Garlic-derived diallyl polysulfides: Biochemical mode of action and application for green insecticides

A thesis submitted for the degree of Masters of Philosophy

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Declaration

This thesis is submitted to the University of East Anglia for the Degree of Masters of Philosophy and has not been previously submitted at this or any university assessment or for any other degree. Except where stated, and reference and acknowledgment is given, this work is original and has been carried out by the author alone.

Emma Gould

Abstract

Garlic (*Allium sativum*) has been used for thousands of years to treat various ailments, due to its bactericidal and fungicidal effects. These protective effects are associated with the presence of organosulfur compounds. Crushing of garlic leads to the production of thiosulfinates such as allicin. Allicin can decompose into diallyl polysulfides and other constituents. The polysulfides can react with glutathione, leading to the generation of reactive oxygen species (ROS) such as hydrogen peroxide. The ROS-generating properties of garlic constituent compounds suggest that it also might have insecticidal effects, and hence might be used to control serious pests such as the cabbage root fly, *Deliaradicum*, which continues to be a significant problem for crops with the genus *Brassica*. New methods for controlling such pests are especially important because current insecticides can cause toxic effects on non-target organisms, one example is chlorpyrifos, which was banned for residential use in 2000. In this thesis *Drosophila melanogaster* was used as a model system to test the insecticidal effects of garlic and garlic derivatives. Insecticidal experiments were performed at all life stages from egg to adults. The garlic products tested were garlic extract, Mexican garlic oil, and China garlic oil, with concentrations of 0 to 5%. Both the garlic extract and garlic oil had concentration- and age-dependent insecticidal effects. The garlic oils were more effective than the garlic extract, due to the presence of greater concentrations of diallyl polysulfides. Eggs and adults were most susceptible to the effects of garlic solutions and these stages are also expected to be easier to target in the field. The pupae were the least susceptible life stage. Biochemical studies showed that diallyl polysulfides lowered glutathione and cysteine levels whilst increasing reactive oxygen species, thus, causing an increase in oxidative stress. Using redox sensitive green fluorescent protein (roGFP) transgenic *Drosophila* demonstrated that garlic oil caused changes in redox couples, thus adversely affecting redox homeostasis. The results are important as they show that botanical insecticides have significant potential for use as alternatives to ecologically harmful insecticides that have traditionally been used in crop protection.

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List of Abbreviations

3-MP- 3-Mercaptopyruvate

3-MST - Mercaptopyruvate sulfurtransferase

AchE - Acetylcholinesterase

ADP - Adenosine diphosphate

ANOVA - Analysis of variance

ASK1 - Apoptosis signal-regulating kinase 1

Bcl-2-B-cell lymphoma 2

cAMP - Cyclic adenosine monophosphate

CAT - Cysteine aminotransferase

CBS - Cystathionine β-synthase

CD - Cyclodextrin

CGO – China garlic oil

CSE - Cystathionine γ-lyase

DAS1 – Diallyl monosulfide

DAS2 – Diallyl disulfide

DAS3 – Diallyl trisulfide

DAS4 – Diallyl tetrasulfide

DAS5 – Diallyl pentasulfide

DAS6 – Diallyl hexasulfide

DCF - Dichlorofluorescein

DCFH-DA - Dichlorodihydrofluorescein diacetate

DDT - Dichlorodiphenyltrichloroethane

DNA - Deoxyribonucleic acid

DTT - Dithiothreitol

EGFR - Epidermal growth factor receptor

GE – Garlic extract

GFP – Green fluorescent protein

GLS–Glutaminase

GO - Garlic oil

GPx - Glutathione peroxidase

GRX - Glutaredoxin

GSH - Glutathione

GSSG – Glutathione disulfide

GST - Glutathione S-transferase

HPLC - High-performance liquid chromatography

JNK - c-Jun N-terminal kinases

MAPK - A mitogen-activated protein kinase

mBBr – Monobrombimane

MeOH - Methanol

MGO – Mexican garlic oil

MIC – Minimum inhibitory concentration

mTOR - mammalian target of rapamycin

nAchR - Nicotinic acetylcholine receptor

NADPH - Nicotinamide adenine dinucleotide phosphate

NEM - N-Ethylmaleimide

NMR - Nuclear magnetic resonance

OxD – Degree of oxidation

PARP - Poly (ADP-ribose) polymerase

Prx - Peroxiredoxins

RNS - Reactive nitrogen species

roGFP – Redox sensitive green fluorescent protein

ROS – Reactive oxygen species

SOD - Superoxide dismutase

SYA - Sugar-yeast-agar

TNFR1 -Tumor necrosis factor receptor 1

TPS – Total polysulfide

Trx – Thioredoxin

TrxR - Thioredoxin reductase

VDE - Vancomycin-dependent enterococci

WIG1 - Wild-type p53-induced gene 1

1 INTRODUCTION

Garlic (*Allium sativum*) has been used to treat a wide range of ailments for thousands of years, including earaches, deafness, severe diarrhoea, constipation, parasitic infections, stomach aches, fever and leprosy (Hahn. 1996). Garlic is commonly used to treat influenza, recurrent upper respiratory tract infections and chronic bronchitis (Shojai, Langeroudi et al .2016). This is due to the belief that garlic has expectorant, antiseptic, antispasmodic, bacteriostatic, antiviral, diaphoretic, hypotensive and antihelminthic effects (Newall, Anderson et al. 1996). Garlic is also reported to have bactericidal, fungicidal (Omar and Al-Wabel 2010) and anticancer effects (Fleischauer and Arab 2001).

In addition to medicinal actions, there is increasing evidence that garlic and its derivatives could have pesticidal properties. This thesis describes investigations into the mechanisms and applications of garlic as a green insecticide

1.1 INSECTICIDES

Below I describe the currently most commonly used insecticides in agriculture and the problems that are associated with their use, to demonstrate the pressing need to develop new insecticides such as those based on garlic and garlic derivatives.

1.1.1 Synthetic insecticides

1.1.1.1 DDT

Dichlorodiphenyltrichloroethane (DDT) (figure 1.1) was originally synthesized in 1874 in Germany as coal tar dye but quickly became popular as an effective insecticide from 1943 (Jarman and Ballschmiter 2012). Approximately 400,000 tons of DDT was used annually in the early 1960s worldwide, and 70-80% of this was used in agriculture. In the early 1970s, the use of DDT was banned in most countries due to its negative effect on wildlife and its carcinogenic activity(Turusov, Rakitsky et al. 2002). DDT has a long persistence in the environment and high fat solubility, which results in long-term accumulation in fatty tissues of non-target organisms (Davies, Field et al. 2007). DDT acts on sodium gates of axons in insects and prevents deactivation of sodium gates after activation and membrane depolarization. This results in a leakage of sodium ions permeating the axon membrane, producing a destabilizing negative after-potential (Narahashi 1986). DTT continues to be used in limited quantities for the control of malaria vectors in some countries (Walker 2000).

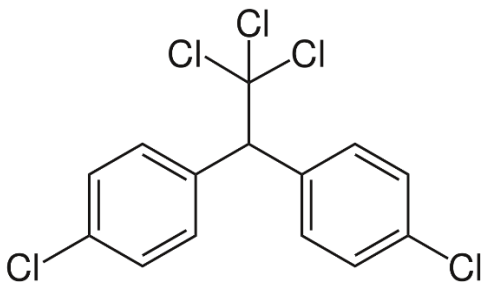
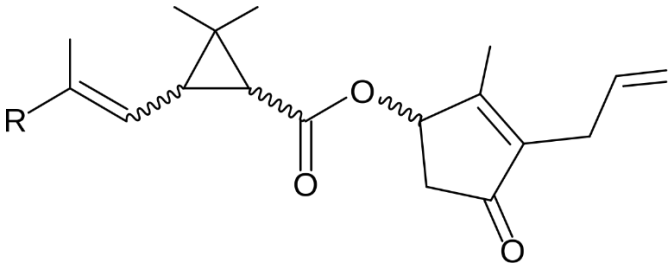


Figure 1.1: Structure of DDT.

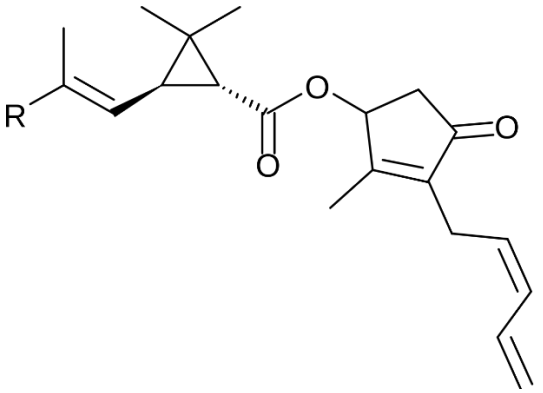
1.1.1.2 Pyrethroids

For over 30 years pyrethroid insecticides have been used in agricultural and residential formulations and comprise approximately a quarter of all insecticides in the world. Pyrethroids are typically sold in combination with other compounds(Casida and Quistad 1998). Human exposure to pyrethroids has been linked to adverse effects, for example exposure during pregnancy can lead to impaired neurodevelopment in developing embryo(Berkowitz, Obel et al. 2003). Pyrethroids are derived from natural compound pyrethrins isolated from plants with the genus *Chrysanthemum*, figure 1.2 (Casida 1980). Pyrethrins do have insecticidal activity but are unstable upon light exposure. Therefore, the structure of pyrethrins was modified to increase stability whilst retaining insecticidal activity (Valentine 1990).

In insects, the main mode of action of pyrethroids involves disruption of neuronal voltage-gated sodium channels. During pyrethroid exposure, these sodium channels carry a slowly inactivating sodium current that increases in amplitude during depolarizations and gives rise to a slowly declining inward sodium tail current during membrane repolarization (Chinn and Narahashi 1986; de Weille and Leinders 1989; Holloway, Salgado et al. 1989; Song and Narahashi 1996; Vais, Williamson et al. 2000). Additionally, pyrethroids shift the voltage-dependent activation and inactivation of sodium channels to hyperpolarized potentials (Narahashi, Frey et al. 1995; Trainer, McPhee et al. 1997). These mechanisms result in prominent neuronal after-depolarizations that induce toxic effects (Song and Narahashi 1996; Lee and Soderlund 2001).



Pyrethroid



Pyrethrin

Figure 1.2: Structures of pyrethroid and pyrethrin.

