dld-1(wr4)* mutant in which the pump is functional. The function of the pump is to eliminate toxins such as arsenite. It seems that 70 ppm phosphine activates the pump, providing protection against arsenite in these two strains by the active exclusion of toxic agents from the cell. This active exclusion is not a resistance mechanism and is unrelated to the resistance caused by mutation of the *dld-1* gene.

The mutation in strain NL147 that misses the activity of the gene *mrp-1*, making the mutant unresponsive to the inclusion of phosphine (Figure 3.3). The functionality of the efflux pump to tolerate arsenite toxicity is dependent on energy efficiency. In the phosphine resistant animals, low oxygen consumption and reduced DLD activity (Schlipalius et al., 2012) made their energy

output is not sufficient as in the wild type nematodes. The wild type has an elevated oxygen consumption and a normal activity of DLD, making them more tolerant to arsenite toxicity and accordingly increasing the arsenite's tolerance-threshold in these worms comparing to the *dld-1(wr4)* strain.

The disparity in the efficacy of the two synergists of phosphine toxicity against the two strains likely relates to the reactivity of the two strains to each of the three compounds. The wild type strain has a normal metabolic rate and is therefore susceptible to phosphine toxicity, which in insects and *C. elegans* is associated with the generation of lethal amounts of ROS (Abdollahi et al., 2004; Banerjee et al., 2001; Price & Dance, 1983; Quistad et al., 2000). In wild type *C. elegans* the efficacy of the synergistic mixture of oxygen plus phosphine is decreased by conditions that suppress metabolism, such as a decrease in temperature, which would also be expected to decrease ROS generation. A mildly stressful increase in temperature, however, increased toxicity despite resulting in a decrease in metabolism. This is likely due to the effect of temperature on enzyme activity including side-reactions that generate ROS. The *dld-1* mutant has a constitutively suppressed metabolism and is therefore resistant to phosphine relative to the wild type strain, which has been shown to be associated with a decrease in oxidative stress. A major unresolved issue from this work is the differential response of the two strains to hyperoxia, which increases the toxicity of phosphine toward the wild type strain, but causes the mutant to become impervious to the negative effects of phosphine at the concentration that was tested. It would seem that the elevated temperature triggers a fundamental change in the physiological state of the mutant under the dual stresses of hyperoxia and exposure to phosphine. In this regard, it is very interesting to note that of three mechanisms that have been proposed to explain the toxicity of phosphine (Nath et al., 2011), the DLD enzyme has the potential to influence each of them; metabolic rate through PDC, the redox state through KGCD and cholinergic signaling through the glycine cleavage system, as each of these enzyme complexes includes DLD as a subunit.

The response to arsenite is quite different as its ability to synergistically enhance the toxicity of phosphine is restricted to the *dld-1* gene mutant. As discussed above, this is likely an indirect effect due to a change in the reactivity of the lipoamide cofactor to arsenite. Lipoamide is a cofactor in each of the enzyme complexes that contain DLD as a subunit. Arsenite is uniquely reactive toward the resistant mutant, which exhibits constitutive suppression of metabolism

through impairment of the DLD enzyme; therefore, phosphine-resistance consequence is low energy outcome leading to reduced activity of active exclusion of toxins especially arsenite.

Future directions

The unique characteristics of the two strains make oxygen an effective synergist against wild type animals and arsenite an effective synergist against the phosphine resistant *dld-1* mutant strain. The synergism between arsenite and phosphine was not previously known but is a particularly valuable discovery. The ability of arsenite to specifically enhance the toxicity of phosphine toward resistant animal provides proof-of-principle and a target site for the development of strategies to actively manage phosphine resistant animals. This may improve pest management and extend the usefulness of phosphine despite widespread resistance.

Acknowledgments

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Chapter 4: Phosphine Cross-resistance with UV and Ionizing Radiation: Attenuation of Radiation Toxicity by the Phosphine Resistance Factor Dihydrolipoamide Dehydrogenase (DLD)[2]

Abstract

Phosphine gas is an excellent fumigant for disinfesting stored grain of insect pests, but heavy reliance on phosphine has led to resistance in grain pests that threatens its efficacy. Phosphine resistance was previously reported to be mediated by the enzyme DLD. Here we explore the relationship between phosphine toxicity and genotoxic treatments with the goal of understanding how phosphine works. Specifically, we utilized mutant lines sensitive or resistant to phosphine, gamma irradiation or UV exposure. The phosphine resistant mutant exhibited cross-resistance to UV and ionizing radiation. The radiation sensitive mutants exhibit sensitivity to phosphine. The radiation-resistant mutant also appeared to be sensitive to phosphine, a phenotype that was statistically distinguishable from the susceptibility of the wild type control.

Keywords: *Ionizing radiation, Gamma rays, UV, Ultraviolet, Phosphine, Cross-resistance, C. elegans, Dihydrolipoamide dehydrogenase, DLD, Pest management.*

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Introduction

The most widely used fumigant globally is hydrogen phosphide (PH_3), commonly known as phosphine. This gas is an ideal fumigant for the control of insect pest infestations in stored commodities, due to the low cost of application, ease of use and lack of chemical residue, as well as the fact that it does not affect seed viability (Chaudhry, 1997). Residue and environmental risks associated with sulfuryl fluoride and methyl bromide have left phosphine as the only general use fumigant (Collins et al., 2001; Jagadeesan et al., 2015). The heavy reliance on phosphine has led to the selection of resistance against phosphine among major insect pests of grain.

The phosphine resistance in insects and in the nematode *C. elegans* can result from mutations in the gene *rph-2* (*dld-1 in C. elegans*) (Jagadeesan et al., 2016; Kaur et al., 2015; Schlipalius et al., 2012), which encodes the enzyme dihydrolipoamide dehydrogenase (DLD). In *C. elegans*, the mutation is associated with a suppressed metabolic rate (Zuryn et al., 2008). Phosphine initiates oxidative stress in exposed organisms due to the induced production of reactive oxygen species as a byproduct of energy metabolism (Chaudhry, 1997; Nath et al., 2011). High level phosphine resistance is now found in several economically important insect species of stored products: the flat grain beetle *C. ferrugineus*, the lesser grain borer *R. dominica*, the rust red flour beetle *T. castaneum*, the psocids *L. bostrychophila*, *L. bostrychophila* (Emery et al., 2011), and the rice weevil *S. oryzae* (Emery et al., 2011; Nguyen et al., 2015; Rajan et al., 2017).

Alternative methods or integrated management is required for controlling these pests. One of the approaches in the integrated pest management in stored products is ionizing radiation. This physical control has gained an excellent reputation in pest management, and it has been suggested as an alternative to methyl bromide (Fields & White, 2002; Ignatowicz, 2004). In addition, gamma irradiation is currently used globally as a quarantine treatment for stored commodities. In the USA Follett (Follett et al., 2013) reported that 120 Gy of gamma radiation is sufficient to disinfest rice from the rice weevil *S. oryzae* adults. Also, adults mortality was immediate after exposure to doses of gamma radiation, of 300 and 500 Gy (Aldryhim & Adam, 1999). Also, the dose 300 Gy has caused complete inhibition of the development process in the immature stages of stored products beetles.

As in gamma irradiation, ultraviolet radiation has been reported as an approach for stored product pest control and as a hygiene treatment (Bruce & Lum, 1978; Collins & Kitchingman,

2010; Faruki et al., 2005; Ghanem & Shamma, 2007). UV radiation can stop the development process of the khapra beetle *T. granarium* at different stages. Also, UV light affected progeny of *O. surinamensis* and *T. castaneum*; there was 21.5% and 53.6% reduction in the mean number of progeny respectively. In the model organism *C. elegans*, UV exposure can trigger different biological responses. It can reduce fecundity by reducing the total production of eggs (Hartman, 1984) as well as by decreasing the hatchability of the eggs that are produced. In addition, 30 and 40 J m^{-2} of UV radiation resulted in 2.5% and 2.4% survivors in the wild type nematodes (Murakami & Johnson, 1996). Exposing the L_4 stage of the wild type nematodes to 40 J m^{-2} of UV has produced 37% mortality (Wang et al., 2010).

Cross-resistance between ionizing radiation and a number of fumigants (ethylene dibromide-carbon tetrachloride; ethylene dibromide; carbon disulfide and methyl bromide) was found in *T. castaneum*. Radiation was also found to induce resistance in insects toward subsequent fumigation. However, exposure to the fumigants did not change sensitivity to ionizing radiation when it was administered after fumigation (Mehta et al., 2004). Phosphine was unique as gamma irradiation did not affect subsequent sensitivity to phosphine fumigation (Cogburn & Gillenwater, 1972). Phosphine resistant individuals of *R. dominica* were found to be more tolerant to ionizing radiation compared with their susceptible counterparts (Hasan et al., 2006).

In this work, we use the free-living nematode *C. elegans* as a model organism to investigate the toxic effect of ultraviolet and gamma irradiation. We also describe the cross-resistance between these treatments and the fumigant phosphine.

Materials and Methods

Nematode cultures

Seven strains of *C. elegans* were subject to this investigation, the wild type (N2) as a sensitive to the treatments and the phosphine resistant strain (*dld-1(wr4)*) (Cheng et al., 2003), these two strains were kept and maintained in the laboratory at the School of Biological Sciences, the University of Queensland. In addition, two UV sensitive strains SP483 and SP488 (Hartman, 1984) were included. Two strains that have been identifying in the *C. elegans* Genetic Center as sensitive to ionizing radiation DW102 and DW103, and finally the strain CE1255 that has been described as resistant to radiation-induced apoptosis. These five strains were provided by the *C. elegans* Genetic Center (CGC), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

The nematodes were maintained on NGM agar plates at 20 °C during their life stage until they reached the early adult stage L_4. All of the animals' exposures were at 20 °C as well.

UV exposure

According to (Hartman, 1984) and our preliminary trials, it is relatively difficult to obtain results from irradiating later stages of *C. elegans* due to the time required for the phenotype to develop and the complication of progeny being produced during that period. Therefore, L_1 stage nematodes on NGM agar plates were treated with a dose range of UV as follows (0, 10, 20, 30, 40, 50 60 and 120 J cm^{-2}) using (XLE-Series UV crosslinker, Spectronics Co.) as described in (Murakami & Johnson, 1996). These doses are within the normal range of UV intensities in nature (Marionnet et al., 2015).

Ionizing radiation

Synchronized L_1 worms on NGM agar plates were irradiated with dose rage of gamma rays (0, 50, 100, 200, 400, 800, 1500, 2000 Gy). A cobalt-60 Gammacell-220 irradiator (Atomic Energy of Canada Ltd.) at The School of Chemistry and Molecular Biosciences, The University of Queensland was used as a gamma rays source.

Phosphine fumigation

The phosphine fumigation was carried out under control environment at 20 °C, the fumigation protocol described in (Valmas & Ebert, 2006) was utilized. The plates with L_4 stage of *C. elegans* were placed in air-tight desiccators into which a measured amount of phosphine gas was injected. In all cases, the volume of gas that was injected into the chamber was less than 0.2% of the volume of the chamber. Phosphine concentrations that were utilized were 0, 50, 100, 200, 400, 800, 1600, 3200 and 6400 ppm. Fumigations were carried out for 24 hours, in line with established resistance monitoring protocols in pest insects. Following the fumigation, the nematodes were transferred to fresh air to recover for 48 hours.

Statistical analysis

Each experiment was repeated thrice, and all the trials contained two technical replicates per strain per treatment's concentration/dose. Each replicate contained ~100 worms for phosphine assay and ~30 worms for the radiation tests. After each treatment, the nematodes were transferred to 20 °C to recover. The recovery period was 48 hours after each treatment. The software WormScan was utilized for mortality scoring as described in (Mathew et al., 2012; Puckering et al., 2017). Briefly, the treated worms in the six centimeters plates were scanned and the individuals that did not respond to the stimulate (not moving) for a ten minutes period were considered dead. Probit analysis (Finney, 1971) was carried out using (LdP Line, copyright 2000 by Ehab Mostafa Bakr, Cairo, Egypt) to calculate the median lethal concentration/dose (LC_{50}/LD_{50}) and the 95% confidence intervals. One way ANOVA followed by Dunnett's multiple comparisons was carried out for comparing the significance of difference between the strains regarding each treatment toxicity. The probit generated data was fit with a modified probit scale using SigmaPlot version 10.0, from Systat Software, Inc., San Jose California USA (SigmaPlot., 2006).

For calculating the growth rate, the WormScan software was also used to measure the length of each individual as described in (Puckering et al., 2017). The average length of each dose of radiation treatments was computed via Microsoft Excel 2016. Two-way ANOVA followed by Dunnett's multiple comparison test was carried out to determine the significance of the difference of the average length between wild type and the other strains at (P= 0.05). The comparisons were performed using GraphPad Prism (Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com).

Results

We have used mutant strains of *C. elegans* that exhibit either resistance or sensitivity toward gamma or UV irradiation to explore the relationship between genotoxic stresses and the toxic stress associated with exposure to phosphine. In this work, we specifically focus on cross-resistance or cross-sensitivity of strains to each of the other stressors.

Phosphine toxicity

Both phosphine resistant, *dld-1(wr4)*, and susceptible, N2, strains of *C. elegans* exhibit concentration-dependent mortality after exposure to phosphine. The LC_{50} of the phosphine resistant mutant was 4-fold higher than that of the susceptible N2 strain, 1282 ppm, and 302 ppm respectively. The UV hypersensitive mutants SP483 and SP488 showed significantly increased sensitivity to phosphine compared to the wild type strain with LC_{50} values of 164 ppm and 174 ppm. The same effect was observed with the ionizing radiation sensitive mutants, DW102 and DW103. Their phosphine LC_{50} values were 225 ppm and 260 ppm, although only DW102 was statistically more sensitive to phosphine than N2. The radiation resistant mutant CE1255 did not exhibit cross resistance to phosphine. In fact, the strain displayed increased sensitivity to phosphine, with an LC_{50} of 239 ppm, which was significantly lower than N2 (Figure 4.1, Table 4.1).