1.1.1.3 Neonicotinoids

Neonicotinoids are one of the most popular groups of insecticides in the world and are licensed for use in over 120 countries (Jeschke, Nauen et al. 2011; van der Sluijs, Simon-Delso et al. 2013; Simon-Delso, Amaral-Rogers et al. 2015). Neonicotinoids can be applied in a variety of methods including by foliar sprays for above-ground plants, as root drenches in the soil, or via trunk injections in trees. The most popular method of application is seed/soil treatment (Jeschke, Nauen et al. 2011). Neonicotinoids are small compounds and are highly water soluble. Neonicotinoids work by blocking insect nicotinic acetylcholine receptors (nAChRs), which results in paralysis and death. The first neonicotinoid was imidacloprid, which was commercially successful in the early 1990s, followed by clothianidin and thiamethoxam and then acetamiprid and thiacloprid in the early 2000s, shown in figure 1.3 (Matsuda, Shimomura et al. 2005). These compounds circulate throughout plants tissues upon uptake and provide protection against sap-feeding insects (Nauen, Reckman et al. 2008; Magalhaes, Hunt et al. 2009). In the last decade, concerns have arisen regarding environmental effects such as soil persistence, effects on non-target pollinator species and the potential contamination of untreated soil during

sowing of treated seeds (Goulson 2013). More recently, acute intoxification of bees has been associated with neonicotinoids seed-coating, with the exposure occurring specifically via pollen, nectar, and guttation droplets, and by direct flying into dust emitted by planters during the sowing of treated seeds (Girolami, Mazzon et al. 2009; Girolami, Marzaro et al. 2011; Tapparo, Giorio et al. 2011; Tapparo, Marton et al. 2012).

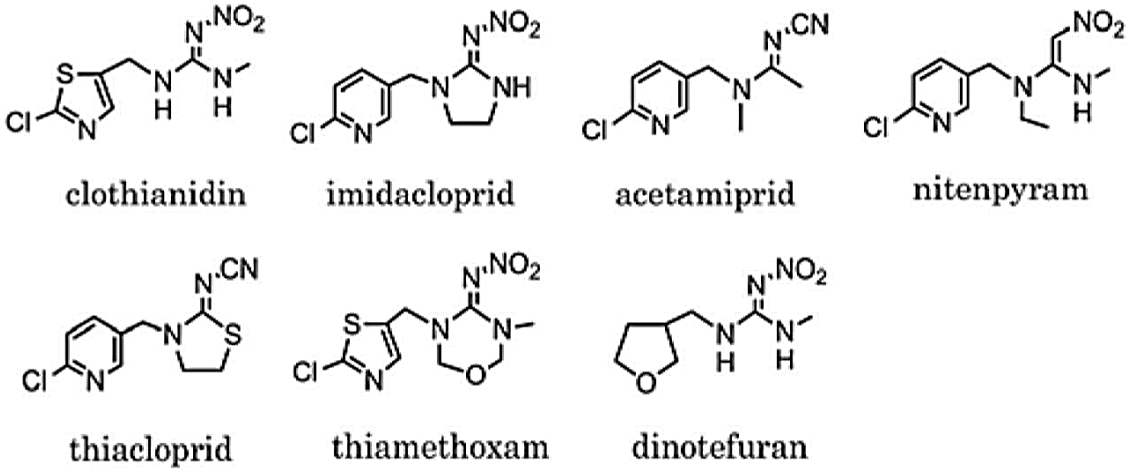


Figure 1.3: Structures of the seven most commonly used neonicotinoids (Uchigashima et al, 2012).

In 2013, reports were released by the European Food Safety Agency (ESFA) assessing the risk of 3 neonicotinoids (imidacloprid, clothianidin, and thamethoxam) to honeybees (Authority 2013). This was followed by the European Union's 2-year moratorium on the use of neonicotinoid insecticides in order to reduce bee losses whilst developing accurate data on the risks of these insecticides. Sublethal concentrations of neonicotinoids can affect forage behaviour in laboratory studies, however, comparable effects have not been observed in field studies when using standard field measurements (Blacquiere, Smagghe et al. 2012).

1.1.1.4 Chlorpyrifos

Chlorpyrifos (*O*, *O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl) is one of the most widely used organophosphorus insecticides in the world, and targets a wide range of insect pests, figure 1.4 (Anwar, Liaquat et al. 2009). It has a half-life of 60 to 120 days, but can persist in the environment for over a year, depending on soil conditions such as pH, temperature, and soil type (Singh, Walker et al. 2006; Gao, Chen et al. 2012). The long persistence of chlorpyrifos has resulted in widespread contamination of soil and water, leading to damaging effects on non-target organisms (Li, Jiang et al. 2008; Xu, Zheng et al. 2008). There have been various methods used for the removal of chlorpyrifos, such as deep ocean dumping, chemical treatment and incineration. However, disposal of this contaminant has been technically and economically challenging (Megharaj, Ramakrishnan et al. 2011; Theriot and Grunden 2011). The environmental fate of chlorpyrifos is dependent on abiotic and biotic processes including microbial degradation, photolysis, and chemical hydrolysis (Xu, Zheng et al. 2008).

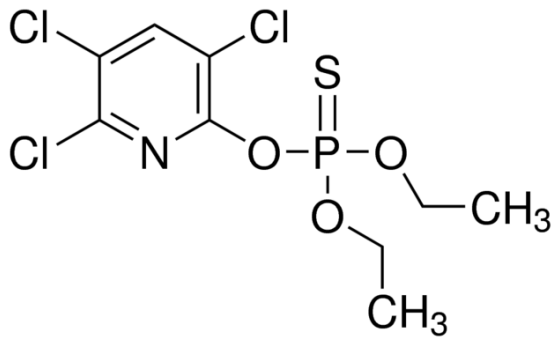


Figure 1.4: Structure of chlorpyrifos.

There is an increase in public concern and the potential health risks from the frequent use of chlorpyrifos, arising from acute or chronic dietary exposure to chlorpyrifos residues in food (Cochran, Kishiyama et al. 1995; Yu, Fang et al. 2006). Chlorpyrifos contains a thiophosphate backbone that is metabolized by cytochrome p450s when consumed into chlorpyrifos-oxon that inhibits acetylcholinesterase (AChE), resulting in severe cholinergic toxicity (Amitai, Moorad et al. 1998). Chlorpyrifos-oxon is subsequently detoxified by A-esterase hydrolysis (Sultatos, Shao et al. 1984; Costa, McDonald et al. 1990). It is suggested that AChE inhibition results in neurotransmitter acetylcholine accumulation in the synapse which leads to hyperstimulation of acetylcholine receptors (Fukuto 1990; Chiappa, Padilla et al. 1995).

1.1.2 Naturally occurring insecticides

Many of the earliest methods of controlling pests, such as the use of natural predators to consume damaging pests, did not did rely on the use of chemicals (Fishel 2013). However, many natural insecticidal (bio-insecticides) secondary metabolites produced as defence mechanisms by plants and microbes can also be exploited to combat pest species. As an example, nicotine extracted from tobacco plants was used in the 18th through to the 20th century to control many agricultural pests (Fusetto and O'Hair 2015). Pyrethrum is another botanical insecticide which attacks the nervous system of insects. Pyrethrum blocks the open position of sodium channels and inhibits voltage-dependent activation (Casida 1973). However, the use of pyrethrum is currently controversial, due to toxicity problems(Jansen, Defrance et al. 2010). The development of naturally occurring toxins have been used and are some of the most effective in controlling pests. One example is the cry toxins, which are a potent insecticide produced by *Bacillus thuringiensis*, and which can be expressed transgenically in plants, to prevent pests from consuming them (Heckel 2012).

One of the major problems with bio-insecticides is their lack of robustness and they are generally more sensitive to variation in temperature, humidity, or light radiation than their synthetic counterparts (Ravenberg 2011). Formulation techniques have been utilized to improve the delivery, shelf life, and field efficacy of bio-insecticides(Leggett, Leland et al. 2011; Ravenberg 2011). Research in this area has led to the emergence of new formulations with improved longevity and precise time and location delivery (Kohl, Gerlagh et al. 1998; Townsend, Ferguson et al. 2004; Lacey 2007; Nuttens, Schampheliere et al. 2009; Hunter 2010). Shorter persistence in general reduces environmental risk but more frequent application is required to maintain a satisfactory level of pest control. Another concern regarding bio-insecticides is the problem of resistance. Over 27 pest species have acquired resistance to the most used bio-pesticide: *Bacillus thuringiensis* (Berling et al., 2009; Bravo et al., 2011).

1.1.2.1 Bacillus thuringiensis

Bacillisthurgingiensis (*Bt*) is an aerobic bacterium that is widely present in soil, water, air, and vegetables, and is the most used bio-insecticide in the world (Raymond, Johnston et al. 2010). This bacteria turns into its sporulated form under tropical environmental conditions and produces Cry and Cyt proteins that are toxic to insects (Bravo, Likitvivatanavong et al. 2011). The insecticidal properties of *Bt* have been known since the 1930s and have been

utilised for pest control since the 1950s. The discovery of serotypes of *Bt* against *Diptera*and *Coleoptera* has allows further usage of specific larvicidal activity against black flies, beetles, and mosquitoes. There are many different *Bt* isolates that have been classified by serology and their toxin protein targets (Lecadet, Frachon et al. 1999).

1.1.2.2 Spinosyns

Saccharopolyspora spinosa is an actinomycete that produces spinosyn molecules that have insecticidal activity. These spinosyns are extracted from the bacteria after fermentation. Spimosyns are commercialized into two active compounds: spinosad and spinetoram (Sparks, Thompson et al. 1998). Spinosad is consisted of a mixture of spinosyns A and D, shown in figure 1.5, which are the most active metabolites produced by *S. spinosa*, and spinosad was first approved for use in 1997. It was first used to control Lepidoptera larvae that were resistant to pyrethroids. This insecticide is used currently in 40 countries for various crops, including apple, grapevine, cotton, crucifer and peach, to control over 50 pest species. Spinetoram was approved in 2007 and was used when it became clear that resistance was occurring in various pests to spinosad itself (Sparks, Dripps et al. 2012). Upon ingestion, spinosyns bind to acetylcholine receptors (nAchR) and induce depolarization of neuron membranes that are connected to muscles. The hyperexcitation of the muscles causes the insect to be paralyzed (Sparks, Dripps et al. 2012).

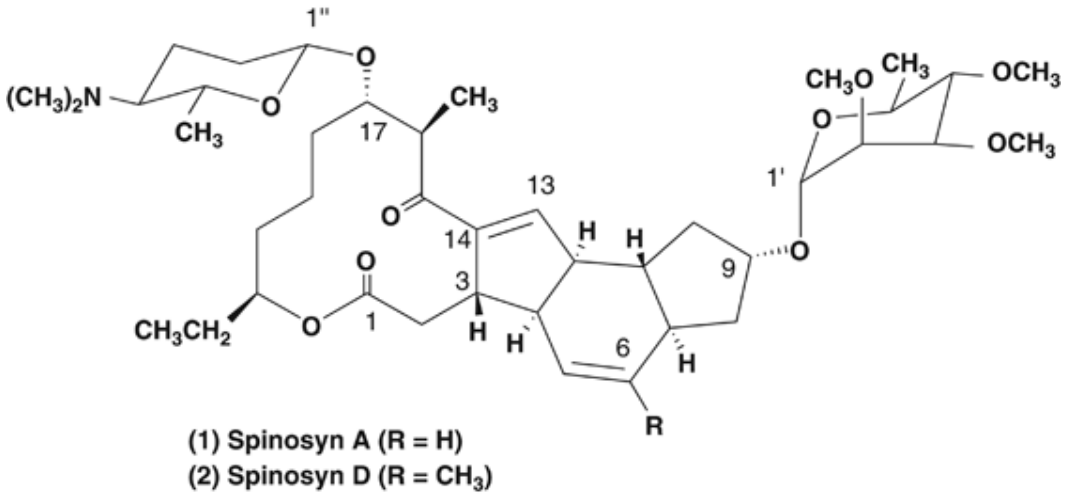


Figure 1.5: Structures of spinosyn A and D (Hirst, 2010).

1.1.2.3 Fungi

There are over 700 entomopathogenic fungal species known and among these, 9 are commercially available and subject to significant study effort for insect pest control: *Paecilomycesfumosoroseus, Lagenidiumgiganteum, Beauveria bassiana, Lecanicillium (Verticillium) lecanii, Entomophagamaimaiga, B. Brongniartii, Hirsutellathompsonii, Aschersoniaaleyrodis,* and*Metarhiziumanisopliae* (Hajek and Stleger 1994). These fungi penetrate into the host via natural breaches or by creating breaches in the cuticle by using enzymes such as chitinases. Chitinases are essential for the pathogenicity of these fungi and are determinants of virulence(Fang, Leng et al. 2005; Bhanu Prakash, Padmaja et al. 2012). Cuticle-degrading proteases are also synthesized by these fungi, these proteases are classified into two families; Pr1 and Pr2 (Castellanos-Moguel, Gonzalez-Barajas et al. 2007).

1.1.2.4 Viruses

There are many virus families that are able to infect arthropods, but Baculoviruses are the only ones used commercially as biological control agents(Huber 1988). Baculoviruses are able to infect Lepidoptera larvae, as well as Diptera, Coleoptera, Trichoptera and Hymenoptera. Baculoviruses are specific by infecting one or very few insect species. However, the *Autographacalifornica* nucleopolyhedrovirus, AcMNPV, is able to infect over 33 species from 7 families of Lepidoptera (Bishop, Hirst et al. 1995). These viruses have a double stranded circular DNA and replicate in the nucleus of the host cell. The genome is 80-200 kb and is packed into a nucleocapsid which contained in a membrane envelope. Baculoviruses have a complex life cycle in which two viral forms are produced (Rohrmann 2013).

1.1.3 Garlic as an insecticide

The data on garlic health benefits show that it uses a pesticide is not likely to represent that same human health concerns as some of the insecticides described above. The toxicity of garlic seems highly dependent on the size of the organism and the amount of garlic used for human consumption/application is not in the same range that results in the insecticidal effects explored in this thesis.

The insecticide trade in the U.K. is a multimillion-pound industry; however, due to changes in legislation outlawing commonly exploited pesticides, it is declining. This provides an opportunity for the development of a new suite of control products that conform to U.K. government requirements for crop production (Prowse, Galloway et al. 2006). Insecticidal properties have been studied in different botanical extracts, including thyme (Mansour, Messeha et al. 2000), neem tree (Dhar, Dawar et al. 1996), citronella (Lindsay et al., 1996), avocado (Rodrigues-Saona and Trumble 1996) and garlic (Birrenkott, Brockenfelt et al. 2000).

There are many botanical pesticides that have been explored for pest management including essential oils, flavonoids, alkaloids, glycosides, esters and fatty acids. These pesticides have various modes of action such as repellents, feeding deterrents/antifeedants, toxicants, growth retardants, chemosterilants, and attractants. Plant extracts and oils have increased in popularity as pesticides in recent years in the agricultural industry, this coincides with increase in research in using more natural, environmentally friendly pesticides. Table 1.1 shows some examples of plant extracts and oils used in research (Don-perdo et al, 1996; Koshier and Sedy, 2001; Sithisut et al, 2011; Tripathi et al, 2000; Trevedi et al, 2017; Showler, 2017; Singh et al, 1989).

Table 1.1: Plant extracts and oils used as insecticides against agricultural pests and their modes of action

Plant extract/oil	Effected species	Mode of action	Reference
Rosmarinum officinalis	Thrips tabaci	Antifeedant and oviposition inhibition	(Koshier and Sedy, 2001)
Limonene (citrus peel)	Callosobruchus maculatus	Fumigant	(Don-perdo, (1996)
E. adenophorum	Rhynchophorus ferrugineus	Antifeedant	(Showler, 2017)
A. nilagirica	Rhynchophorus ferrugineus	Antifeedant	(Showler, 2017)
Mentha citrate	*Sitophilus oryzae* (L.)	Fumigant	(Singh et al, 1989)
Pinus longifolia	*Sitophilus oryzae* (L.)	Reproduction retardant	(Singh et al, 1989)
Zingiberaceae	Sitophilus zeamais	Contact toxicity, antifeedant and repellent.	(Sithisut et al, 2011)
Zingiberaceae	Tribolium castaneum	Contact toxicity, antifeedant and repellent.	(Sithisut et al, 2011)
Mentha arvensis	Tribolium castaneum Callosobruchus maculatus	Fumigant Fumigant	(Tripathi et al, 2000)
Cinnamomum zeylanicum	Callosobruchus chinensis	Fumigant	(Trevedi et al, 2017)
Syzygium aromaticum	Callosobruchus chinensis	Fumigant	(Trevedi et al, 2017)
Citrus Bergamia	Callosobruchus chinensis	Fumigant	(Trevedi et al, 2017)

Garlic extracts have shown to be toxic to various different invertebrate pest species at all life stages. It has been shown that aqueous garlic extracts can inhibit egg hatching in mosquitoes (Jarial 2001). They can have antifeedant and toxic effects on both Coleoptera(Chaim, Huang et al. 1999) and Lepidoptera(Gurusubramanian and Krishna 1996) and repellent and toxic effects against Hemiptera (Flint, Parks et al. 1995; Gurusubramanian and Krishna 1996).

Garlic oil has been shown to possess fumigant activity against the larval sciarid fly *Lycoriella ingénue* (Park, Choi et al. 2006), the adult German cockroach *Blattellagermanica* (Tunaz, Er et al. 2009) and various other grain storage insects (Huang, Chen et al. 2000; Mikhaiel 2011). Garlic extract and garlic oil have already been formulated into different pest control products for crop protection for use against various pests. In a study that compared two commercial products, ENVIRepel (Cal Crop USA, Greeley, CO) and Garlic Barrier Ag (Garlic Barrier AG, Glendale, CA), with garlic extract and steam-distilled garlic oil, for control of *Bemisiaargentifolii* (whitefly), it was found that ENVIRepel and Bellows & Perring Garlic oil sprays gave no protection. However, a solution of Garlic Barrier Ag of 10% reduced the number of insects, and a 10% garlic extract solution provided the greatest protection (Flint, Parks et al. 1995). In contrast (Liu and Stansly 1995) showed that Garlic Barrier Ag did not appear to repel whiteflies. The conflicting results could be due to batch inconsistency (Prowse, Galloway et al. 2006). A product known as Alsa (DeruNed B.V., Bleiswijk, The Netherlands), which consists of 26 % garlic extract, has also been tested for control of grapefruit pest thrips *Pezothripskellyanus*. However, there was no significant difference between the garlic extract treated and untreated groups over 3 years (Vassiliou 2011). A concentration of 26 % garlic extract is relatively high compared to other studies; the authors do not state how the garlic extract ingredient of the Alsa was manufactured which could alter insecticidal effect.

The garlic constituent compounds diallyl disulfide and diallyl trisulfide show an acute toxicity effect against *Callosobruchuschinensis,* with diallyl trisulfide having stronger toxicity than diallyl disulfide and crude garlic oil. Diallyl trisulfide also has strong toxicity against other Colleoptera such as *S. oryzae, T. castaneum*, and *S. zeamais*(Huang, Chen et al. 2000; Koul 2004) and is reported as the strongest fumigant constituent in garlic oil and more potent than diallyl disulfide against *Bursaphelenchusxylophilus* (pine wood nematode). Structure-activity studies suggest that the higher molecular weight constituents

in garlic oil should be more potent against nematodes and insects. Diallyl trisulfides also have a repellent effect against yellow mosquitoes (*Aeses aegypti*) (Campbell, Gries et al. 2010). The active and inactive components in garlic oil have a synergistic effect in *Rosmarinus officinalis, Litseapungens*, and *Litseacubeba* (Miresmailli, Bradbury et al. 2006; Jiang, Akhtar et al. 2009).

The efficiency of the garlic oil and garlic extract is variable across different life stages, which will significantly affect its efficacy in the field. It has been reported that the adults of *D. radicum* are more susceptible than the other life stages to garlic-based products (Prowse, Galloway et al. 2006). However, sensitivity to garlic treatment can also vary across different insect species (table 1.2). Therefore, in each application, there needs to be consideration of the targeted pest life cycle.

Table 1.2: Efficacy of different garlic compositions on a range of plant pathogenic insects and their different life stages.