UV Radiotoxicity

We also monitored the growth and mortality responses of *C. elegans* to ultraviolet radiation. Lethality at forty-eight hours after UV treatment was dose-dependent. Using log dose probit (LDP) analysis, the median lethal dose (LD_{50}) was 18 J cm^{-2} for the wild type strain and 31 J cm^{-2} for the *dld-1(wr4)* mutant (Figure 4.2, Table 4.1). Thus, the *dld-1(wr4)* mutant that was initially selected for its phosphine resistance phenotype, also exhibits 1.7-fold cross-resistance to UV radiation.

The UV-sensitive strain SP483 carries a mutation in the *lem-3* gene that participates in recombination repair of damaged DNA (Dittrich et al., 2012; Hartman, 1984; Hong et al., 2018), whereas strain SP488 carries a mutation in the *smk-1* gene that encodes a kinase that activates the general stress response transcription factor, DAF-16 (Wolff et al., 2006). Under our assay conditions, these two strains only showed an apparent sensitivity to UV radiation that did not

reach the level of statistical significance. The LD_{50} values for UV-radiation exposures were 15 J cm^{-2} and 14 J cm^{-2}, for the two mutants, respectively, and 18 J cm^{-2} for the wild type strain. The two mutants were statistically more sensitive to UV exposure than either the *dld-1(wr4)* mutant or the *cep-1* strain (CE1255). CEP-1 encodes an ortholog of the human tumor suppressor p53. Mutation of this gene in *C. elegans* results in an elevated resistance to radiation-induced apoptosis (Derry et al., 2001). This strain also shows a significant increase in resistance to UV-radiation with an LD_{50} of 41 J cm^{-2}, a 2.3-fold increase relative to wild type (Figure 4.2, Table 4.1).

UV radiation causes a dose-dependent inhibition of the growth rate, which we quantified for each of the strains. Under normal conditions, the average length of the five strains was similar, with the exception of SP483 (*lem-3*) that is significantly shorter than the wild type at (P= 0.05). The average animal length after 48 hours of exposure of L_1 nematodes to 10 to 60 J cm^{-2}, did not differ significantly between the wild type strain and the *dld-1(wr4)* mutant except at 60 J cm^{-2} (Figure 4.4). The sizes of the other mutants differed significantly from the wild type strain at most doses. At the highest dose of 60 J cm^{-2}, the loss of *lem-3* function resulted in a 70% reduction in the average body length of the SP483 strain, from 0.64±0.01 mm to 0.19±0.03 mm. This is not surprising as LEM-3 is directly involved in the DNA-damage response, which is essential for normal cell division (Dittrich et al., 2012). The SP488 strain is defective for activation of the DAF-16 stress response transcription factor via SMK-1. This mutation results in a 79% reduction in the average animal length. from 0.91±0.07 to 0.19±0.05 mm at 60 J cm^{-2}.

The UV-induced reduction in animal length after 48 hours from exposing to 60 J cm^{-2} was expressed in the wild type with 0.38±0.16 mm in average animal length representing 59% reduction from 0.92±0.07 mm at 0 J cm^{-2}. In comparison, the *cep-1* mutants (CE1255) were statistically similar to the wild type after exposure to the same dose with an average length of 0.22±0.17 mm, with 77% reduction from 0.95±0.01 mm (Figure 4.4A). On the other hand, the *dld-1(wr4)* mutants showed a significant increase in tolerance to the UV-inhibition of growth comparing to the wild type. The average length decreased from 0.92±0.07 mm to 0.56±0.13 mm after 60 J cm^{-2} of UV radiation with only 39% reduction.

As anticipated the magnitude of the dose-dependent reduction in growth was more apparent after 72 hours but were consistent with the 48 hour data. When compared to the unexposed strains, exposure to 60 J cm^{-2} reduced growth to 44.6%, 40.7%, 74.5%, 82.5% and 55.4% for N2, *dld-1(wr4)*, SP483, SP488 and CE1255 respectively (Figure 4.4B).

Gamma Radiotoxicity

Forty-eight hours after exposure of L_1 nematodes to gamma radiation, dose-dependent mortality was apparent. The *dld-1*(*wr4*) mutant exhibited resistance to gamma radiation, with an LD_{50} of 655 Gy, that was statistically equivalent to the resistance of the *cep-1* mutant (CE1255) LD_{50} of 602 Gy. The two strains did, however, differ significantly from the wild type N2 strain LD_{50} of 401 Gy.

The wild type nematodes and the ionizing radiation sensitive mutant DW102 were significantly different in their ability to survive radiation exposure, with LD_{50} values of 401, 334 Gy respectively (Figure 4.3, Table 4.1). The DW102 mutation is at *brc-1*, a gene who's function is required for DNA-damage repair (Adamo et al., 2008; Polanowska et al., 2006). *brc-1* mutants of *C. elegans* have an abnormal increase in apoptosis and RAD51 foci after being exposed to ionizing radiation. In addition, *brc-1* in *C. elegans* has an essential role in DNA double-strand break (DSB) repair (Adamo et al., 2008). The DW103 strain has been mutated at the gene *brd-1*. The BRD-1 protein forms a heterodimer with BRC-1, which plays a significant role in coordinating the repair of damaged DNA and associated processes in *C. elegans* and human cells that have been exposed to radiation (Boulton, 2006). The sensitivity to phosphine of DW103 did not differ statistically from susceptibility of the wild type strain, with an LD_{50} of 344 Gy.

As with UV, ionizing radiation inhibited the growth of the nematodes in a dose-dependent manner. The average animal length under normal conditions for the mutants is statistically indistinguishable from the wild type. Exposure to ionizing radiation resulted in a dose-dependent decrease in average animal length, as determined after a 48 hour recovery period following the exposure, The *dld-1(wr4)*, *brc-1* and *brd-1* mutants each responded to the growth-inhibition induced by gamma-radiation in a similar manner with no significant difference between the mutants and the wild type nematodes across all doses (Figure 4.5A). The same result was observed after 72 hours post exposure (Figure 4.5B). On the other hand, at 800 Gy the radiation-resistant mutant (*cep-1*) was more tolerant of gamma-radiation-induced inhibition of growth. The *cep-1* nematodes were significantly longer than the wild type strain throughout the exposure's dosage range, and the trend continued after 72 hours (Figure 4.5).

Table 4.1: LC_{50}/LD_{50} values of *C. elegans* strains after 24 hours phosphine fumigation, UV and gamma irradiation. Values were computed from probit analysis for each strain and treatment. One way ANOVA followed by Dunnett's multiple comparison test was performed to identify significant differences in LC_{50} values due to exposure of each treatment between the wild type and the other strains.

Treatment	Strain†	LD_{50}/LC_{50} (CI#)‡	R	Slope ± SE	X^2	*df*
PH_3 (ppm)	N2	302 (270-337)	0.98	2.89±0.19	6.03	6
	dld-1(wr4)	1282 (739-2143)****	0.97	3.21±0.23	19.36	5
	SP483	164 (93-266)*	0.99	1.99±0.16	19.86	6
	SP488	174 (153-196)*	0.99	2.39±0.15	6.47	6
	DW102	225 (203-249)*	0.99	3.32±0.23	4.56	6
	DW103	260 (233-290)	0.98	2.93±0.19	9.32	6
	CE1255	239 (215-264)*	0.99	3.06±0.18	2.81	6
UV (J cm^{-2})	N2	18 (13-22)	0.97	2.47±0.20	12.95	7
	dld-1(wr4)	31 (25-37)****	0.97	2.84±0.19	16.81	8
	SP483	15 (14-17)	0.98	1.96±0.11	13.86	10
	SP488	14 (13-15)	0.99	2.68±0.15	11.97	9
	CE1255	41 (35-48)****	0.97	4.18±0.33	16.68	7
γ (Gy)	N2	401 (363-443)	0.99	3.29±0.21	3.61	7
	dld-1(wr4)	655 (604-709)****	0.99	5.20±0.44	0.72	4
	DW102	334 (302-369)	0.99	3.33±0.21	1.62	7
	DW103	344 (309-383)	0.99	2.87±0.17	5.77	7
	CE1255	602 (551-655)****	0.99	4.44±0.32	7.33	5

†N2 (wild type), *dld-1(wr4)* (phosphine-resistant), SP483 & SP488 (UV-sensitive), DW102 & DW103 (ionizing radiation-sensitive) and CE1255 (resistant to radiation-induced apoptosis).
#CI = confidence interval
‡*$p < 0.05$, ****$p < 0.0001$

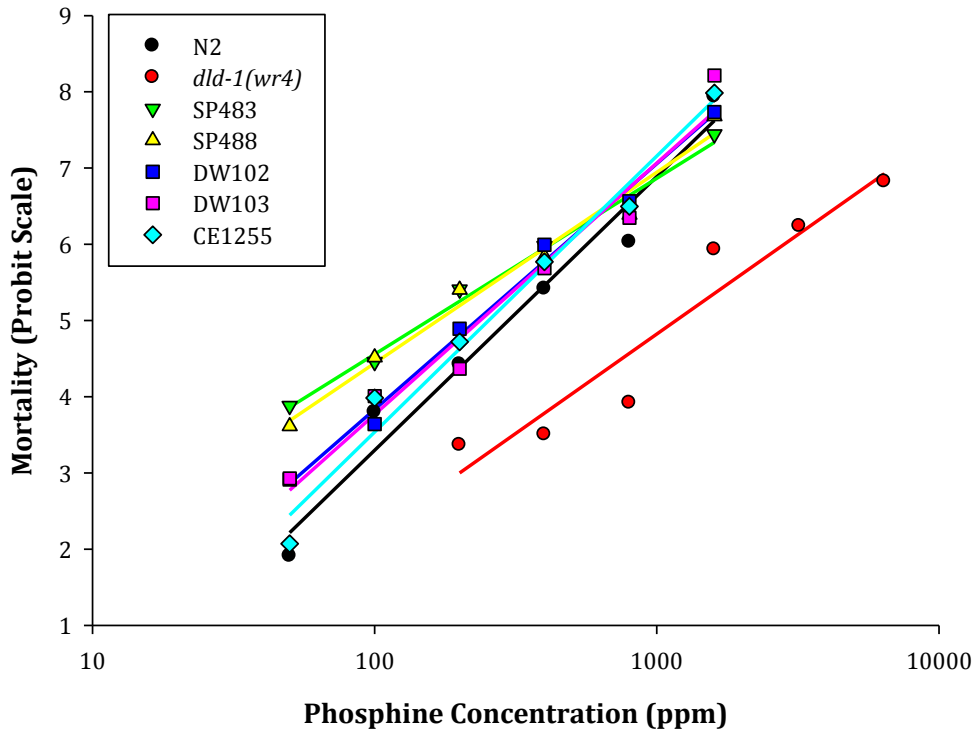

Figure 4.1: Phosphine-induced mortality in *C. elegans* strains: N2 (wild type), *dld-1(wr4)* (phosphine-resistant), SP483 & SP488 (UV-sensitive), DW102 & DW103 (ionizing radiation-sensitive), CE1255 (radiation-resistant). Mortality scoring was calculated after 48 hours recovery from 24 hours of phosphine fumigation. Fumigation was repeated three times then averaged for each concentration.

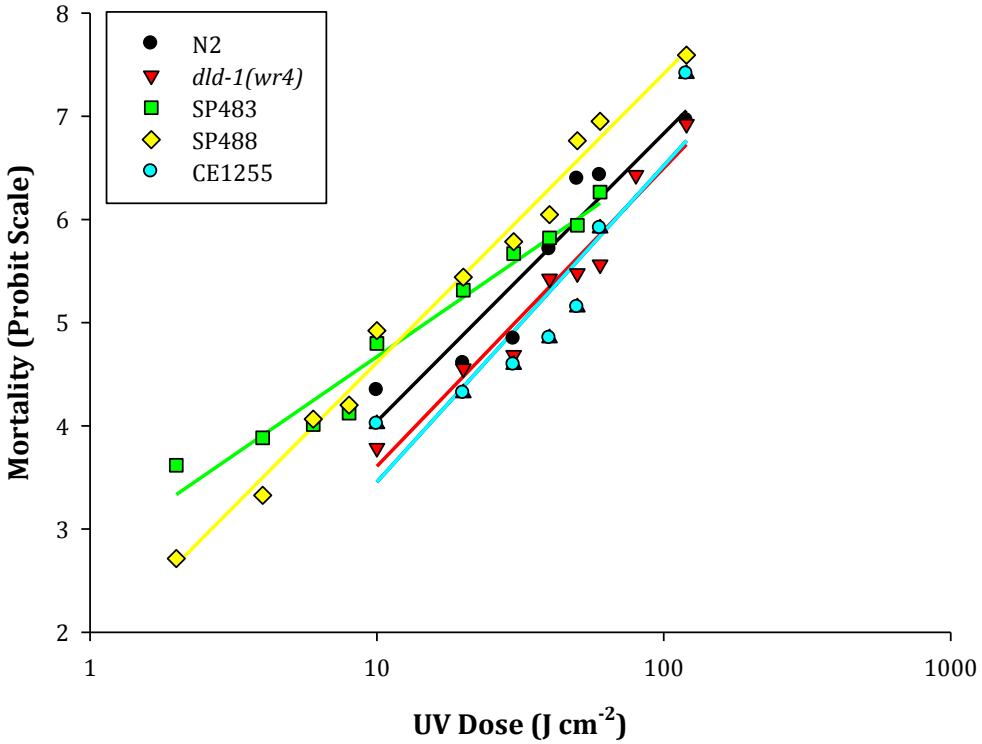

Figure 4.2: UV-induced mortality in *C. elegans* strains: N2 (wild type), *dld-1(wr4)* (phosphine-resistant), SP483 & SP488 (UV-sensitive), CE1255 (radiation-resistant). Mortality scoring was scored after 48 hours recovery from UV exposure. UV treatment was repeated three times then averaged for each dosage.