Insect	**Effected Crops**	**Garlic Composition [a]**	**Life Stage**	**LC50 (mg/L) [b]**	**Reference**
Agrotisipsilon *(Black cutworm)*	Strawberries, rice, tobacco, cotton, sugar beet	Garlic oil	Eggs	60	(Sharaby and El-Nojiban 2015)
			Larvae	190	(Sharaby and El-Nojiban 2015)
			Pupae	90	(Sharaby and El-Nojiban 2015)
Bemisiatabaci *(Silverleaf whitefly)*	Tobacco, tomatoes, brassica, cucumber, pumpkin, cotton, melons	Garlic oil	Adults	150	(Kim, Chae et al. 2011)
Cacopsyllachinensis	Pears	Garlic oil	Adults	142	(Zhao, Zhang et al. 2013)
		DAS2	Adults	1104	(Zhao, Zhang et al. 2013)

		DAS3	Adults	640	(Zhao, Zhang et al. 2013)
Callosobruchus maculatus *(Cowpea weevil)*	Cowpea beans	Garlic oil	Adults	0.25	(Douri, Bougdad et al. 2013)
Deliumradicum *(Cabbage root fly)*	Brassica	Garlic extract	Eggs	800	(Prowse, Galloway et al. 2006)
			Larvae	2.64×10^5	(Prowse, Galloway et al. 2006)
			Adults	1600	(Prowse, Galloway et al. 2006)
Heteracrislittoralis *(Grasshopper)*	Corn, rice, cotton, vegetables	Garlic oil	First instar larvae	670	(Hincapie, Lopez et al. 2008)
Lycoriella ingénue *(Sciarid fly)*	Mushrooms, herbs	DAS1	Larvae	0.25	(Park, Choi et al. 2006)
		DAS2	Larvae	0.087	(Park, Choi et al. 2006)
		DAS3	Larvae	0.25	(Park, Choi et al. 2006)
Pezothripskellyanus *(Kelly's citrus thrips)*	Citrus fruits	Garlic extract	Larvae	2.6×10^5	(Vassiliou 2011)
Sitophilus oryzae *(rice weevil)*	Rice	Garlic oil	Adults	0.017	(Chaubey 2016)
Sitophilus zeamais *(Maize weevil)*	Maize	DAS3	Adults	5.54	(Machial, Shikano et al. 2010)
Triboliumcastaneum *(Red flour beetle)*	Flour, cereals	DAS3	Adults	1.02	(Machial, Shikano et al. 2010)

[a]Activity depends on how garlic extract or oil is manufactured. Various papers representing the biological activity of polysulfides do not mention the characterisation of the preparations. The polysulfide concentration in an extract or oil depends on method of extraction or distillation. This could explain the large differences in order of magnitude and can make comparing results difficult.
[b] Original units were converted for ease of comparison, refer to reference for original values and units

The above findings show the potential of using garlic oil and its major constituents, especially diallyl disulfide and diallyl trisulfide, as a possible natural insecticide for controlling pests, especially given that currently used insecticides can be highly toxic to humans and non-targeted organisms. It has been proposed that garlic oil would have little deleterious effect on the agricultural environment (Saeed, Zayed et al. 2010). Garlic oil and its constituents also have limited persistence in field conditions so will not affect reinvading predators and parasitoids after one day of treatment, a problem that frequently occurs with currently used insecticides (Isman 2000). Hence garlic oil has the potential as a novel insecticide and further studies for formulation development are required to improve the effectiveness and stability (Zhao, Zhang et al. 2013).

1.2 PEST ORGANISMS OF INTEREST IN THE UK

Below are potential pests that could be targeted by diallyl polysulfides, including focal pest species of interest for this thesis research.

1.2.1 Delia radicum

Delia radicum, the cabbage root fly, is a serious pest species of brassicaceous crops such as cabbage, turnip, cabbages, and swede. For untreated brassica crops, up to 60-90% can be damaged by an infestation of cabbage root flies(Finch 1989; Finch and Collier 2000). The females lay eggs in the soil near the plant stem and, once hatched, the larvae feed on the roots before pupating in the surrounding soil. Damage caused by the larvae leads to plant mortality which can lead to a significant reduction in crop yield, seed production and flowering(Jong and Stadler 1999).The larvae and the long emergence period of the adult makes control by using pesticides ineffective and difficult(Zoephel, Reiher et al. 2012).Cabbage root flies are also a well characterised model for plant mediated above-belowground interactions. It has been shown that *D. radicum* and *D. floralis* that feed on the roots induce changes in levels of primary and secondary non-volatile compounds in both cultivated and wild *Brassica* species(Birch, Griffiths et al. 1992; Hopkins, Griffiths et al. 1998; Hopkins, Griffiths et al. 1999; Dam, Raaijmakers et al. 2005; Solar, Bezemer et al. 2005; Dam and Raaijmakers 2006; Pierre, Dugravot et al. 2012). Root feeding of *D. radicum* was shown to induce both a systemic (shoots) and local (roots) increase in the levels of aliphatic and indole glucosinolates (GLS) in Brassica species(Dam and Raaijmakers 2006). The systemic induced response could contribute to reduced performance of leaf feeding *Pieris brassicae* on feral *B. nigra* plants(Solar, Bezemer et al. 2005).

Most control methods rely on chemical insecticides for *D. radicum*, however, cultural practices are still in use, such as crop rotation, cultivation and row covers (Jyoti et al, 2001). Chlorpyrifos (Lorsban), an organophosphate, is the most commonly used pesticide for *D. radicum* control in the Pacific Northwest (Quarles, 2000). However, chlorpyrifos has been banned for residential use in 2000 due to human toxicity and its commercial use is under restrictions. Therefore, if chlorpyrifos were to be fully banned from use for crop protection against *D. radicum*, growers would need alternative methods for control, and these are currently very limited (Bruck, 2005). Another chemical insecticide used for *D. radicum* control is neonicotinoids. As described above, these insecticides have been

banned due to their toxicity to bees, which are vital for pollination of wild plants and crops (Rundlof, Andersson et al. 2015).

Alternative natural methods of controlling *D. radicum* would be intercropping, for example clover has shown to be efficient as a pest management strategy for reducing oviposition by *D. radicum* (Finch and Collier, 2000a; Hooks and Johnson, 2003). Another method would be the use of natural predators. *D. radicum* eggs are preyed on by several small ground dwelling beetles such as staphylinids and carabids (Wishart et al, 1956; Hughes, 1959; Finch, 1996; Prasad and Snyder, 2004). Egg predation could contribute to biological control of *D. radicum* (Finch, 1996; Bjorkman et al, 2010). Additionally, *D. radicum* larvae are predated by *Aleocharabilineata* (Gyll.) (Coleoptera: Staphylinidae) and *Trybiographarapae* (Westwood) (Hymaenoptera: Figitidae) (Finch and Skinner, 1980; Jones et al, 1993). These parasitoids species usually accompany one another and hence could jointly contribute to *D. radicum* regulation (Bonsall et al, 2004); Hummel et al, 2010).

1.2.2 Otiorhynchussulcatus

The vine weevil (*Otiorhynchussulcatus*, Coleoptera: Curculionidae) is a significant horticultural crop pest that causes serious damage (Moorhouse, Charnley et al. 1992; Cross and Burgess 1997; Lola-Luz, Downes et al. 2005). Plant damage is primarily caused by the larvae feeding on roots, which results in reduced plant vigour and, in severe infestations, plant death. The adults feed on the leaves which also affects a plant's decorative appearance (Garth and Shanks 1978; Bedding and Miller 1981). The known host plant range of the vine weevil includes 140 plant species, especially strawberries and blackcurrants (Penman and Scott 1976; Warner and Negley 1976).

The vine weevil reproduces asexually by parthenogenesis and can rapidly establish itself in new areas (Moorhouse, Charnley et al. 1992). There are six larval instar stages and the pupation takes place in the soil. The pupal stage lasts from three weeks to several months, depending on the temperature of the soil. One generation of black vine weevil takes a year to develop in the outdoor habitat(Gill, Lutz et al. 2001). The vine weevil originated from mountainous areas of central Europe and then spread to North Europe, where it prefers certain plant species such as horticultural crops for oviposition and feeding (Hanula 1988; tol and Visser 1998; Tol, Dijk et al. 2004).

Vine weevil larvae used to be controlled by incorporating the organochlorine aldrin. This was effective; however, in the 1990s, due to environmental and toxicological reasons, the use of this agent was no longer permitted in Europe and the U.S.A. Due to the prohibition of organochlorine compounds there has been an increase in the number and economic importance of vine weevils. Other chemicals have also been used to control vine weevil larvae, such as chlorpyrifos but this is not as effective as aldrin (Cross, Buxton et al. 1995). Imidacloprid has also been used to control vine weevil larvae in non-food crops (Shah, Ansari et al. 2007).

Many chemical control agents have been studied for their effectiveness for controlling black vine weevil larvae (Georgis and Poinar 1984; Halfhill 1985; Nielsen and Boggs 1985; Blackshaw 1986; Moorhouse, Charnley et al. 1992). Blackshaw (1986) assessed seven different insecticides where all were effective at controlling the number over larvae. Chlorpyrifos, fonofos, and aldrin provided 100% control. Halfhill (1985) attained the greatest black vine weevil control with bendiocarb, carbofuran, oxamyl, and chlordane. Nielsen and Boggs (1985) assessed the effectiveness of many pesticides and found the highest control of the vine weevil was with carbofuran. However, any of these insecticides have now been removed and are no longer in use (Gill, Lutz et al. 2001).

More recently, there has been an increased interest in using more environmentally friendly alternatives to control vine weevil larvae, such as biological control(Shah, Ansari et al. 2007). Biological control is particularly useful with food crops, for example strawberries and blackcurrants, in which chemical insecticides cannot be applied near to harvest. One biological control method for controlling vine weevils in potted plants and in glasshouse crops is the use of entomopathogenic nematodes (Bedding and Miller 1981; Simons 1981; Georgis and Poinar 1984). However, nematodes have a relatively short shelf life which results in inconsistent effectiveness, especially at low temperatures, and high application cost (Tol, Dijk et al. 2004). This has encouraged the research for alternative control methods (Georgis, Koppenhofer et al. 2006).

1.2.3 Drosophila suzukii

Drosophila suzukii (spotted wing fruit fly) is evolving to be a globally pervasive pest due to its rapid range expansion. *Drosophila suzukii* is thought to have originated in South East Asia, possibly in South China-Northern India. It then spread across Japan in the early 1900s and reached Hawaii in the 1980s but was not considered a pest. It then expanded to a Santa Cruz County raspberry field in California, USA, in 2008(Hauser 2011). It was reported from Spain within the same year(Calabria, Maca et al. 2012). By 2009, it had spread across the USA including California, Washington, Oregon, Florida, and British Columbia in Canada (Hauser 2011). It has continued to spread across northern America and Europe, including Italy, France, Mexico, Germany, Austria, Croatia, Spain and the United Kingdom (Lee, Bruck et al. 2011; Cini, Ioriatti et al. 2012; Rouzes, Delbac et al. 2012).

Drosophila suzukii is one of two species of *Drosophila*, the other being *Drosophila pulchella Tan*, known to oviposit in healthy fruit instead of damaged or ripened fruit (Mitsui, Takahashi et al. 2006; Walsh, Bolda et al. 2011). The females have prominent serrated ovipositors and egg insertion into the fresh fruit can cause damage. These oviposition wounds are susceptible to secondary infection by other insects and microorganisms causing further damage (Camargo and Phaff 1957; Molina, Harrison et al. 1974; Louis, Girard et al. 1996; Atallah, Teixeira et al. 2014). Additionally, the developing larvae remain in, and feeding upon, the fruit cause more damage, potentially resulting in a reduction in crop yields of up to 80%, and significant financial loss (Walsh, Bolda et al. 2011).

In order to have effective pest management it is necessary to have reliable and rapid identification of the pest, and this is not often possible for emerging species. Accurate identification is near impossible unless the pests have been reared to adults. However, rearing to adults for identification can be a lengthy task, and producers may lose a large amount of gross income during these periods (Dhami and Kumarasinghe 2014). Fortunately, there are detailed morphological identification keys available for the species that belong to the *melanogaster* group (Yang, Hou et al. 2012), and more recently a more resolved identification guide for *D. suzukii*was produced (Hauser 2011). However, identification keys are only available for adults and not for larvae, pupae and eggs (Dhami and Kumarasinghe 2014).

Currently, the most effective insecticides for controlling *D. suzukii* are conventional broad spectrum products such as organophosphates and pyrethroids, which are unfavoured by integrated pest management programs, (Beers, Steenwyk et al. 2011; Haviland and Beers 2012; Timmeren and Isaacs 2013). Neonicotinoids are also used, but to a lesser extent as they are suggested to be less effective (Bruck, Bolda et al. 2011). One type of insecticide that is specific to control of *D.suzukii* is the spinosyns, such as spinosad and spinetoram, which are in limited use to minimise the spread of resistance (Beers, Steenwyk et al. 2011; Bruck, Bolda et al. 2011; Haviland and Beers 2012). In conclusion, there are limited options for managing *D. suzukii*, thus, garlic-based products could be a promising alternative insecticide.

1.3 ANTIMICROBIAL ACTIVITY OF GARLIC

1.3.1 Effect of garlic compounds on bacteria

Garlic is also well known to be an anti-bacterial agent and it is effective against both gram positive and gram negative bacteria such as *Escherichia coli (E. coli), Helicobacter pylori,* and *Lactobacillus casei* (table 1.3). Allicin is the garlic constituent compound that is mostly responsible for these anti-bacterial effects (Cellini, Di Campli et al. 1996; Lemar, Passa et al. 2005). There are many reports demonstrating the antimicrobial effects of garlic including against *Salmonella enteritidis, Staphylococcus aureus, Salmonella typhimurium, Brusella abortus, Bacillus anthracis,* and *Vibrio*(Fromtling and Bulmer 1978; Prasad and Sharma 1980; Weber, Andersen et al. 1992; Lemar, Passa et al. 2005).

Many studies report the use of garlic on hospital-acquired bacterial infections that are drug resistant. For example, dried garlic has an effect on vancomycin and vancomycin allicin, and can affectbacteriostaticity in vancomycin-dependent enterococcus (VDE)(Jonkers, Sluimer et al. 1999).*Klebsiella pneumoniae* and *Pseudomonas aeruginosa* represent common hospital-acquired infections that can be effectively treated with a combination of diallyl trisulfide and diallyl tetrasulfide with cephtazidim, gentamicin, meropenem, and imipenem (Weber, Andersen et al. 1992; Tsao and Yin 2001).

Limura*et al* (2002) studied the effect of garlic against *Helicobacter pylori*, the causative agent of gastritis and stomach cancer. It was observed that mice infected with *H. pylori* and given garlic extract had increased gastritis symptoms but that stomach cancer did not occur. The control, which was not given the garlic extract, developed both gastritis symptoms and stomach cancer (Iimuro, Shibata et al. 2002). However, these results contrast with human studies of *H. pylori* infections in which there was a prevention in stomach cancer, gastritis, and gastrointestinal infections following treatment with garlic extract (O'Gara, Maslin et al. 2008).

The antibacterial mechanism of garlic compounds remains unclear. There has been studies using allicin to inhibit Salmonella typhimurium which suggested RNA synthesis inhibition as a primary target (Feldberg et al, 1988). Other studies demonstrate that allicin and other thiosulfinates from garlic react to cysteine and inhibit acetyl-CoA synthases (Fujisawa et al, 2009; Focke et al, 1990). More recent studies show the mode of action of allicin and diallyl polysulfides is a combination of a decrease in glutathione levels and S-allylmercapto-modification of cysteines causing inactivation of metabolic enzymes; both

inducing thiol stress in bacteria (Muller et al, 2016). Diallyl polysulfides have strong reactivity towards thiol groups which is important for their antibacterial activity. These compounds bare an electrophilic sulfur centre which forms an S-allylmercapto adduct upon reacting with thiols, therefore, enzymes containing important thiol groups are oxidized and inhibited (Leontiev et al, 2018). The ability of allicin to permeabilize cell membranes could possibly contribute to its antimicrobial activity (Gruhlke et al, 2015).

Table 1.3: Minimum inhibitory concentration (MIC) values (mg/L) of diallyl polysulfides active against different pathogenic bacteria. MIC values have been converted to mg/L for comparison across studies and to facilitate an overview of the sensitivity of the different bacterial species to the effects of polysulfides. DAS1: diallyl sulfide; DAS2: diallyl disulfide; DAS3: diallyl trisulfide; DAS4: diallyl tetrasulfide.

Redox modulator	**Organism**	**Dose (MIC) mg/L**	**Reference**
Allicin	*H. pylori*	6-12 12.5	(O'Gara, Hill et al. 2000) (Aala, Yusuf et al. 2012)
	T. rubrum	12.5	(Aala, Yusuf et al. 2012)
	Pseudomonas spp. *Streptococcus spp.* *Staphylococcus spp.* *P. aeruginosa* *S. pneumonia* *S. pyogenes* *S. aureus* *A. baumannii*	64 64 64 64 32 32 32 16	(Reiter, Levina et al. 2017)
	E. coli	23	(Muller, Eller et al. 2016)
DAS1	*H. pylori*	2,074- 4,148	(O'Gara, Hill et al. 2000)
	B. cereus *C. jejuni* *C. botulinium* *E. coli* *L. monocytogenes* *S. enteric* *S. aureus* *V. cholerae*	64 56 64 72 48 54 64 72	(Rattanachaikunsopon and Phumkhachorn 2008)
DAS2	*S. aureus* *H. pylori*	2 100	(O'Gara, Hill et al. 2000)
	B. cereus *C. jejuni* *C. botulinium*	14 12 20	(Rattanachaikunsopon and Phumkhachorn 2008)

	E. coli *L. monocytogenes* *S. enteric* *S. aureus* *V. cholerae*	20 8 12 16 24	
DAS3	*S. aureus* *H. pylori*	0.5 13-25	(O'Gara, Hill et al. 2000)
	B. cereus *C. jejuni* *C. botulinium* *E. coli* *L. monocytogenes* *S. enteric* *S. aureus* *V. cholera*	4 2 4 12 2 2 8 12	(Rattanachaikunsopon and Phumkhachorn 2008)
	M. tuberculosis	2.5	(Oosthuizen, Arbach et al. 2017)
DAS4	*H. pylori*	3-6	(O'Gara, Hill et al. 2000)
	B. cereus *C. jejuni* *C. botulinium* *E. coli* *L. monocytogenes* *S. enteric* *S. aureus* *V. cholerae*	1 1 1 4 0.5 0.5 2 4	(Rattanachaikunsopon and Phumkhachorn 2008)
Garlic oil[a]	*H. pylori*	8-32	(O'Gara, Hill et al. 2000)
	B. cereus *C. jejuni* *C. botulinium* *E. coli* *L. monocytogenes* *S. enteric* *S. aureus* *V. cholera*	40 36 32 48 20 32 36 40	(Rattanachaikunsopon and Phumkhachorn 2008)
	S. boydii *S. flexnar* *S. fluvialis* *V. metschnikovii* *V. parahaemoyticus* *V. enterocolittica* *C. coli* *C. lari* *B. fragilis* *B. subtilis* *E. aerogenes* *E. faecalis* *K. aerogenes*	2.75 2.75 2.75-5.5 0.34 0.08 0.68 0.49 0.49 0.04 0.17-0.68 0.68 0.34 0.17	(Ross, O'Gara et al. 2001)

	P. vulgaris *L. acidophilus* *S. faecalis* *S. mutans* *S. pyogenes*	2.74 0.34-2.75 0.34 0.08 0.04	
	R. fascians, *A. tumefaciens* *E. amylovolora* *P. solanacerum*	250 300 300 750	(Soltan, et al. 2016)
Garlic Extract[a]	*P. gingivalis* *A. actinomycetemcomitans*	16.6 62.5	(Shetty, Thomas et al. 2013)
	S. aureus *E.coli* *P. aeruginosa*	4 7 6	(Saha, Saha et al. 2016)
	B. subtilis *K. pneumonia* *S. epidermidis* *S. typhi*	0.1 0.2 0.9 0.02	(Gull, Saeed et al. 2012)
	Proteus spp.	7-21	(Durairaj, Srinivasan et al. 2009)
	H. pylori	2-5	(Cellini, Di Campli et al. 1996)
	S. epidermidis *S. pneumoniae* *S. pyogenes* *H. influenzae* *Shigella spp.* *P. aeruginosa*	22.9 30.3 33 30.5 15.6 3.5	(Iwalokun, Ogunledun et al. 2004)
	S. mutans	4-32	(Fani, Kohanteb et al. 2007)
	B. cenocepacia *B. stabilis* *B. cepacia*	8-16 16 62	(Wallock-Richards, et al. 2014)

[a]Activity depends on how garlic extract or oil is manufactured. Various papers representing the biological activity of polysulfides do not mention the characterisation of the preparations. The polysulfide concentration in an extract or oil depends on method of extraction or distillation.

* Note there has been a lack of studies in recent years on the antimicrobial activity of garlic, possibly due to the large amount of studies that has been done previously.

1.3.2 Effect of garlic compounds on Viruses

Published literature on the effects of garlic against viral infections is scarce. It has been reported that garlic is effective against influenza B, herpes simplex types 1 and 2, human cytomegalovirus, viral encephalitis, and pertinacious virus (Weber, Andersen et al. 1992; Hanafy, Shalaby et al. 1994; Josling 2001). Weber et al (1992) demonstrated this by virucidal assays. There is evidence that allicin and its condensation products are the main

components in garlic that are accountable for antiviral activity. Allimax® Liquid and Capsules, an allicin-containing supplement, has been shown to be effective against herpes simplex types 1 and *Molluscum contagiosum* infections, which was shown in a placebo-controlled survey. However, the authors did not state a possible mechanism(Josling 2001).

1.3.3 Effect of garlic compounds on Fungi

The treatment of fungi can be difficult due to the development of resistance that can occur with long term medication as well as the difficulty of treating disease-causing agents mediated by eukaryotic organisms. It is reported that garlic has fungistatic effects with different fungal genera such as *Candida*, *Aspergillus* and *Cryptococci* (table 1.4). Intravenous application of garlic extract is reported to have a greater lethal effect against *Cytococcal meningitis* than Amphotericin-B, which is a standard antifungal treatment for the most severe invasive mycoses(An, Shen et al. 2009; Ogita, Fujita et al. 2009).The antimicrobial activity of garlic is especially effective against *Candida albicans*, *Histoplasma capsulatum, Aspergillus, Trichophytum species*, and *Penicillum*. These effects of garlic extract can also be obtained when using pure allicin (Adetumbi and Lau 1983).