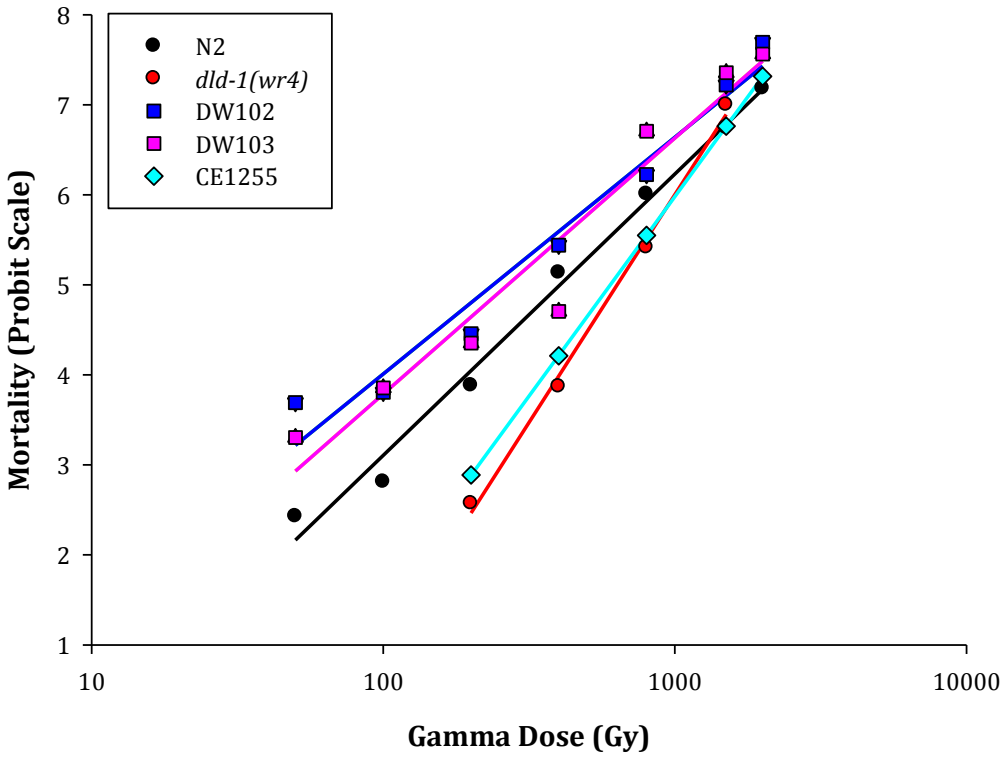

Figure 4.3: Gamma-induced mortality for the N2 (wild type), *dld-1(wr4)* (phosphine-resistant), DW102 & DW103 (ionizing radiation-sensitive), CE1255 (radiation-resistant). Mortality scoring was after 48 hours recovery from exposing L_1 nematode to doses of gamma radiation. Irradiation was repeated three times then averaged for each dose (Gy).

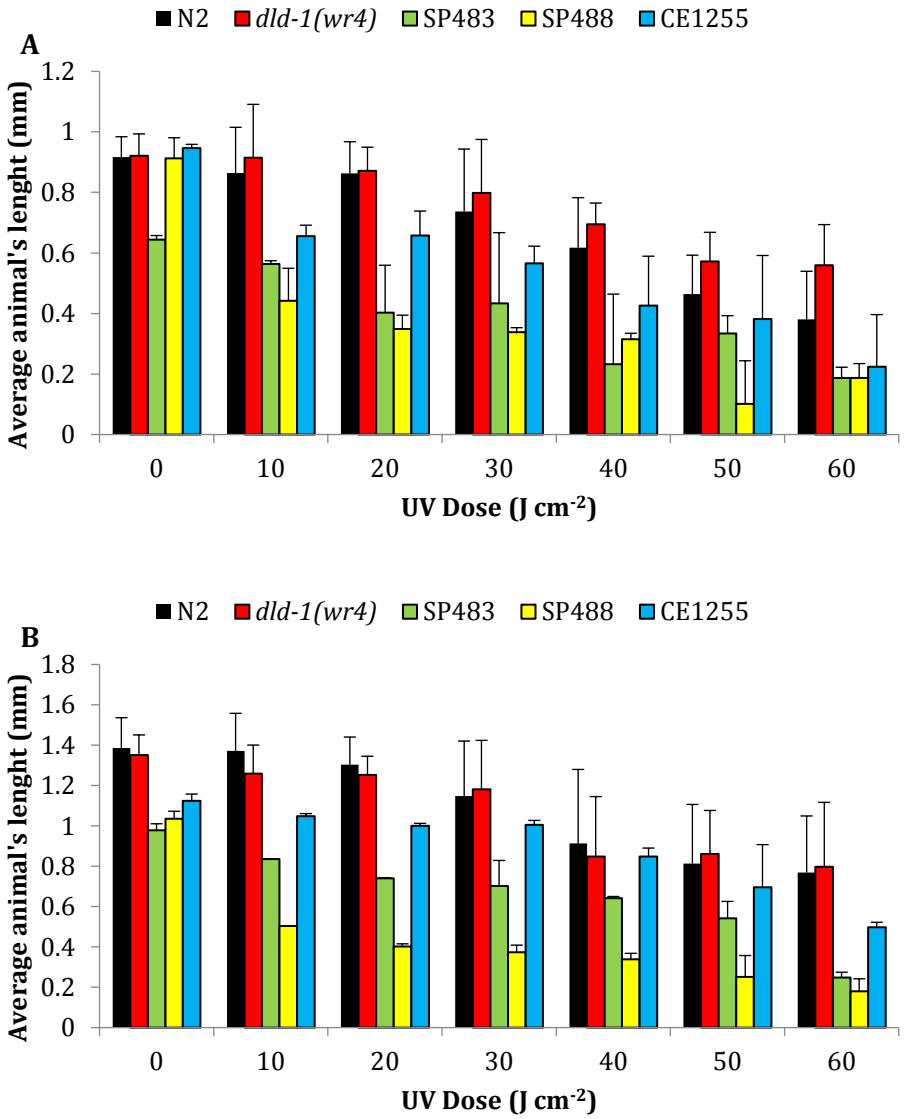

Figure 4.4: Inhibition of growth-induced by UV after (A) 48hrs and (B) 72hrs from irradiating L_1 stage. Two-way ANOVA followed by Dunnett's multiple comparisons test to compare the average animal length between N2 the wild type and the other strains, ***dld-1(wr4)*** (phosphine-resistant), SP483 & SP488 (UV-sensitive), DW102 & DW103 (ionizing radiation-sensitive) and CE1255 (resistant to radiation-induced apoptosis).

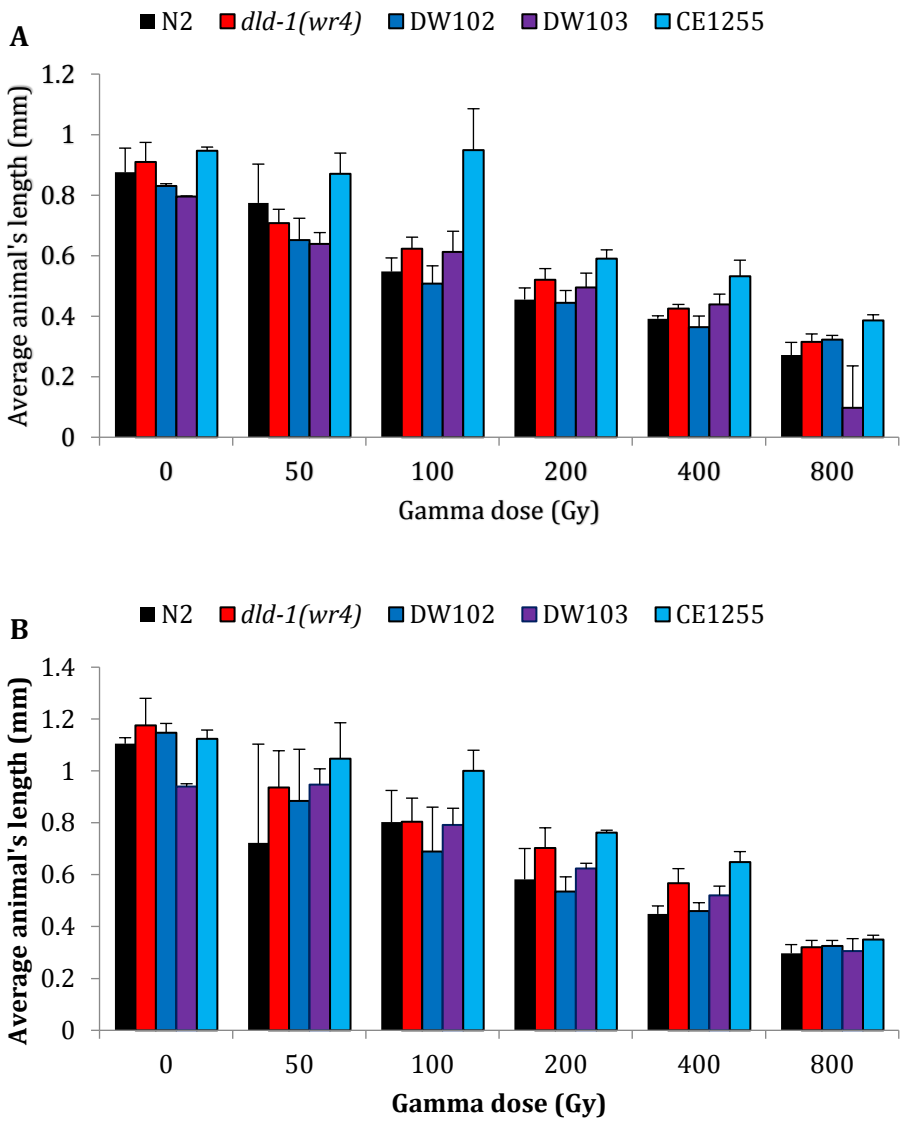

Figure 4.5: Inhibition of growth-induced by gamma irradiation after (A) 48hrs and (B) 72hrs from irradiating L_1 stage. Two-way ANOVA followed by Dunnett's multiple comparisons test to compare the average animal length between N2 the wild type and the other strains, *dld-1(wr4)* (phosphine-resistant), SP483 & SP488 (UV-sensitive), DW102 & DW103 (ionizing radiation-sensitive) and CE1255 (resistant to radiation-induced apoptosis).

Discussion

Irradiation treatments for stored products disinfestation have been employed in countries worldwide including Saudi Arabia, Brazil, China, India, Russia, France, Turkey and the United States (Hallman, 2013). The co-existence of phosphine fumigation and ionizing radiation as pest management tools in the grain storage system raises the concern of cross-resistance between the two treatments. In this investigation, we tested mutant strains of *C. elegans* that had been selected for resistance or susceptibility to either phosphine, gamma radiation or UV radiation for their response to each of the other control measures.

Phosphine is a reducing agent that interferes with cellular respiration. Exposure to phosphine can initiate oxidative stress by excessive production of reactive oxygen species (ROS) (Chaudhry, 1997; Nath et al., 2011). ROS are generated naturally as a byproduct of metabolic electron transfer reactions, notably from the mitochondrial electron transport chain (ETC). ROS react aggressively with other molecules including proteins, lipids and DNA eventually leading to cell death (Hsu et al., 2000; Hsu et al., 2002; Morrell, 2008).

Phosphine-resistance in *C. elegans* and insects is mediated by genetic modification of DLD (Schlipalius et al., 2012). The DLD enzyme is a subunit of four enzyme complexes that feed metabolites of carbohydrate and amino acids into aerobic energy metabolism (Carothers et al., 1989). In *C. elegans*, a mutation in the *dld-1* gene causes not only phosphine resistance but also a 75% decrease in aerobic respiration, monitored as a decrease in oxygen consumption (Zuryn et al., 2008). Aerobic respiration is essential to phosphine toxicity (Bond & Monro, 1967; Bond et al., 1967; Hobbs & Bond, 1989; Kashi, 1981a) and is a significant source of ROS. It is likely that the resistance of the *C. elegans* mutant is mediated by a decrease in ROS generation on exposure to phosphine as a direct result of the suppressed metabolism (Table 4.1).

The primary injurious effect of UV and ionizing irradiation on living organisms is DNA-damage. This includes single or double-strand DNA breaks (SSBs, DSBs) (Jackson & Bartek, 2009; Stergiou & Hengartner, 2004). In *C. elegans*, mutations in *lem-3* and *smk-1* genes, that encode proteins required for DNA repair, made the animals more sensitive to the genotoxic effect of UV radiation, X-rays and other DNA-damaging chemicals (Hartman, 1984; Sadaie & Sadaie, 1989). Interestingly, these mutants exhibited an elevated sensitivity to the fumigant phosphine. Mutations in *brc-1* and *brd-1* that encode heterodimers vital to DSB repair (Adamo et al., 2008; Boulton, 2006; Polanowska et al., 2006) resulted in an increased sensitivity to ionizing

radiation-induced damage as well as increased sensitivity to phosphine exposure. The hypersensitivity to phosphine gas displayed by each of the four mutants (Table 4.1) implicates phosphine as a DNA-damaging agent. This is relatively consistent with the report of phosphine causing oxidation of DNA in the brain tissue of rats that had been exposed to phosphine orally (Hsu et al., 2002).

The respond to DNA damage includes three types of proteins, sensors that can detect the damage, transducers that transmit signals throughout the cell to coordinate the response and the effectors that elicit the proper biological response (Stergiou & Hengartner, 2004). A key component of the response is to halt or delay the progression of the cell cycle to allow DNA repair prior to DNA replication and cell division. CEP-1 (the homolog of mammalian p53) is the cell cycle checkpoint protein that halts cell division, allowing DNA repair and promoting radioresistance (Wong et al., 2011). As an alternative protective pathway when DNA damage is too severe, p53 can promote the elimination of the damaged cell through apoptosis. A mutation in the p53 homolog in *C. elegans*, *cep-1* that causes resistance to radiation-induced apoptosis did not elevate phosphine resistance, but rather, increased sensitivity to exposure to low concentrations of phosphine. This contradicts the reported implication of phosphine as a factor of apoptosis in phosphine-insulted mitochondria (Anand et al., 2012; Iyanda, 2012). However, the previous studies have been performed on rats with extremely high doses of phosphine, whereas we have used much lower concentrations. Our experimental conditions may have been insufficient to trigger cell death.