Table 1.4: MIC (mg/L) of diallyl polysulfides against different pathogenic fungal species. MIC values have been converted to mg/L for comparison across studies and to facilitate an overview of the sensitivity of the different bacterial species to the effects of polysulfides. DAS1: diallyl sulfide; DAS2: diallyl disulfide; DAS3: diallyl trisulfide; DAS4: diallyl tetrasulfide.

Redox modulator	**Organism**	**Dose (MIC) mg/L**	**Reference**
Allicin	*C. albicans* *C. neoformans* *C. parapsilosis* *C. tropicalis* *C. krusei* *T. glabrata*	0.8 0.3 0.15 0.3 0.3 0.3	(Ankri and Mirelman 1999)
	Aspergillus spp.	8-32	(Shadkchan, Shemesh et al. 2004)
DAS2	*C. krusei* *C. albicans* *C. krusei* *C. glabrate* *A. niger* *A. fumigates*	8 1 8 4 2 8	(O'Gara, Hill et al. 2000)

	A. flavus	4	
DAS3	*C. krusei* *C. albicans* *C. krusei* *C. glabrate* *A. niger* *A. fumigates* *A. flavus*	2 0.5 4 2 1 4 2	(O'Gara, Hill et al. 2000)
Garlic oil[a]	*C. albicans* *P. funiculosum*	0.35 0.69	(Li, Shi et al. 2016)
Garlic Extract[a]	*Candida spp*	14.9	(Iwalokun, Ogunledun et al. 2004)

[a] Activity depends on how garlic extract or oil is manufactured. Various papers representing the biological activity of polysulfides do not mention the characterisation of the preparations. The polysulfide concentration in an extract or oil depends on method of extraction or distillation.
* Note there has been a lack of studies in recent years on the antimicrobial activity of garlic.

1.3.4 Effect of garlic compounds on protozoa

Several studies show the effectiveness of garlic extract against protozoa, including*Trypanosomes, Balantidium entozoon, Entamoeba histolytica, Leishmania, Scedosporiumprolificans, Leptomonas, Opalinaranarum,* and*Crithidia*(Davis, Perrie et al. 2003; Bayan, Koulivand et al. 2014). It has been recommended that garlic should be used for treatment of giardiasis due to the side effects of, and the increase in resistance, to other treatments. Crude garlic extract concentration of 25 pg/mL is sufficient enough to inhibit the activity of giardia and approximately 50 pg/mL is the lethal dosage for the infective agent. It was established that garlic is effective as an antigiardial since in 24 hours it had removed symptoms from all patients and within 72 hours it had completely removed any indication of giardiasis from stool samples. The patients had been given 0.6 mg/mL prepared garlic capsules or 1 mg/mL twice daily aqueous extract. It was suggested that the antiprotozoal effects are due to allicin, ajoene and organosulfides in the garlic (Soffar and Mokhtar 1991). In conclusion, garlic compounds have a wide range of anti-microbial effects. However, as explored in the next section, they also have potentially potent activity as insecticides.

1.4 THE CHEMISTRY OF GARLIC: HOW ITS CONSTITUENTS (DIALLYL POLYSULFIDES) WORK

Crushing of garlic causes the production of thiosulfinates from sulfur-substituted cysteine sulfoxides, such as allicin. This occurs via the activity of alliinase and the highly reactive intermediate sulfur-substituted sulfenic acids (figure 1.7 and 1.8) (Block, 1992). The thiosulfinates are compartmentalized with alliinase in the cell vacuole. Other thiosulfinates such as allyl-methyl-, methylallyl-, and trans-1-propenyl-thiosulfinate has also been found in garlic, and these are like allicin, also unstable (Lawson, Wang et al. 1991; Lawson and Wang 2005). Allicin can decompose into diallyl sulfide (DAS1), diallyl disulfide (DAS2), diallyl trisulfides (DAS3), and sulfur dioxide, figure 1.9. Allicin is highly reactive with amino acids and proteins where a –SH group is created (Rose et al, 2005; Omar and Al-Wabel 2010). Allicin cannot be detected in the blood when raw garlic or pure allicin is ingested (Lawson and Wang 2005). There is evidence that allicin can affect DNA processing, signal transduction, RNA synthesis and apoptosis (Feldberg, Chang et al. 1988; Rabinkov, Miron et al. 2000). It has been reported that these allicin effects may be mediated via nitric oxide formation. The inhibitory effect of these garlic compounds is mediated via a reduction of nitric oxide synthase mRNA expression rather than a direct effect on nitric oxide synthase enzymatic activity (Dirsch, Kiemer et al. 1998). The biological effects of allicin are associated with both its antioxidant and SH-modifying activities, which have been demonstrated in model systems (Wills 1956; Han, Lawson et al. 1995; Ankri, Miron et al. 1997; Miron, Rabinkov et al. 1998; Rabinkov, Miron et al. 1998). It has also been shown that allicin is able to permeate the cell membrane and still affect intracellular activities (Miron, Rabinkov et al. 2000). Figure 1.6 summarizes biological effects of garlic constituents.

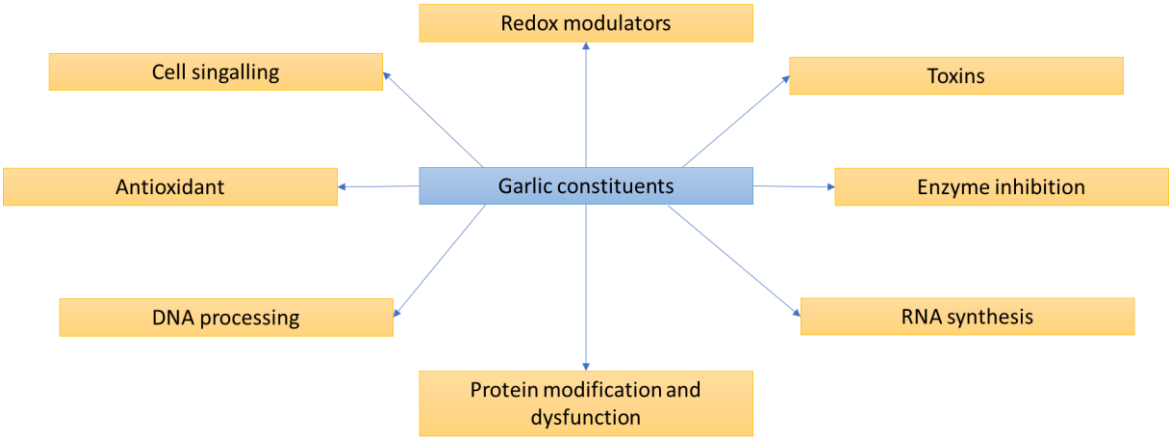


Figure 1.6: Summarized illustration of known biological properties of garlic constituents.

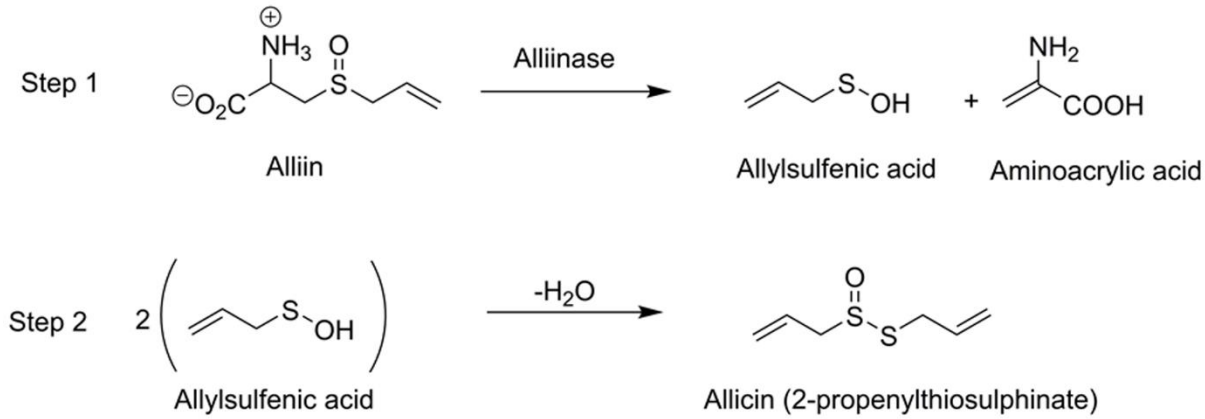


Figure 1.7. Chemical structure of allicin and mechanism of formation from alliin by the enzyme alliinase. Step 1. Alliin is hydrolysed by to produce allylsulfenic acid which, in step 2, react together spontaneously to produce allicin with the loss of water (Wallock-Richards et al, 2014).

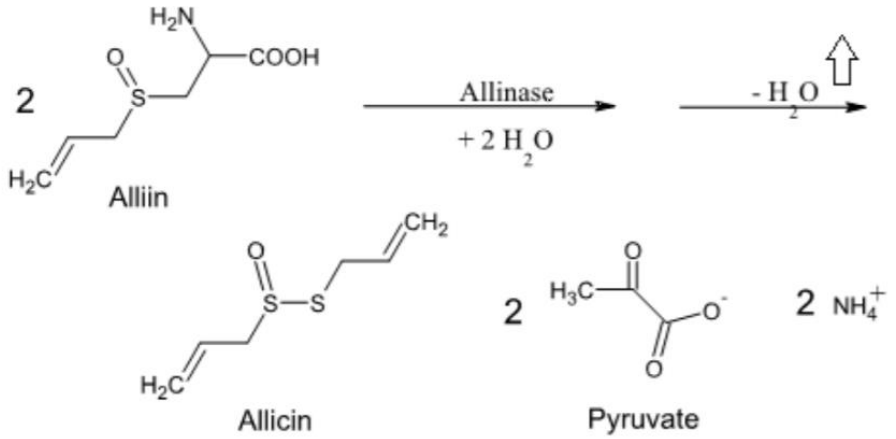


Figure 1.8: Reaction of alliinase with alliin (Weiner et al., 2009)

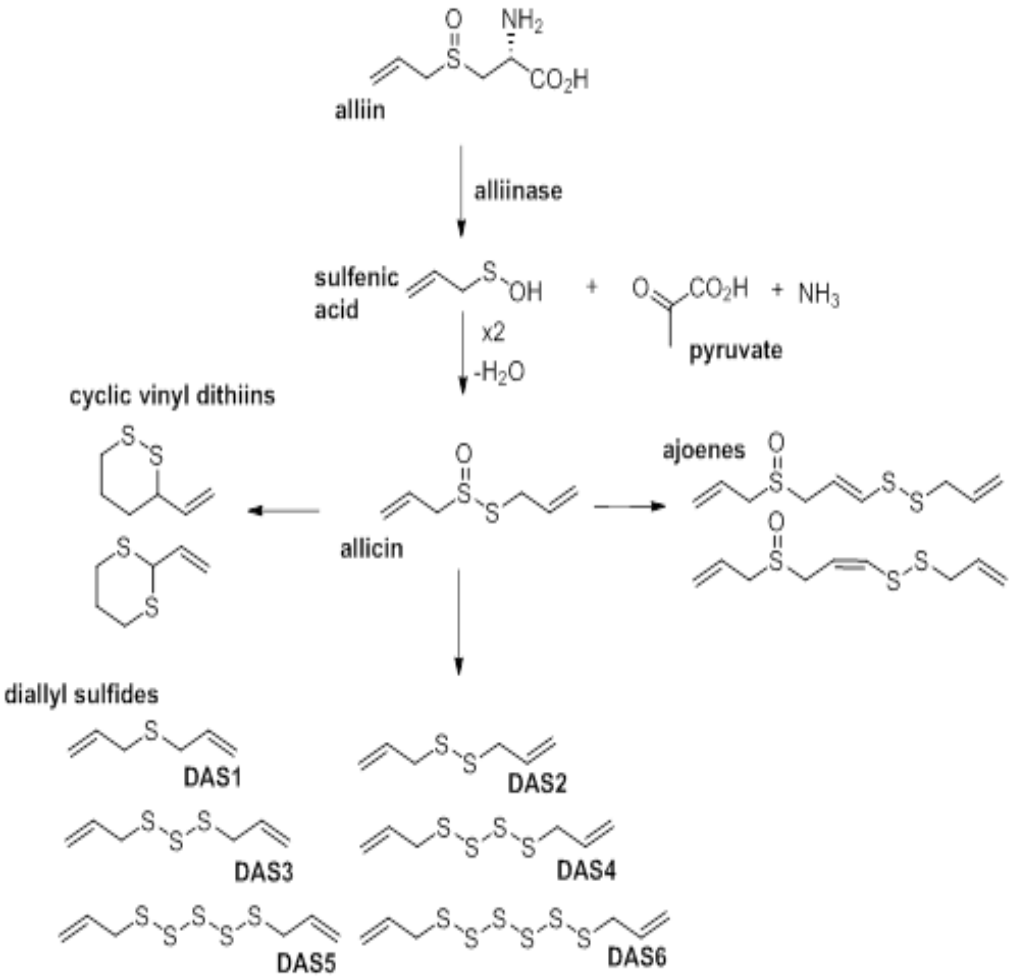


Figure 1.2

Figure 1.9: Allicin can decompose into different organosulfur compounds. Alliinase reacts with alliin to produce sulfenic acid which then produces allicin. Allicin is very unstable and can decompose into cycle vinyl dithiins, ajoenes, and diallyl sulfides.

Alliin is the major odour precursor of garlic, and is also known as S-(+)-allyl-l-cysteine sulfoxide S-2-propenyl cysteine sulfoxide or ACSO, figure 1.10. Alliin is decomposed upon cell damage to produce DAS1, DAS2, and DAS3 and gives garlic its distinct odour (Palani et al, 2014; Rose et al, 2005).

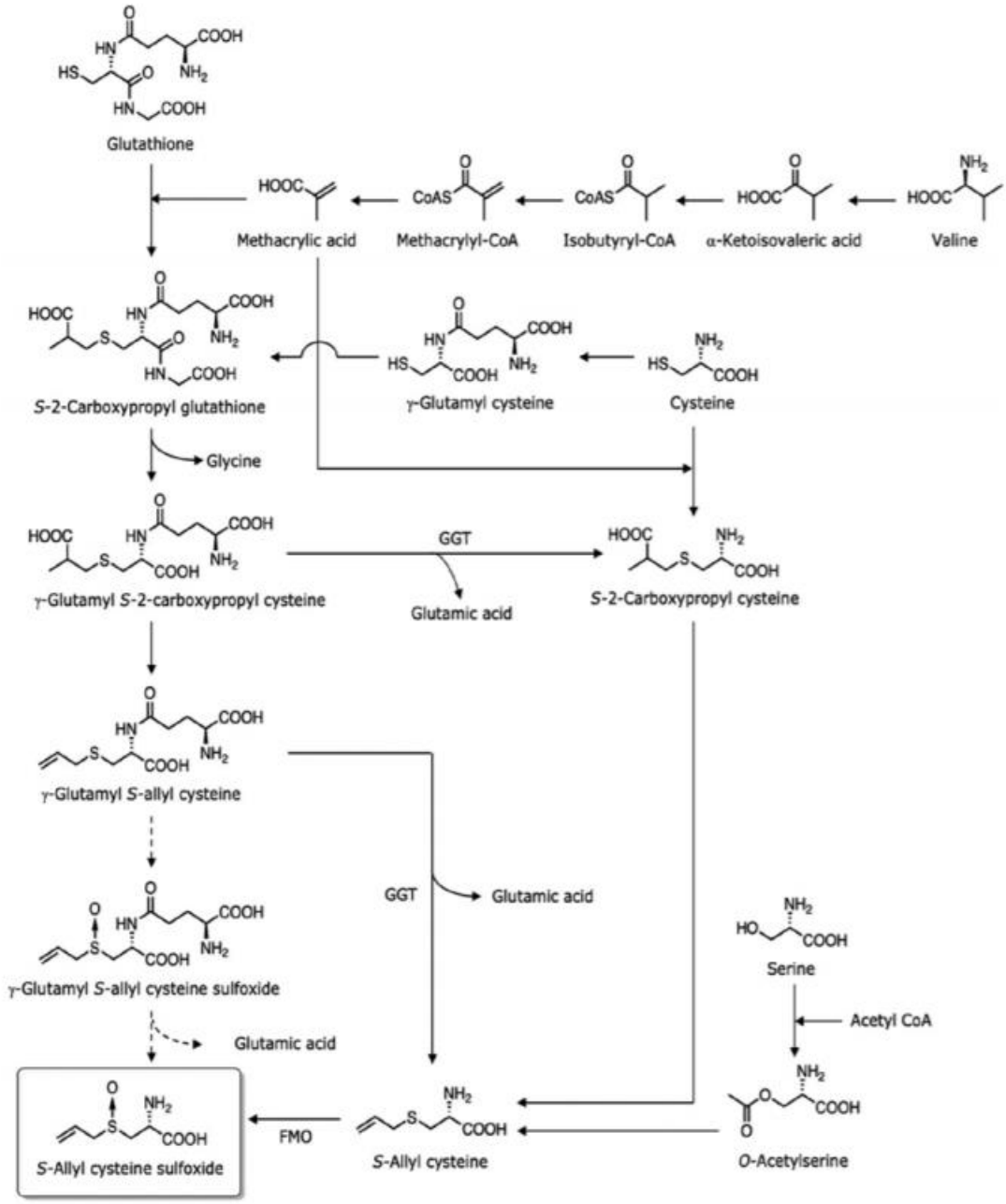


Figure 1.10: The proposed pathways for the biosynthesis of alliin (ACSO, S-allyl-l-cysteine sulfoxide) (Yamaguchi and Kumagai, 2020)

Over 20 sulfides in garlic oil and garlic extract have been identified, some chemical compositions are shown in table 1.5 and 1.6. Many of these, in particular sulfides that have an allyl group, are responsible for the characteristic taste and smell of ingested garlic. The major sulfides in garlic oil are diallyl sulfides, allylmethyl, and dimethyl mono- to hexasulfides. Some garlic oils can also contain small amounts of allyl 1-propenyl and methyl 1-propenyl di-, tri-, and tetrasulfides (Lawson and Hughes 1992). The most abundant in fresh garlic oil is diallyl trisulfide. However, garlic oil products that are

commercially available contain higher amounts of diallyl disulfide, which is thought to be due to the disproportionate level of diallyl trisulfide in the oil, shown in table 1.7 as examples. Variation in the amount of these different sulfides is associated with temperature or garlic oil preparation time (Amagase 2006).

Table 1.5: Chemical compounds of interest found in garlic bulb (Omar and Al-Wabel, 2010).

Chemical compound	**Amount (ppm)**
2-Vinyl-4H-1,3-dithiin	2–29
3,5-Diethyl-1,2,4-trithiolane	0.15–43
3-Vinyl-4H-1,2-dithiin	0.34–10.65
Alanine	1320–31,168
Allicin	1500–27,800
Alliin	5000–10,000
Allyl-propyl-disulfide	36–216
Cystine	650–1560
Diallyl-disulfide	16–613

Diallyl-sulfide	2–99
Diallyl-trisulfide	10–1061
S-allo-mercapto-cysteine	2
S-allyl-cysteine	10

Table 1.6: Chemical composition of three different garlic oils (Chekki et al, 2014).

Chemical compounds	**Tunisian garlic oil (%)**	**Seoulean garlic oil (%)**	**Argentinean garlic oil (%)**
Diallyl sulfide	4.1	-	2.2
Allyl methyl disulfide	6.5	0.13	-
Allyl methylsulfide	-	-	0.9
Dimethyl trisulfide	-	0.51	2.3
Diallyl disulfide	44.6	32.8	34
Allyl methyl trisulfide	11.8	7.4	13.1
3-vinyl-1,2-dithiin	4.04	1.99	2.1
2-vinyl-1,3-dithiin	1.2	5.9	1.6
Diallyl trisulfide	27.7	29.1	24

The beneficial effects of garlic are associated with the presence of organosulfur compounds, in particular, allyl derivatives. One of the constituents of garlic is alliin (S-allyl cysteine sulfoxide) which is a non-protein amino acid. Biosynthesis of alliin is initiated by *S*-alkylation of glutathione, which is followed by the removal of glycyl and γ-glutamyl groups, and then *S*-oxygenation. Most of the enzymes involved in the biosynthesis of *S*-alkyl-L-cysteine sulfoxides in *Allium* plants have not been identified. Figure 1.11 shows the proposed biosynthesis pathway of alliin by Yoshimoto et al (2014) and figure 1.12. Is the proposed biosynthesis of alliin and other sulfoxides by Lawson (1996). There is approximately 1% of alliin in dried garlic but this decreases to non-detectable amounts within a few days due to instability (Omar and Al-Wabel 2010). Other major sulfur-containing compounds are γ-glutamyl-S-allyl-L-cysteines. Alliin is odorless until it is converted into thiosulfinates, such as allicin, via enzymatic reactions by alliinase when the raw garlic is cut or crushed. Therefore, thiosulfinates are not found in intact garlic (Amagase 2006).