Radiation causes oxidative stress by generating ROS in the damaged cells (Baverstock, 1985; Rajagopalan et al., 2002; Ye et al., 2010), leading to a similar effect caused by phosphine exposed animals (Chaudhry, 1997; Nath et al., 2011). We have demonstrated that a phosphine resistant mutant provides cross-protection against radiation-induced damage. This was previously observed in an insect pest, the lesser grain borer *R. dominica,* in which a phosphine resistant strain was more resistant to ionizing radiation than their phosphine-sensitive counterparts. The authors discuss that the phosphine resistant insects have the genetic ability to counter oxidative damage caused by phosphine, and can tolerate exposure to ionizing radiation since it has been reported to cause oxidative stress (Hasan et al., 2006). They also conclude that antioxidants would provide protection against phosphine. Antioxidants were reported in a number of studies as protecting agents from phosphine exposure. One of them is melatonin, which can attenuate phosphine-induced oxidative damage (Hsu et al., 2000; Hsu et al., 2002).

UV radiation affects DNA via reactive radicals that cause oxidative damage to the macromolecules in the cell including DNA. The antioxidant vitamins, C and E, significantly reduce the UV radiation-induced damage in mammalian skin cells (Stewart et al., 1996). In addition, even chromosome aberrations caused by double-stranded DNA breaks caused by exposure to gamma-rays are significantly reduced by the antioxidant resveratrol (Carsten et al., 2008), which also increases survival rates following gamma ray exposure of *C. elegans* (Ye et al., 2010). Evidently, both UV and gamma radiation-induced damage can be ameliorated by antioxidants. Seemingly, toxication pathways of phosphine and radiation are overlapping in oxidative damage

We also monitored growth inhibition in response to radiation exposure. This inhibition is most likely due to cell cycle arrest as a result of DNA damage as the cell cycle arrest mutant CE1255 had a normal growth rate. The cell cycle arrest is a primary defense mechanism in living organisms against radiation damage (Stergiou & Hengartner, 2004) as stopping the cell cycle allows the cell to repair the DNA, preventing the replication and inheritance of damaged DNA by the daughter cells. In our results, this stoppage was expressed in the exposed nematodes as a growth inhibition where the surviving nematodes were shorter than the unexposed worms due to a delay in their growth.

Conclusions

Radiotoxicity and phosphine toxicity both involve oxidative stress. Phosphine resistant animals are able to resist radiation-induced damage. However, a mutation that results in resistance to radiation-induced apoptosis and cell cycle inhibition does not provide resistance to phosphine. Likewise, mutations that are defective in repair of double and single stranded breaks to DNA are sensitive to gamma radiation, whereas phosphine susceptibility in not affected. In contrast, mutation of an activator of the general stress response transcription factor, DAF-16 causes greater susceptibility to both UV light and phosphine. These results indicate that phosphine poisoning does not involve DNA damage, but mutations that affect more general stress responses can exhibit cross-reactivity to both phosphine and radiation-induced damage.

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Chapter 5: Preconditioning Effect on Phosphine Toxicity: Stress Pre-conditioning with Temperature, UV, and Gamma Radiation Induces Tolerance against Phosphine Toxicity[3]

Abstract

Phosphine is the only general use fumigant for the protection of stored grain, though its long-term utility is threatened by the emergence of highly phosphine-resistant pests. Given this precarious situation, it is essential to identify factors, such as stress pre-conditioning, that interfere with the efficacy of phosphine fumigation. We used *C. elegans* as a model organism to test the effect of pre-exposure to heat and cold shock, UV and gamma irradiation on phosphine potency. Heat shock significantly increased tolerance to phosphine by 3-fold in wild type nematodes, a process that was dependent on the master regulator of the heat shock response, HSF-1. Heat shock did not, however, increase the resistance of a strain carrying the phosphine resistance mutation, *dld-1*(*wr4*), and cold shock did not alter the response to phosphine of either strain. Pretreatment with the LD_{50} of UV (18 J cm^{-2}) did not alter phosphine tolerance in wild type nematodes, but the LD_{50} (33 J cm^{-2}) of the phosphine resistant strain (*dld-1*(*wr4*)) doubled the level of resistance. In addition, exposure to a mild dose of gamma radiation (200 Gy) elevated the phosphine tolerance by ~2-fold in both strains.

Keywords: *Heat shock, Cold shock, UV radiation, Gamma radiation, Phosphine, C. elegans*

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Introduction

Phosphine (PH_3) gas is an ideal fumigant for the control of insect pests of stored commodities due to the low cost of application, ease of use and the lack of chemical residues. Phosphine is also environmentally benign as it decomposes to phosphate. These properties are not matched by any other potential fumigant, leading to heavy dependence on phosphine fumigation around the world (Collins et al., 2001). Phosphine is a metabolic poison that affects cellular respiration (Cheng et al., 2003; Zuryn et al., 2008). It may also disrupt neural acetylcholine signaling (Nath et al., 2011) or cause damage to DNA (Hsu et al., 2002). In addition, phosphine is known to cause oxidative damage (Nath et al., 2011). The diversity of potential mechanisms makes it difficult to predict interactions between phosphine and other treatments.

We have chosen to investigate the effect of pre-exposure to diverse stressors for two purposes: firstly, to increase our understanding of the toxic mechanism of phosphine, and secondly, to identify positive interactions that might be useful in practice to improve the efficacy of phosphine. In this work, we use the free-living nematode *Caenorhabditis elegans* as a model organism to investigate the effect of pretreatment with heat, cold, UV and gamma radiation on phosphine sensitivity. Each of these treatments has been used commercially to protect stored commodities except UV radiation. High temperature is used to control pest infestation whereas cooling of grain in warm climates is typically used to suppress growth and reproduction of pest insects to slow infestation (Fields & White, 2002). Gamma irradiation is used on a limited scale as a quarantine treatment for stored grain (Follett, 2004; Follett et al., 2013; Ignatowicz, 2004).

These stressors may interact with phosphine toxicity and genetic resistance to phosphine in a variety of ways. These include hormesis, a phenomenon where a living organism acquires tolerance to a stressor following challenge with a sublethal dose/concentration of the same or a different stressor (Cypser & Johnson, 2002). In addition, synergism, cross-resistance, and sensitization to phosphine have each been observed under various conditions that are discussed below.

The response to heat stress has been more exhaustively studied and in a wider range of species than has any other stress response. Pretreatment with heat increases the thermotolerance of *C. elegans* and results in an extended lifespan (Cypser & Johnson, 2002). Heat shock, where organisms are expose to lethal high temperature for a short nonlethal period, also enhances the resistance to a variety of chemical and physical stressors (Wang et al., 2004). Pre-exposure to

cold stress can also induce tolerance to subsequent stress in the exposed organism (Le Bourg, 2007). The protective response that is induced by pre-exposure to extreme temperature is mediated by the production of heat shock proteins (*HSP*s). These proteins protect cells from not only extreme temperature but also other stressors (Moskalev et al., 2009; Wang et al., 2004; Yanase et al., 1999).

Exposure of *C. elegans* to ultraviolet radiation at low doses inhibits their fertility and at high doses is lethal (Hartman, 1984). When wild type *C. elegans* is exposed to 40 J m^{-2} of UV light, the survival rate is 2.4% (Murakami & Johnson, 1996). The nematodes that do survive exhibit an increase in lifespan, indicating that exposure to UV light has triggered a protective defense mechanism. A mild dose of UV also induces a protective response in *C. elegans* against oxidative damage caused by exposure to heavy metals (Wang et al., 2010). Furthermore, exposure of *C. elegans* to UV radiation early in development inhibits aerobic respiration throughout development as determined by decreased oxygen consumption (Leung et al., 2013).

Cross-resistance between phosphine and gamma radiation has been observed in a phosphine-resistant strain of the lesser grain borer relative to its susceptible counterpart. The resistant strain was able to withstand the DNA damage caused by gamma irradiation as assessed by single-cell electrophoresis (comet assay). Furthermore, cross-adaptation of *Drosophila* sp. to heat and oxidative stress was observed after pretreatment with gamma radiation. The flies became more tolerant of oxidative stress induced by superoxide radical (O^{-}_{2}) after exposing to gamma rays at an early life stage (Moskalev et al., 2009).

In this paper, experiments were designed to identify major stress response pathways that interact with the response to phosphine exposure in either phosphine susceptible or resistant animals. The approach was to subject *C. elegans* to a shock of lethal magnitude, but for a sublethal period. The response to a subsequent exposure to phosphine was then monitored relative to the unshocked control to identify preconditioning effects. We test high and low temperature stress as well as exposure to UV and gamma radiation in both a wild type and a phosphine-resistant strain. We find that preconditioning due to heat stress is mediated through heat shock response factors.

Materials and Methods

C. elegans strains and culture conditions:

The strains of *C. elegans* used in this study are the phosphine susceptible wild type strain N_2 and the phosphine-resistant strain *dld-1*(*wr4*) (Cheng et al., 2003). Also, three strains with a genetically modified heat shock response, The three strains, (RB791 (*hsp-16.48*), RB1104 (*hsp-3*) and PS3551 (*hsf-1*)), were provided by the *C. elegans* Genetic center (CGC), which is funded by the National Institutes of Health (NIH) Office of Research Infrastructure Programs (P40 OD010440). Before phosphine treatment, Synchronized L_1 worms were prepared as previously described in (Stiernagle, 1999). The L_1 nematodes were cultured on 6 cm NGM agar plates (0.3 % NaCl, 0.25 % peptone, 5 mg/ml cholesterol, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1.7 % agar) seeded with *E. coli* strain OP50 and grown at 20 °C for 24 hours (L_2 stage) or 48 hours (L_4 stage).

Chemical:

The phosphine gas used in these experiments was generated from aluminum phosphide tablets (570g/kg aluminum phosphide, BEQUISA Co. (GASTION), Brazil). A fragment of aluminum phosphide tablet was placed in one liter of 5% sulfuric acid in a Valmas chamber (Valmas & Ebert, 2006). The gas was collected in an air-tight receptacle sealed with silicon septum that allowed gas to be withdrawn with a syringe.

Pretreatments

Heat and cold shock

Prior to phosphine fumigation, developmentally synchronized L_4 stage *C. elegans* were incubated on NGM agar for 4 hours at 30 °C. In the case of cold shock, the nematodes were maintained in an incubator at 10±0.5 °C for 4 hours. The stressed nematodes were then moved to the normal temperature of 20 °C for 4 hours.

Ultraviolet radiation

UV irradiation was carried out as described by Wang et al. (Wang et al., 2010). Synchronized L_2 stage nematodes of wild type (N2) and phosphine-resistant (*dld-1*(*wr4*)) strains were irradiated with 18 and 33 J cm^{-2} UV light respectively (XLE-Series UV crosslinker, Spectronics Co.). These doses represent the LD_{50} for each strain (Figure 4.2, Table 4.1). After irradiation, the worms were allowed to recover at 20 °C for 24 hours at which point they had reached the L_4 stage and were ready for phosphine fumigation as described below.

Gamma radiation

L_1 stage nematodes of wild type (N2) and phosphine-resistant (*dld-1*(*wr4*)) strains were irradiated with gamma rays as described in (Cypser & Johnson, 2002). The gamma dosage was 200 Gy utilizing a cobalt-60 Gammacell-220 irradiator (Atomic Energy of Canada Ltd.). Subsequent to gamma irradiation, the nematodes were incubated at 20 °C for 48 hours to reach the L_4 stage for the phosphine fumigation.

Phosphine fumigation

Phosphine exposure was carried out as previously described in (Valmas & Ebert, 2006) and in the previous chapters. The concentrations of gas that were used were 0, 50, 100, 200, 400, 600, 800, 1000, 1200,1600, 2000, 2500, 3200 and 6400 ppm. Fumigations were carried out for 24 hours, in line with established resistance monitoring protocols in pest insects. Following the fumigation, the nematodes were transferred to fresh air to recover for 48 hours.

Statistical analysis

Each experiment was repeated three times, and each trial contained two replicates per strain per phosphine concentration. After the forty-eight hour recovery period, Automated WormScan was utilized for mortality scoring as described in (Mathew et al., 2012; Puckering et al., 2017). Briefly, the treated worms in the six centimeters plates were scanned and the individuals that did not respond to the light stimulus in the ten minutes period between scans were scored as dead. To determine the median lethal concentrations (LC_{50}) of phosphine, probit analysis was carried out on exposures that resulted in 0.1% to 99.9% average mortality (Finney, 1971). The analysis and the graphs' generation were carried out using the software

LdP Line (copyright 2000 by Ehab Mostafa Bakr, Cairo, Egypt). The effects of pretreatments on the LC_{50} of phosphine were computed by one-way ANOVA followed by Dunnett's multiple comparisons. An unpaired t-test was performed to compare the heat shock effect on phosphine resistance in each of the heat shock mutants. These comparative tests were performed using GraphPad Prism (version 7.03 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com).

Results

The following experiments were designed to identify major stress response pathways that interact with the response to phosphine exposure in either phosphine susceptible or resistant animals. The approach was to subject *C. elegans* to a shock of lethal magnitude, but for a sublethal period. The response to a subsequent exposure to phosphine was then monitored relative to the unshocked control to identify pre-conditioning effects.

Heat shock

Whereas the wild type animals showed strong heat shock preconditioning, the phosphine-resistant *dld-1(wr4)* mutant exhibited a mild increase in resistance that was not statistically significant. Without heat shock, the wild type animals showed a normal concentration-dependent response to phosphine exposure - an LC_{50} of 229 ppm after fumigation for 24 hours at 20 °C. On the other hand, if wild type animals were given a 4-hour heat shock at 30 °C, then allowed to recover for four hours prior to fumigation, the LC_{50} increased to 625 ppm phosphine. The 2.7 fold induced tolerance was statistically significant at $p = 0.05$ (Figure 5.1, Table 5.1). In contrast, exposing the phosphine-resistant strain to the same 30 °C heat shock before fumigation resulted in a statistically insignificant increase in the LC_{50} from 1227 ppm in the absence of heat shock to 1456 ppm when heat shock was applied (Figure 5.1, Table 5.1). The slope of the response curve of the wild type strain decreased following heat shock, indicating an increase in phenotypic diversity within the population. The result was a slope that was similar to that of the phosphine response curves of the resistant strain, either with or without

heat shock. These slopes differed markedly from the response of the wild type strain to phosphine in the absence of heat shock (Figure 5.1 and 5.2, Table 5.1).

We then tested the effect of mutations in two heat shock response effector genes on the heat shock-induced tolerance toward phosphine. As with the wild type strain, the *hsp-16.48* mutant strain (RB791) showed increased tolerance to phosphine after heat shock. The LC_{50} of this strain toward phosphine in the absence of heat preconditioning was 271 ppm, whereas after exposure to a 30 °C shock the LC_{50} almost doubled to 539 ppm (Figure 5.1B, Table 5.1). Similarly, the *hsp-3* strain (RB1104) showed induced resistance due to heat preconditioning, but the magnitude of the response was only 1.4-fold, from an LC_{50} of 596 ppm to 854 ppm phosphine (Figure 5.1C, Table 5.1). The slope of the response curve, once again, was shallower when individuals of this strain were exposed to heat shock preconditioning. On the contrary, elimination of the master regulator of the heat shock response, HSF-1, completely eliminates the heat shock preconditioning effect. Thus, the LC_{50} of the *hsf-1* mutant strain (PS3551) was 437 ppm phosphine in the absence of heat shock at 30 °C and 444 ppm phosphine following heat shock, which is statistically indistinguishable (Figure 5.1A, Table 5.1).

Cold shock

The pre-treatment of wild type animals with a 4-hour cold shock at 10 °C resulted in no significant increase in the LC_{50} (304 ppm) in response to phosphine relative to the LC_{50} of the untreated control (229 ppm). Unlike heat shock, cold shock of the phosphine-resistant strain may have caused slight sensitization to phosphine, with a decrease in the LC_{50} from 1227 to 1044 ppm, though the effect did not reach statistical significance (Figure 5.3, Table 5.1).

Ultraviolet radiation

The response to UV radiation manifests over an extended period. To determine whether exposure to UV light results in induced preconditioning to phosphine. L_2 stage nematodes, wild type, and mutant were given a burst of UV radiation at their respective LD_{50} values, 18 and 33 J cm^{-2} (Figure 4.2, Table 4.1), after which they were allowed to grow under standard conditions to the L_4 stage (Stiernagle, 1999). UV pretreatment did not affect the response to phosphine of

the wild type strain. The LC_{50} for the wild type control was 195 ppm, which increased to 266 ppm following UV pre-treatment, but the difference was not statistically significant. On the contrary, the phosphine-resistant strain showed pre-conditioning against phosphine toxicity in response to UV exposure. The LC_{50} with UV pre-treatment significantly increased to 2607 ppm from 1291 ppm without UV pre-treatment (Figure 5.4 and 5.5, Table 5.2).

Gamma radiation

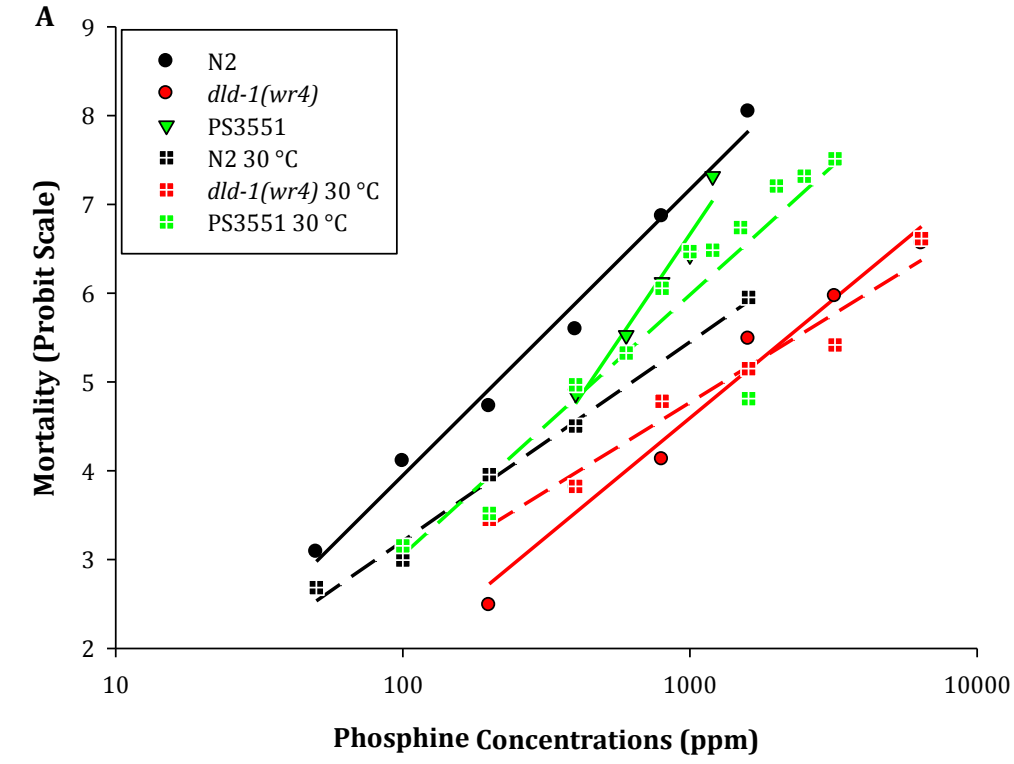
Wild type nematodes exposed to a pre-treatment of 200 Gy of gamma radiation show an increase in the LC_{50} of about 1.4-fold compared to nematodes that have not been pre-treated with gamma radiation. The LC_{50} rose from 195 to 346 ppm in response to pre-treatment, which was statistically significant. In the phosphine-resistant strain, the same effect was observed with a slightly greater magnitude. Gamma radiation pre-treatment increased the LC_{50} to phosphine 2-fold compared to nematodes that had not been pre-treated, with an increase in LC_{50} from 1291 to 2518 ppm. Once again, the observed difference was statistically significant (Figure 5.4 and 5.5, Table 5.2). In both strains, the slopes of the response curves became significantly shallower in response to gamma radiation pre-treatment.

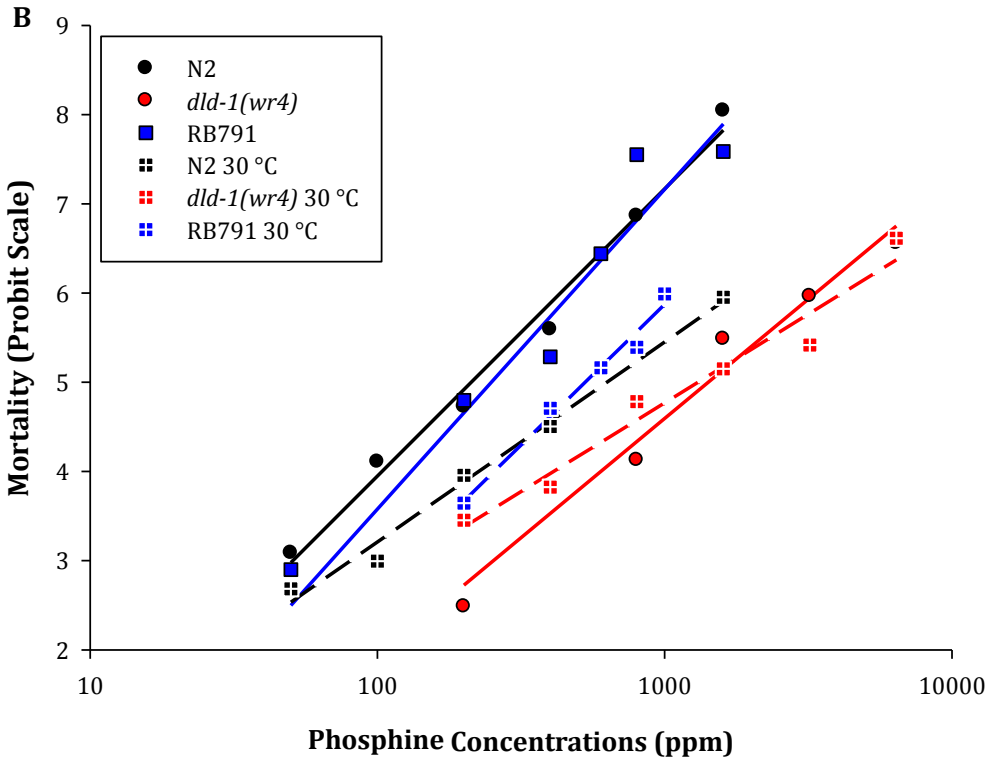
Table 5.1: Phosphine LC_{50} values and resistance factor for *C. elegans* strains with and without preconditioning. One-way ANOVA followed by Dunnett's multiple comparison test was used to identify significant differences in phosphine LC_{50} values between pretreated and unpretreated animals for the wild type and *dld-1(wr4)* strains. An unpaired t-test was used to compare the LC_{50} values between pretreated or unpretreated heat shock response mutants, PS3551, RB1104, and RB791.

Strain	Pre-treatment temperature (°C)	LC_{50} Phosphine (ppm)†	Slope±SE	X^2	R	RF‡
Wild type (N2)	Not pre-treated	229 (206-255)	2.88±0.17	3.12	0.99	
	30	625 (534-749)****	2.28±0.18	1.24	0.99	2.7
	10	304 (181-518)	1.96±0.13	22.47	0.93	1. 3
Phosphine-resistant *(dld-1(wr4))*	Not pre-treated	1227 (1064-1409)	2.47±0.17	5.35	0.99	
	30	1456 (927-2266)	1.96±0.14	7.89	0.98	1.2
	10	1044 (892-1221)	1.75±0.14	2.44	0.99	0.85
PS3551 (*hsf-1*)	Not pre-treated	444 (397-485)	4.42±0.38	1.82	0.98	
	30	437 (401-473)	3.41±0.19	11.68	0.99	
RB1104 (*hsp-3*)	Not pre-treated	596 (544-653)	3.29±0.27	1.48	0.99	
	30	854 (763-962)***	1.83±0.14	9.42	0.98	1.4
RB791(*hsp-16.48*)	Not pre-treated	271 (206-344)	3.78±0.24	22.48	0.97	
	30	539 (492-589)****	3.17±0.29	3.16	0.99	2

† ***$p < 0.001$, ****$p < 0.0001$, ppm parts per million

‡ Resistance Factor


A
N2
dld-1(wr4)
PS3551
N2 30 °C
dld-1(wr4) 30 °C
PS3551 30 °C
Mortality (Probit Scale)
Phosphine Concentrations (ppm)


B
N2
dld-1(wr4)
RB791
N2 30 °C
dld-1(wr4) 30 °C
RB791 30 °C
Mortality (Probit Scale)
Phosphine Concentrations (ppm)

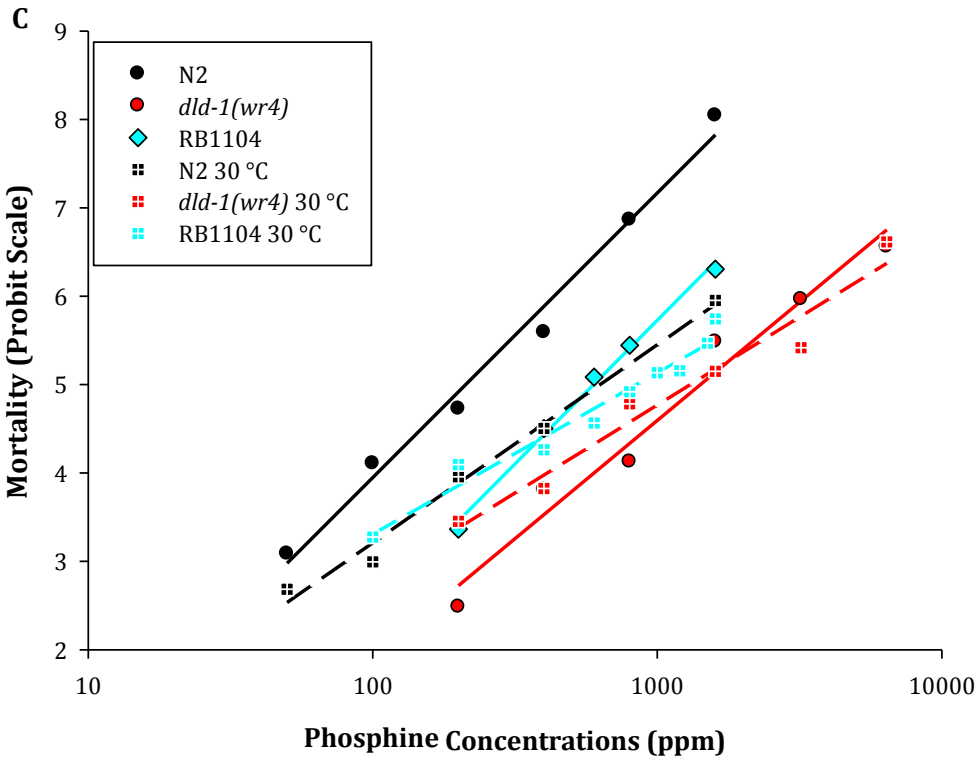


Figure 5.1: Heat shock preconditioning against phosphine-induced mortality of *C. elegans* in wild type, phosphine-resistant, and heat shock response mutants. A four-hour heat shock at 30°C was followed by a 4 hour recovery period, after which the nematodes were subjected to 24 hour exposure to phosphine. Mortality was scored after a further 48 hour recovery period, either without or with heat shock preconditioning. Wild type (N2), phosphine-resistant (*dld-1(wr4)*). (A) PS3551 (*hsf-1*) (B) RB791 (*hsp-16.48*) (C) RB1104 (*hsp-3*).

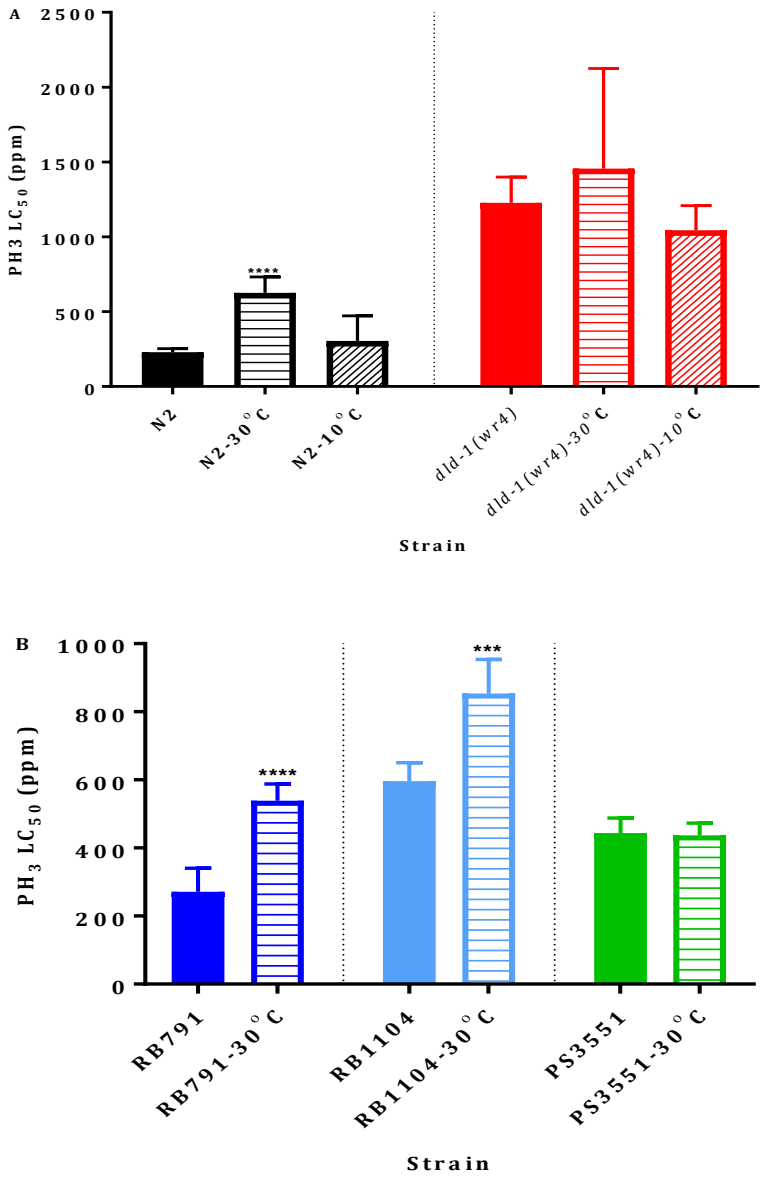


Figure 5.2: Analysis of LC_{50} value for Heat shock preconditioning against phosphine-induced mortality of *C. elegans*. (A) Comparison of the wild type and the phosphine-resistant mutant and (B) heat shock mutants either without or with heat or cold preconditioning. A four-hour heat shock at 30°C was followed by a 4 hour recovery period, after which the nematodes were subjected to phosphine exposure for 24 hours. Mortality was scored after a further 48 hour recovery period. The LC_{50} value for each strain is shown, either without or with heat shock preconditioning. Error bars represent the 95% confidence intervals for each LC_{50} data point. One-way ANOVA followed by Dunnett's multiple comparisons for the LC_{50} values, ****$p < 0.0001$. Wild type (N2), phosphine-resistant (*dld-1(wr4)*). Unpaired t-test was used to compare the LC_{50} values of each heat shock mutant strain, ****$p < 0.0001$, ***$p < 0.001$. RB791 (*hsp-16.48*), RB1104 (*hsp-3*) and PS3551 (*hsf-1*).

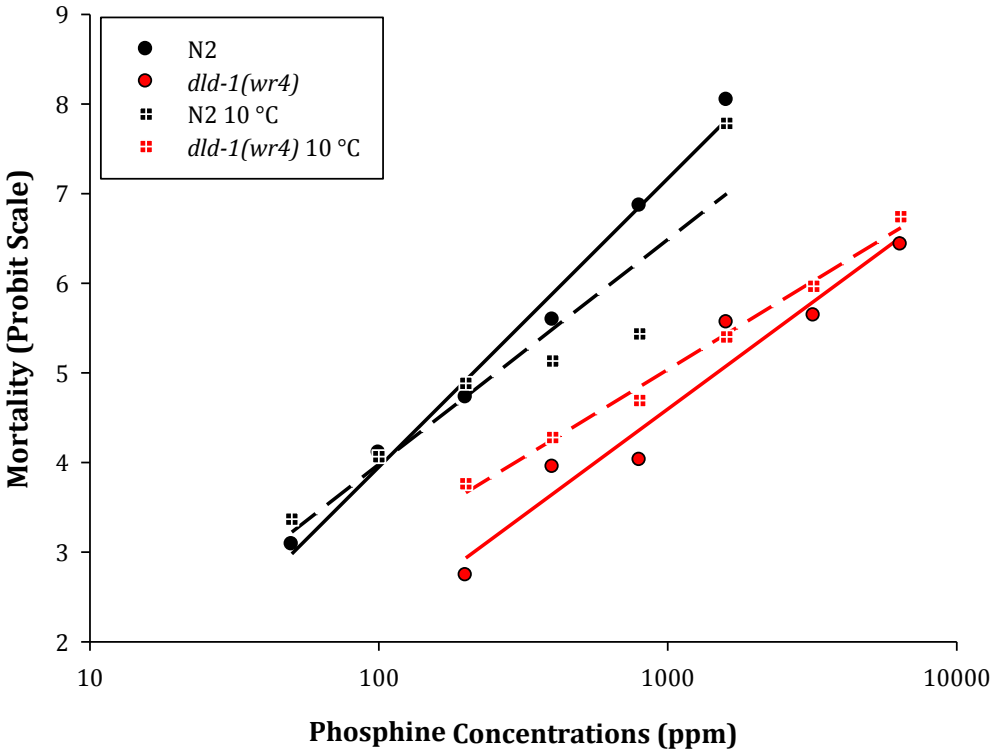


Figure 5.3: Effect of cold shock on phosphine-induced mortality of wild type *C. elegans* and the phosphine-resistant *dld-1* mutant. A four-hour cold shock at 10°C was followed by a 4 hour recovery period, after which the nematodes were subjected to 24 hour exposure to phosphine. Mortality was scored after a further 48 hour recovery period. Wild type (N2), phosphine-resistant (*dld-1(wr4)*).

Table 5.2: Phosphine LC_{50} values and resistance factor for *C. elegans* strains with and without radiation preconditioning. One-way ANOVA followed by Dunnett's multiple comparisons to compare the LC_{50} with the pretreatments LC_{50}s for the wild type and *dld-1(wr4)*.

Strain	Pre-treatment	LC_{50} Phosphine (ppm)†	Slope±SE	X^2	R	RF‡
Wild type		195 (115-276)	2.46±0.20	9.75	0.98	
	UV	266 (228-312)	1.96±0.13	4.84	0.98	1.4
	γ-rays	346 (294-404)**	1.80±0.16	3.91	0.99	1.8
Phosphine-resistant (*dld-1(wr4)*)		1291(1130-1476)	2.12±0.14	7.35	0.97	
	UV	2607 (1950-3705)**	2.48±0.186	11.37	0.99	2
	γ-rays	2518 (2098-3108)**	1.51±0.13	3.47	0.99	2

† ***$p < 0.001$, ****$p < 0.0001$, ppm parts per million
‡ Resistance Factor

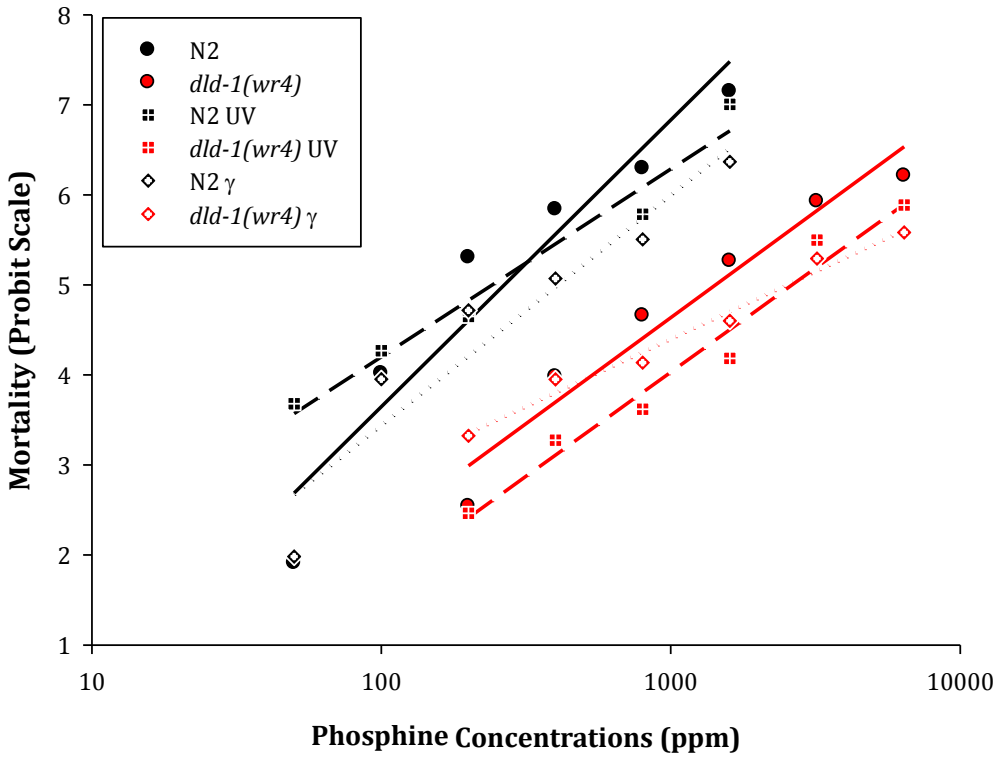


Figure 5.4: Effect of UV light and gamma radiation on phosphine-induced mortality of wild type *C. elegans* and the phosphine-resistant *dld-1* mutant. *C. elegans* were exposed to 18 and 33 J cm^{-2} UV radiation or 200 Gy gamma radiation 24 hours, after which the nematodes were subjected to 24 hour phosphine exposure. Mortality was scored after a further 48 hour recovery period. Wild type (N2), phosphine-resistant (*dld-1(wr4)*).

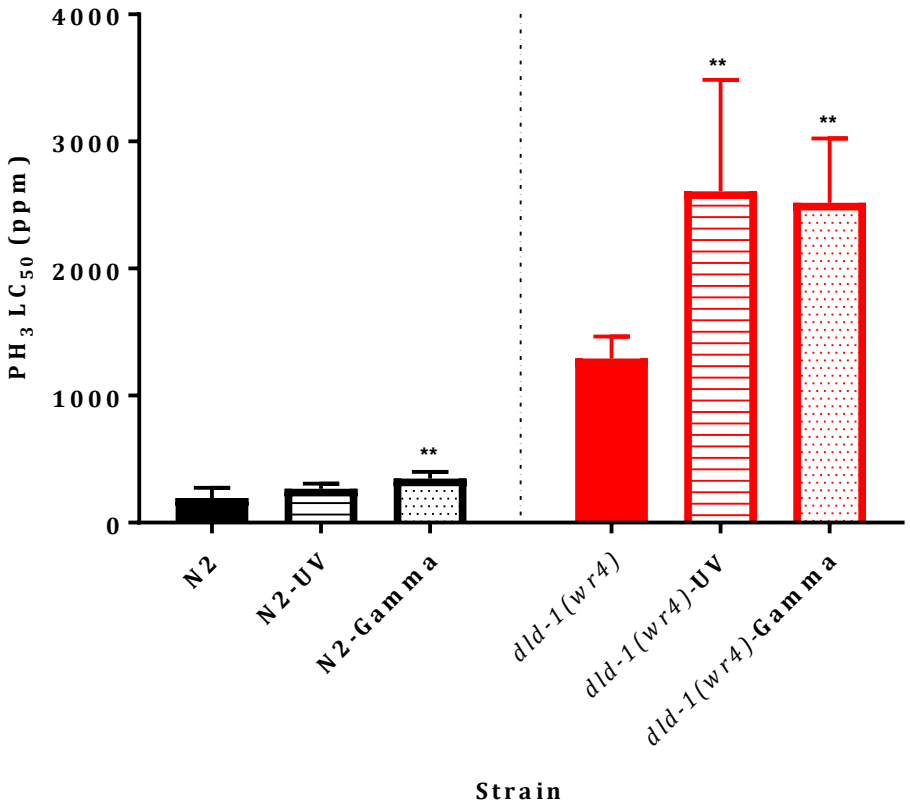


Figure 5.5: Effect of UV and gamma radiation on phosphine-induced mortality of *C. elegans* in wild type and the phosphine-resistant *dld-1* mutant. Nematodes were exposed to a range of dosages of either UV light or gamma radiation at the L_1 stage. Mortality was assessed 48hrs after exposure as lack of movement in response to a bright light stimulus. LC_{50} values are shown, and the error bars represent the 95% confidence intervals for each LC_{50} data point. Wild type (N2), phosphine-resistant (*dld-1(wr4)*). One-way ANOVA followed by Dunnett's multiple comparison test for the LC_{50} values, **$p < 0.01$.

Discussion

The adaptive response of organisms to stress has been widely studied, including communication between and cross-induction of stress response pathways (Cypser & Johnson, 2002; Moskalev et al., 2009; Wang et al., 2010; Yanase et al., 1999). In this research, we tested the ability of four distinct stresses to induce cross-resistance against the agriculturally important fumigant phosphine, with the goal of gaining a deeper understanding of how resistance is mediated. We monitored the effect of preconditioning against the toxicity of phosphine in a wild type strain of *C. elegans* (N_2), as well as in a phosphine-resistant mutant (*dld-1(wr4)*). The orthologue of the *dld-1* gene is also a major phosphine resistance factor in pest insects of stored grain, making these studies of stress-induced tolerance of practical importance to pest control.

Whereas exposure to cold did not alter the response to phosphine, we found that pre-treatment with heat, UV light and gamma radiation each altered the response of the nematodes to subsequent exposure to phosphine gas, but in unique ways. We chose heat shock-induced preconditioning for further study because it has been exhaustively investigated and because heat stress is a condition likely to be encountered by pest insects in the field. We initially screened seven strains that had been mutated in various heat shock response genes to identify genes important to heat shock pre-conditioning against phosphine toxicity. Three mutants were selected for further study, as heat pre-conditioning influenced their responses to phosphine, each in a unique way. Two of the strains carried a mutation in one of the heat shock response effector genes, whereas the third had a mutation that disrupted HSF-1, the master regulator of the heat shock response (Brunquell et al., 2016) (Appendix II, S Table 2).

When organisms are exposed to heat shock, their cells produce heat stress defense proteins as an adaptive mechanism (Parsell & Lindquist, 1993). The expressed proteins provide not only protection against heat stress, but also to a range of other stressors as well. Thus, heat shock is known to induce cross-tolerance to secondary abiotic stresses (Wang et al., 2004). We observe that heat pre-treatment made wild type nematodes more resistant to phosphine by 2.7-fold. The phosphine-resistant strain, however, did not exhibit heat-shock induced preconditioning against phosphine. This result suggests that the heat-shock inducible defenses that lead to phosphine resistance are constitutively up-regulated in the strain carrying the phosphine-resistance mutation *dld-1*(*wr4*) that was used in this study. The fact that strongly phosphine resistant pest insects also carry mutations in the *dld-1* orthologue suggests that heat stress may

induce resistance in susceptible insects, but is unlikely to exacerbate the resistance phenotype in insects that already exhibit strong resistance.

When an organism is exposed to heat stress, the endoplasmic reticulum (ER) environment is disturbed, an event that can result in interruption of the protein folding process. The accumulation of unfolded proteins in the ER triggers the unfolded protein response (UPR). One of the primary processes of the UPR is the upregulation of chaperones that will bind to the unfolded proteins and prevent their transport (Ma & Hendershot, 2001). *HSP* genes are transcriptionally upregulated in response to ER stress (Shen et al., 2001). There is also a distinct UPR in the mitochondria that includes a unique set of chaperones that protect against protein unfolding in that cellular compartment (Yoneda et al., 2004).

We included in this study, three strains each of which contained a mutation in one of three genes, *hsp-16.48*, *hsp-3* and *hsf-1*. The HSP-16 protein is a member of the α-crystallin family of small heat shock proteins (sHSPs). These proteins are strongly induced in *C. elegans* in response to heat stress and contribute to stress resistance and longevity (Kourtis et al., 2012; Morley & Morimoto, 2004). One of the effects of phosphine poisoning is necrosis of tissues in the exposed organism (Saleki et al., 2007). Kourtis et al. (Kourtis et al., 2012) concluded in their study that a single sHSP is sufficient to protect against necrotic insults, it may be that phosphine-induced necrosis is prevented by upregulation of chaperones, thereby increasing tolerance toward phosphine.

We have demonstrated a clear relationship between the heat shock response and the induction of resistance to phosphine. Heat shock is able to induce phosphine resistance in wild type nematodes, but only in the presence of HSF-1. This supports the notion that the induced resistance to phosphine occurs through the heat shock response system. Heat shock is, however, unable to further increase the resistance level of the phosphine resistant strain. It is interesting to note that reactive oxygen species (ROS) are the mediators of phosphine toxicity (Nath et al., 2011). ROS can also induce the heat shock response and the response itself protects against the damaging effects of ROS. In the wild type animals, it seems that induction of anti-ROS defenses provides a significant level of resistance against phosphine exposure. Our results also indicate that the heat shock response is either constitutively activated in the resistant strain or that an alternative anti-ROS defense is used that makes induction the heat shock response redundant. An alternative explanation is that phosphine does not induce ROS

generation in the mutant, which would simply make the heat shock inducible anti-ROS defense system unnecessary (Baler et al., 1993; Nishizawa et al., 1999).

In the case of cold shock, we observed no significant difference in phosphine sensitivity. This could be due to the fact that 10 °C is within the normal temperature range that *C. elegans* experiences in the environment (Félix & Braendle, 2010), which makes it not stressful enough to trigger a temperature stress defense mechanism.

Pretreatment with ultraviolet radiation has no hormetic effect on phosphine resistance in wild type nematodes, which is consistent with previous findings (Cypser & Johnson, 2002). Their proposed explanation is that *C. elegans* is a soil-borne organism that is not exposed to damaging amounts of UV radiation in its natural habitat. As a result, there was insufficient selective pressure to drive the evolution of a genetic response to resist UV stress. Others (Wang et al., 2010), however, have reported that pretreating *C. elegans* with UV light increases the resistance of worms to neurotoxic metals and decreases the level of oxidative stress resulting from exposure to these metals. It is important to note that the authors assessed the effect of these neurotoxins on the locomotory behavior, whereas we assessed the effect on mortality due to phosphine exposure. As a result, the two results are not directly comparable.

In this research, pre-treatment with gamma radiation produced cross-protection against phosphine in the wild type strain (1.4-fold) as well as the phosphine-resistant mutants (2-fold). Similar to UV exposure, gamma radiation pretreatment of the phosphine-resistant mutants has doubled their resistance to phosphine. As with heat shock, gamma radiation can trigger living cells to produce heat shock proteins and these proteins are responsible for cross-tolerance to a variety of stressors (Moskalev et al., 2009), though our experiments do not rule out alternative explanations.

Controlled storage temperature and gamma radiation are used to disinfest stored products of insect pests. In addition, the insects may encounter temperature extremes and exposure to UV light in the environment. We find that these stresses can significantly affect the efficacy of phosphine fumigation and we identify a stress response pathway through which tolerance to phosphine can be induced. Our findings can contribute to more effective phosphine fumigation by taking into account any planned or unplanned pre-exposure to environmental stresses.

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Chapter 6: General Discussion and Conclusion

Continuous use of phosphine, due to the lack of suitable alternatives for fumigating stored grain, has resulted in highly resistant insect pests. Therefore, understanding phosphine's mode of action and the resistance mechanisms motivated this work, to contribute to the protection of stored grain. The precise mode of action of phosphine is still not fully understood. The mode of action and mechanisms of phosphine resistance are discussed in regard to the findings of this study.

Energy crisis and oxidative stress as a phosphine mode of action

The results of this work relate phosphine toxicity strongly with energy metabolism, ensuring phosphine as metabolic poison (Anand et al., 2012; Chaudhry, 1997; Nath et al., 2011; Sciuto et al., 2016). The correlation between phosphine toxicity and energy metabolism was clear since agents like temperature and oxygen that enhance metabolism through increasing oxygen consumption, increase the toxicity of phosphine as well.

Phosphine in the absence of oxygen is not toxic (Kashi, 1981a), this makes oxygen an essential partner in phosphine toxication. Exposure of living cells to phosphine can result in the production of the oxygen free radical superoxide (O_2^-) (Nakakita & Kuroda, 1986; Pratt, 2003), which causes cellular oxidative damage. Culturing *C. elegans* at the optimal growth temperature stimulates elevated energy metabolism to achieve that growth. In the presence of phosphine, this elevated energy metabolism will accelerate the generation of reactive oxygen species such as superoxide.

The apparent involvement of energy metabolic rate in phosphine toxicity aligns with the discovery that the DLD enzyme is a phosphine resistance factor (Schlipalius et al., 2012). PDC contains the DLD enzyme as a subunit, and represent a linkage between aerobic and anaerobic respiration (Kim et al., 2006; Sugden & Holness, 2003). The ability of PDC to control the flow of metabolites from glycolysis to the TCA cycle, allows it to act as a switch between active and suppressed aerobic respiration, as occurs during hibernation in mammals (Andrews, 2007). The ability to toggle between anaerobic and aerobic respiration could explain the relationship between DLD and resistance to phosphine as the mutation in DLD could predispose PDC to restrict the rate of aerobic respiration, which is an observed characteristic of the *dld-1*(*wr4*) mutant strain.

The product of the DLD enzyme is NADH, which feeds electrons into the mitochondrial ETC. The mitochondrial ETC is reported to be the site of action of respiratory uncouplers as well as the primary site of oxygen consumption, both of which can synergize phosphine toxicity. During electron flow through the ETC to molecular oxygen, the superoxide radical O_2^- is generated, but in amounts that are not damaging to the cell. The presence of uncouplers increases the flow of electrons through the ETC resulting in an increase in the amount of the superoxide byproduct that is produced. The DLD enzyme itself is also a significant source of ROS. When oxygen levels are elevated, the potential for generating partially reduced oxygen such as superoxide from either the ETC or DLD is increased. Inhibiting DLD will likewise inhibit its associated enzyme complexes such as PDC, as well as the flow of electrons through the ETC, hence, DLD plays a major role in phosphine toxicity/resistance (Anand et al., 2012; Chen et al., 2015; Koçak et al., 2015; Oppert et al., 2015; Park et al., 2008; Schlipalius et al., 2008; Schlipalius et al., 2012; Valmas et al., 2008; Zuryn et al., 2008).

The phosphine resistant nematodes of *C. elegans* have a mutation in the *dld-1(wr4)* gene that encodes the DLD enzyme. The mutation causes a decline in energy metabolism as indicated by a low rate of oxygen consumption in the resistant mutant. With the low oxygen consumption, the mutation naturally produces lesser amounts of ROS, which makes the interaction of phosphine with PDC less toxic. This is consistent with my observation that oxygen is not an effective phosphine synergist against *dld-1(wr4)* mutant animals.

On the other hand, the mutation in the resistant animals makes these organisms vulnerable to arsenite (As_3^{-3}). Phosphine resistant animals are more sensitive to arsenite than the wild type and very close to an arsenite sensitive mutant. Arsenite reacts with the lipoic acid cofactor that passes electron to DLD when it is in the recued state. The sensitivity of the *dld-1(wr4)* mutant toward arsenite suggests that the mutation inhibits the transfer of electrons to DLD, resulting in the lipoioc acid cofactor being in a reduced state for a more extended period of time making it susceptible to a covalent chemical reaction with arsenite.

The arsenite sensitive mutant line that I used in my studies disrupts the *mrp-1* gene, which encodes an ATP binding cassette efflux pump that removes toxic chemicals from the cell (Leslie et al., 2001). Interestingly, phosphine stimulates MRP-1 activity. When a sublethal concentration of phosphine was combined with arsenite, the wild type nematodes and the *dld-1(wr4)* mutant were able to tolerate arsenite toxicity up to a breaking point. The wild type animals were more tolerant to the combination than the phosphine resistant nematodes,

despite the fact that the stimulation of the efflux pump seemed to be the same between the two strains. The difference in the response of the two strains seems to be due to an independent factor. It is entirely likely that this is due to the increased reactivity of the lipoic acid in the *dld-1*(*wr4*) mutant toward arsenite. Instead, active exclusion of phosphine by MRP-1 contributes to the basal tolerance toward phosphine in both phosphine resistant and wild type strains.

Oxidative stress and DNA-damage in phosphine toxicity

It is well established that phosphine induces ROS, which is, at least in part, responsible for the toxic effect of phosphine exposure (Chaudhry, 1997; Nath et al., 2011; Sciuto et al., 2016). The enhancement of phosphine toxicity by oxygen in chapter three supports that suggestion. Hyperoxia can cause oxidative stress, and combining it with phosphine may intensify the production of ROS. Therefore, the phosphine resistant genotype of the *dld-1(wr4)* mutant provided cross resistance to the synergistic combination of oxygen plus phosphine. The genetic adaptation to resist oxidative damage caused by phosphine in the *dld-1(wr4)* mutant extends to resistance against radiation as well.

I determined that the *dld-1(wr4)* mutation resulted in not only a 4-fold increase in phosphine resistance but also a 2-fold increase in resistance to UV and gamma radiation indicating that this mutation may be able to counter multiple stressors. The cross resistance to both phosphine and radiation is not related to DNA repair. Instead, both type of stressor can cause oxidative stress and the damage caused by both phosphine and radiation can be attenuated by, antioxidants (Hsu et al., 2000; Ye et al., 2010).

While there is evidence that phosphine exposure also leads to DNA damage, it is reported to be a secondary effect of oxidative stress (Hsu et al., 2000; Hsu et al., 2002). When four mutations, each of which has a direct role in DNA repair, were tested for their ability to survive exposure to phosphine, only two gave a statistically significant response to phosphine in the anticipated direction. While the fifth mutant line was sensitive to both radiation and phosphine exposure, the mutation was not specific to DNA repair as it disrupted a general stress response transcription factor. In chapter four, the phosphine sensitivity displayed by the radiation resistant mutant (*cep-1*) supports the involvement of ROS in DNA-damage. This mutant is able to resist DNA damage induced by radiation but was vulnerable to phosphine toxicity, indicating that phosphine toxicity does not lead to programmed cell death.

The ROS generated in response to phosphine exposure seems to have its toxic effect through oxidative stress that has the potential to cause widespread cellular damage. Given the close association between phosphine toxicity and resistance with aerobic respiration, the primary effect of phosphine is likely on the mitochondria. I do see, however, that DNA repair is a significant contributor to tolerance toward phosphine, but this is likely due to a secondary effect of oxidative stress on DNA.

Heat shock response a defense mechanism against phosphine toxicity

Phosphine may disturb the ER environment and the homeostasis of the cell most likely via the generation of ROS, interrupting the protein folding process. This event, as discussed in chapter five, will trigger the unfolded protein response through the upregulation of heat shock response proteins. The widespread damaging effect of phosphine on the cell is consistent with the increased phosphine tolerance most likely by the upregulation of heat shock proteins in chapter five. The master regulator HSF-1 regulates heat shock protein expression, and it seems that they provide a defense mechanism against phosphine toxicity.

Phosphine tolerance is induced by heat shock pre-conditioning, with an absolute requirement for the master, cell-wide regulator of the heat shock response, HSF-1. The mitochondria have a set of chaperones that can be upregulated in response to a decrease in the organelle function. Therefore, phosphine inhibition of cellular respiration can lead to the mitochondria chaperones upregulation.

Heat shock proteins are reported to provide protection against ROS. As such, the involvement of the heat shock defense mechanism in phosphine resistance is consistent with the main role of ROS in phosphine toxicity. Induced resistance was only observed in the phosphine susceptible strains. Which implies that the resistant strain benefits from pre-induced defenses, possibly a component of the heat shock response itself, that precludes the need for further induction of a heat shock response

Conclusion

Phosphine seems to target multiple sites in exposed organisms. The sites that dependent on oxidative phosphorylation are the primary effectors of phosphine toxicity. It interrupts cellular respiration by interacting with the ETC generating injurious amounts of ROS. Therefore, it can be synergized by respiration-increasing agents. With DLD as a natural source for ROS, a genetic mutation in the encoding gene of the enzyme (*dld-1*), can provide resistance against phosphine and the synergistic action of oxygen. However, the mutation causes a reduction in energy output and exposing lipoamide in the DLD complex in its reduced state for a more extended period, resulting in susceptibility to arsenite.

The ability of the mutation to counter oxidative stress caused by phosphine also results in cross-protection against other ROS-generating agents, in this study UV and ionizing radiation. Another potential site of phosphine action is the DNA. Several mutants of *C. elegans* that are sensitive to radiation with deficiencies in DNA repair expressed cross-sensitivity to phosphine implicating it as a DNA damaging chemical, but this damage may be indirectly mediated through oxidative stress. Furthermore, the DNA damage caused by phosphine does not trigger apoptosis. Pretreatment with heat shock results in the upregulation of HSPs, which provide protection against ROS, leads to elevated tolerance against phosphine. With a genetic resistance to phosphine, HSPs induction of phosphine-tolerance was not required.

Summary of key findings

1. Oxygen enhances phosphine toxicity in susceptible animals.
2. Optimal temperature encourages oxygen consumption leading to phosphine sensitivity.
3. Arsenite and phosphine toxicity is negatively correlated.
4. Arsenite synergizes phosphine against phosphine-resistant nematodes.
5. Active exclusion is involving in phosphine detoxification.
6. Cross-resistance between phosphine and radiation is present.
7. Phosphine is a DNA damaging agent.

8. Radiation inhibits growth in a dose-dependent manner.

9. Heat shock induces tolerance against phosphine toxicity in susceptible worms, and the master regulator HSF-1 mediates it.

10. Pre-conditioning with UV and gamma radiation increases tolerance against phosphine in susceptible and resistant animals.

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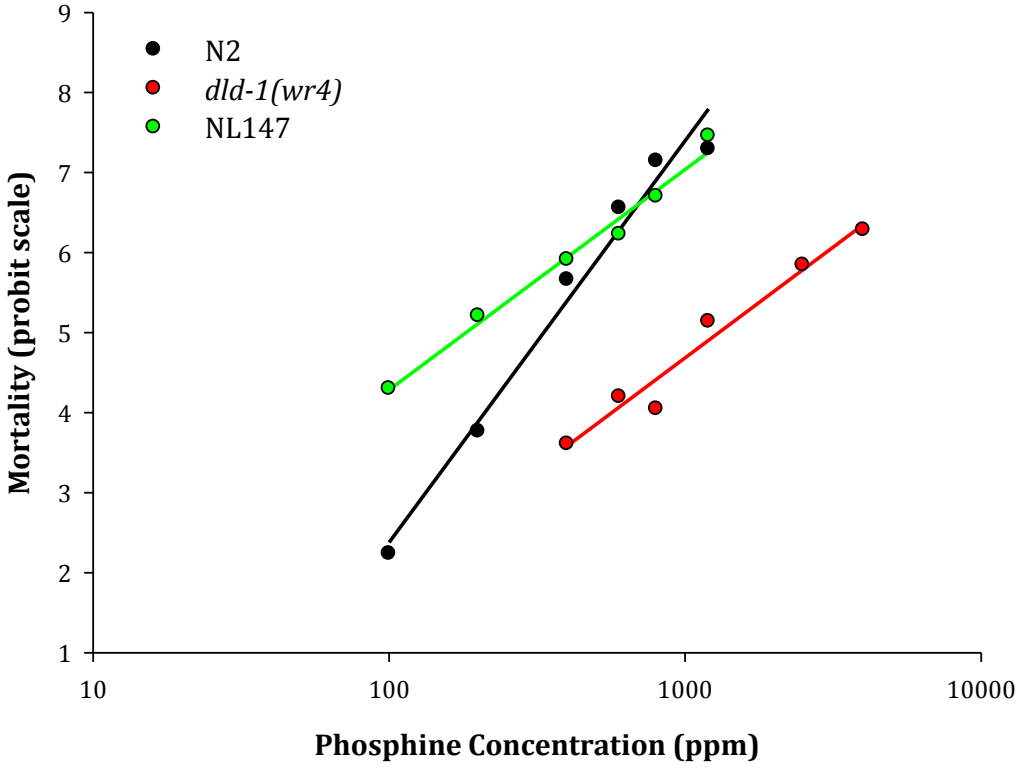
Appendices

Appendix I: Supplementary Information for phosphine synergism (Chapter three).

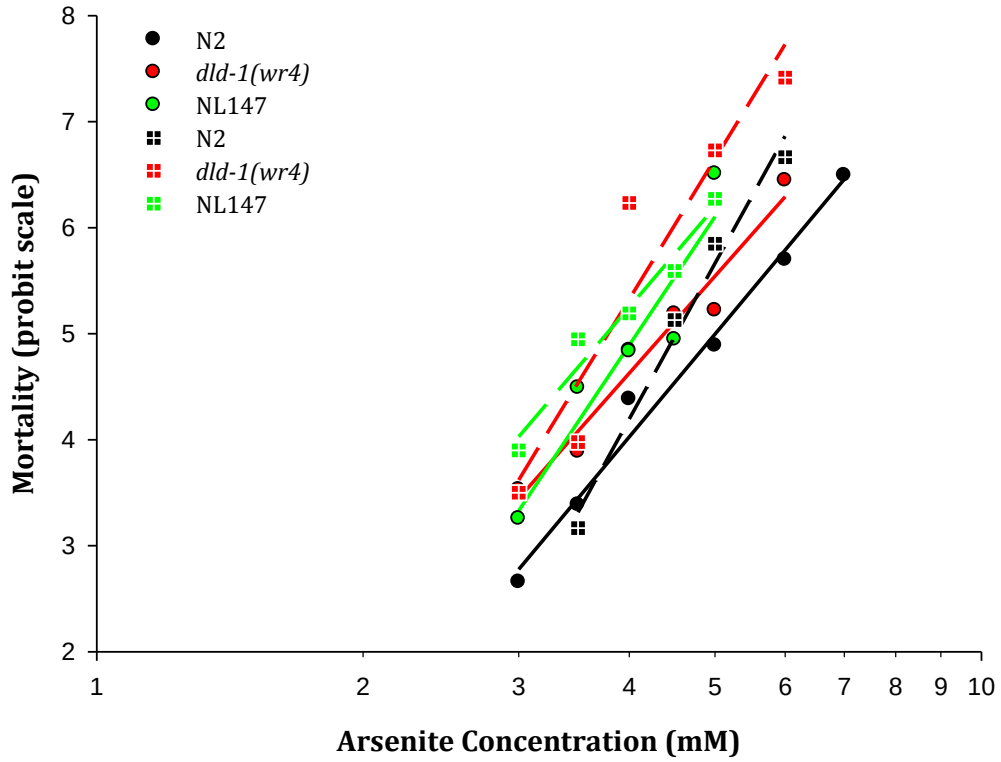
S Table 1: LC_{50} values from probit analysis for the mortality induced in *C. elegans* mutants after exposing to a range of concentrations from phosphine and arsenite. One way ANOVA followed by Dunnett's multiple comparison test was performed to identify significant differences in LC_{50} values due to exposure of each treatment between the wild type and the other strains.

Treatment	Strain	LC_{50}†	R	Slope ± SE	X^2	*df*
Phosphine (ppm)	N2	321 (298-344)	0.998	5.58±0.40	1.35	4
	dld-1(wr4)	1283 (1165-1419)****	0.990	2.83±0.20	6.04	6
	NL147	184 (159-209)*	0.993	2.51±0.20	3.57	6
Arsenite (mM)	N2	4.99 (4.83-5.15)	0.992	9.38±0.61	7.29	6
	dld-1(wr4)	4.04 (3.62-4.52)*	0.980	10.28±0.77	12.09	5
	NL147	3.97 (3.87-4.07)*	0.994	13.39±0.99	6.78	5
Arsenite+70 ppm Phosphine (mM)	N2	4.48 (3.86-4.95)	0.955	12.33±0.87	17.92	5
	dld-1(wr4)	3.73^{b**}	0.927	17.35±1.33	125.11	6
	NL147	3.76 (3.64-3.87)**	0.980	9.42±0.85	6.79	5

† $^{***}p < 0.001$, $^{****}p < 0.0001$, ppm parts per million



S Figure 1: Mortality induced for the three strains after exposure to a range of phosphine concentrations. Forty-eight hours after the exposure, mortality was scored for each treatment. Regression lines are based on average mortality from three replicates.



S Figure 2: Mortality induced of the three nematode strains by exposing to a range of arsenite concentrations, with or without 70 ppm phosphine. Regression lines are based on average mortality from three replicates. Solid lines represent arsenite; dotted lines represent the mixture of arsenite and 70 ppm phosphine.

Appendix II: Supplementary Information for preconditioning effect on phosphine toxicity (Chapter five).

S Table 2: *C. elegans* mutants of the heat shock response were screened for a change in induced tolerance toward phosphine. A screening phosphine-bioassay in *C. elegans* mutants, that have been characterized in the *C. elegans* Genetic Center with genetic-mutated background in regard to heat shock response. Mutants with unique heat shock response to phosphine toxicity were chosen.

Strain	Description	Genotype	Survival (%)							
			Normal				Heat Shock			
			Control	LC_{10} (80ppm)	LC_{50} (230ppm)	LC_{90} (1000ppm)	Control	LC_{10} (80ppm)	LC_{50} (230ppm)	LC_{90} (1000ppm)
VC281	F38E11.2. Superficially wild type. Attribution: This strain was provided by the C. elegans Reverse Genetics Core Facility at the University of British Columbia, which is part of the international C. elegans Gene Knockout Consortium.	*hsp-12.6(gk156) IV*	91	100	41	3	100	100	54	10
PS3551	Defects in egg laying. Do not grow at 25C. Do not distribute this strain; other labs should request it from the CGC.	*hsf-1(sy441) I*	100	100	87	4	100	100	56	19

RB791	T27E4.3, T27E4.8. Homozygous. Outer Left Sequence: TGGCATTCCTTCCTTATTGC. Outer Right Sequence: TGAGAAGCCGAGTAGCTGGT. Inner Left Sequence: GTAAGGCTTTCTGCCGTTTG. Inner Right Sequence: TGAGGGCCCTGTAGAAGTTG. Inner primer WT PCR product: 3051. Attribution: This strain was provided by the C. elegans Gene Knockout Project at the Oklahoma Medical Research Foundation, which was part of the International C. elegans Gene Knockout Consortium.	*hsp-16.48(ok577) V*	100	100	59	1	100	100	100	63
RB1098	F38E11.2 Homozygous. Outer Left Sequence: GTGACGATTCGAGAGCAACA. Outer Right Sequence:	*hsp-12.6(ok1077) IV*	100	79	77	1	100	100	77	72

	CGTGCGAAGATTGAACAGAA. Inner Left Sequence: TTCGAAGCTCAATGAACGAA. Inner Right Sequence: AGCCCAAGATGACAATGGAC. Inner Primer PCR Length: 2303. Estimated Deletion Size: about 700 bp. Attribution: This strain was provided by the C. elegans Gene Knockout Project at the Oklahoma Medical Research Foundation, which was part of the International C. elegans Gene Knockout Consortium.									
RB1104	C15H9.6 Homozygous. Outer Left Sequence: GGGGTAGGAGAGCCATTTTC. Outer Right Sequence: ACTTGGCCTTTTCCGATTTT. Inner Left Sequence: CGATCGTTTAGAGCTCGTCC. Inner	*hsp-3(ok1083) X*	76	100	54	52	100	99	66	56

	Right Sequence: CCTGCCGTTTCCATAACAGT. Inner Primer PCR Length: 2947. Estimated Deletion Size: about 1300 bp. Attribution: This strain was provided by the C. elegans Gene Knockout Project at the Oklahoma Medical Research Foundation, which was part of the International C. elegans Gene Knockout Consortium.									
RB2600	22A3.2 Homozygous. Outer Left Sequence: ttgaaaatgtttcttcgggg. Outer Right Sequence: aattacaactgactcggcgg. Inner Left Sequence: tgccagaaacttccagttca. Inner Right Sequence: gccccttcagcataacgat. Inner Primer PCR Length: 1319. Estimated Deletion Size: about 400 bp. Attribution: This strain was	*hsp-12.1(ok3622) I*	100	100	82	0	100	100	60	22

	provided by the C. elegans Gene Knockout Project at the Oklahoma Medical Research Foundation, which was part of the International C. elegans Gene Knockout Consortium.									
RB2612	C14B9.1 Homozygous. Outer Left Sequence: tttcaggtccacaacaccaa. Outer Right Sequence: aaaatcatccctcgatgtgc. Inner Left Sequence: agttcgaggtcggacttgac. Inner Right Sequence: cattattcgtgcgttgatgc. Inner Primer PCR Length: 1096. Estimated Deletion Size: about 400 bp. Attribution: This strain was provided by the C. elegans Gene Knockout Project at the Oklahoma Medical Research Foundation, which was part of the	*hsp-12.2(ok3638) III*	100	100	94	9	100	100	100	27

	International C. elegans Gene Knockout Consortium.									