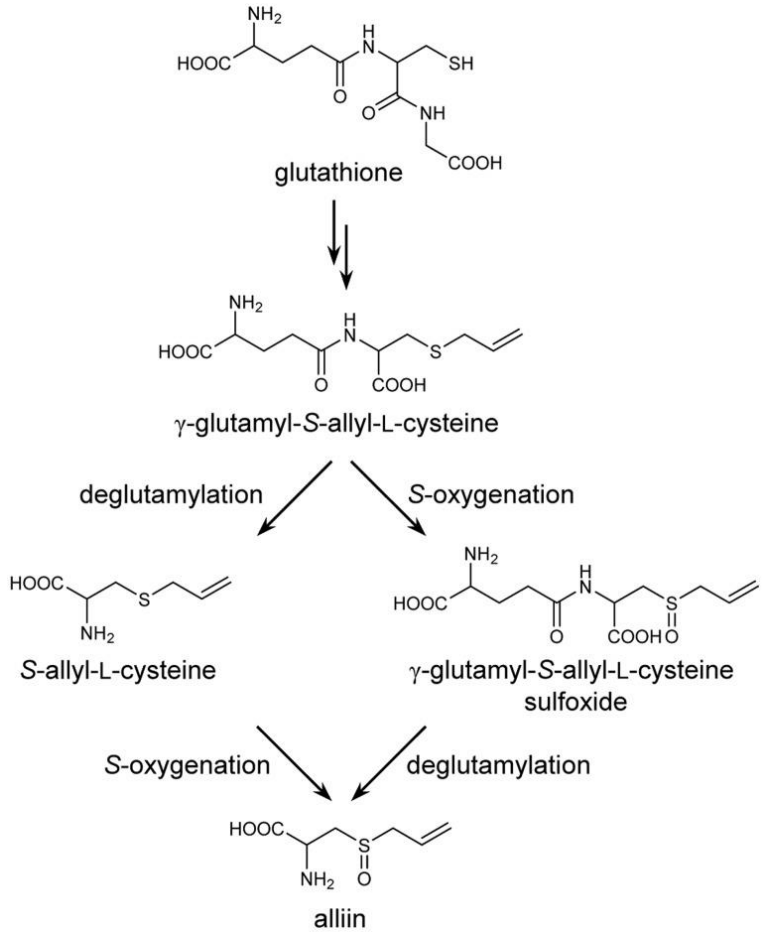


Figure 1.11: Proposed biosynthetic pathway for alliin in garlic by Yoshimoto et al (2014).

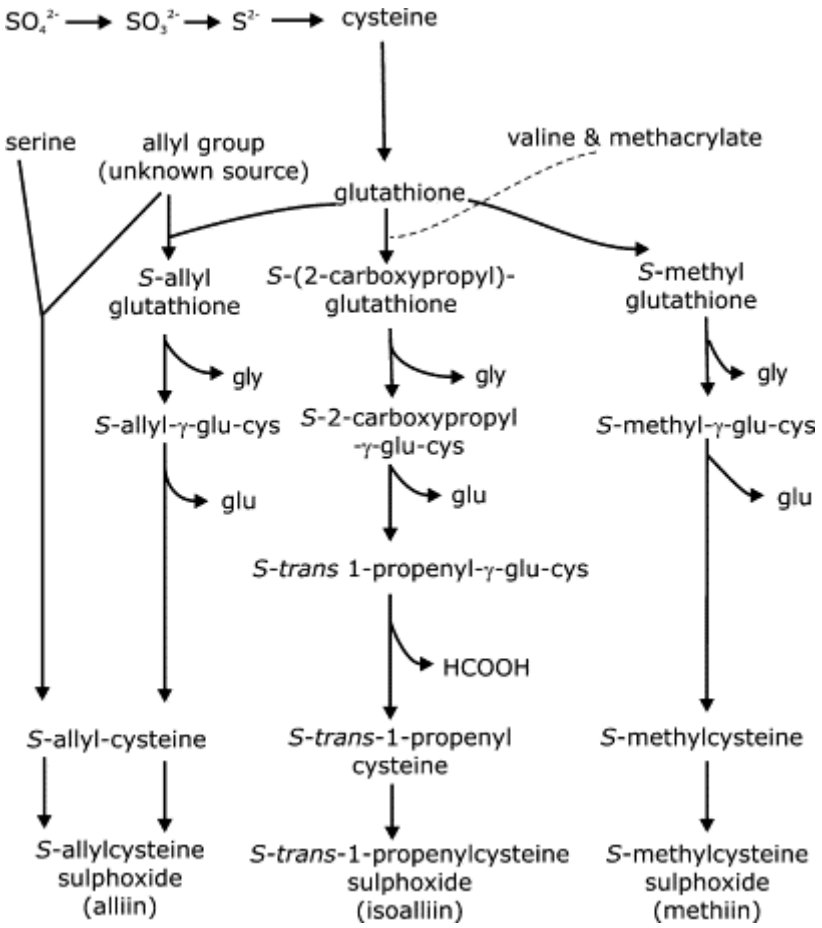


Figure 1.12: Proposed biosynthesis of sulfoxides in garlic (Lawson, 1996)

Many biosynthesis pathways have been proposed for alliin (such as those in figure 1.11 and 1.12) (Hughes et al, 2005). The location of the biosynthesis of alliin has not been identified. γ-Glutamyl cysteines are located in the chloroplast, γ-glutamyl peptides are located in the cytoplasm, and cysteine sulfoxides are located in the cytoplasm or cytoplasmic vesicle. Whereas for the location of the biosynthesis enzymes required, γ glutamyl cysteine synthetase resides in the chloroplast, and γ glutamyl transpeptidase and flavin containing mono oxygenase resides in the cytoplasm (Jones et al, 2004). The decomposition of alliin is supported by the work of Yamaguchi et al (2019) where alliin is decomposed into allyl-sulfenic acid via allicin, and then two allyl-sulfenic acid compounds produce diallyl polysulfides. Derivatives of alliin in garlic consists of many organofulfur compounds, S-allyl-cysteine, S-allyl-mercapto cysteine, and N-acetylcysteine (Tran et al, 2018; Asdaq and Inamdar, 2011). S-allyl-cysteine has many properties including antioxidant, signalling, anti-inflammation, regulated redox, and antiapoptotic activities (Zeng et al, 2017). S-allyl-mercapto cysteine has been shown to have anticancer capabilities by preventing cancer cell proliferation (Liu et al, 2015).

The therapeutic role of allicin in anticancer and antibiotic treatments has been challenged by reports that have confirmed that diallyl polysulfides have a similar or even superior antibiotic and anticancer effects when directly compared (O'Gara, Hill et al. 2000; Tsao and Yin 2001; Tsao and Yin 2001; Munday, Munday et al. 2003; Xiao, Herman-Antosiewicz et al. 2005). Diallyl trisulfide and diallyl tetrasulfide are breakdown products of allicin and occur naturally in garlic. There have been many studies that show that diallyl trisulfides and diallyl tetrasulfides have a wide range of activity including antibacterial, antimicrobial, antifungal, and anticancer (Wills 1956; Antosiewicz, Herman-Antosiewicz et al. 2006; Xiao and Singh 2006). However, diallyl trisulfides and diallyl tetrasulfide are less reactive to thiols in comparison to allicin. Allicin has the ability to rapidly modify thiol groups of peptides and proteins (Rabinkov, Miron et al. 2000; Munchberg, Anwar et al. 2007).

Alliinase is the main enzyme involved in the transformation of cysteine sulfoxides to thiosulfinates. The substrate for alliinase is S-methyl-L-cysteine and alliinase has an optimum pH of 6.5 (Mazelis and Crews 1968). The optimum temperature of alliinase in Allium species varies between 30-40℃ and the optimum pH for alliinase enzymatic activity is 6.7-7 (Jansen et al,1989; Keusgen et al, 2002) Alliinase activity has shown to be pH dependent, with allicin and other thiosulfinates being released during incubation of garlic powder in buffer solutions with a pH range of 2 to 10. It has also been shown that thiosulfinates cannot be produced with pH below 3.6 and hence are not found in the stomach (Lawson and Hughes 1992). Alliinase uses pyridoxal-5′-phosphate (PLP) as a cofactor to convert alliin to allicin, pyruvate, and ammonia. Alliinase has been crystallized and its three-dimensional structure solved. Alliinase is a family of isozymes with differing substrate specificity for thiosulfinates among Allium species (Weiner, Shin et al 2009). The affinity of the enzyme for substrate also varies depending on the alliinase source, with Km ranging from 2.7 mM for Chinese chive to 13 mM for leek (Musah, He et al 2009). Krest et al (2000) found the highest specific alliinase activity amongst the Allium family was found for garlic at 178 μmol min-1 mg-1 (Krest,Glodek et al 2000).

Allicin can decompose into cycle vinyl dithiins, ajoenes, and diallyl sulfides (Munchberg, Anwar et al. 2007). Cycle vinyl dithiins are volatile artefacts of allicin. The vinyl dithiins generated are 2-vinyl-[4H]-1,3-dithiin and 3-vinyl-[4G]-1,2-dithiin (Yu, Wu et al. 1989). Ajoenes are a well-known antiplatelet agents, capable of inhibiting the aggregation of platelets, and have been studied in both *in vivo* and *in vitro* experiments (Apitz-Castro,

Cabrera et al. 1983; Apitz-Castro, Escalante et al. 1986; Apitz-Castro, Jain et al. 1991; Apitz-Castro, Badimon et al. 1994). Ajoenes are also potent modulators of membrane-dependent functions of immune cells that are regulated by signal transduction systems (Romano, Montano et al. 1997). Ajoene is also reported to induce apoptosis and inhibit proliferation in many cancer cell lines. This leads to the reduction of tumour growth *in vivo* (Li, Ciu et al. 2002).

1.5 CHEMISTRY OF GARLIC: GSH AND OXIDATIVE STRESS

It has been established that diallyl polysulfides and other sulfur containing compounds derived from garlic can react with reduced glutathione (GSH) (Munchberg, Anwar et al. 2007). GSH is the most abundant non-protein thiol in mammalian systems and has the potential to interact with allicin (Khramtsov, Yelinova et al. 1989). GSH is the main cofactor for instantaneous interaction with allicin in living cells (Meister and Anderson 1983). GSH is converted to GSSG by GSH peroxidase via the reduction of H_2O_2 and other peroxides. The conversion of GSH to GSSG can also occur by transhydrogenation. GSSG reductase uses NADPH to mediate the reduction of GSSG to GSH. The conversion of GSH to GSSG can also occur extracellularly, a process that requires O_2 and leads to H_2O_2 formation. Free radicals can also react with GSH to form GSSG (Meister and Anderson 1983). γ-Glutamyl transpeptidase catalyses the breakdown of GSH, and also GSSG and S-substituted GSH, by transferring the γ-glutamyl moiety to acceptor amino acids such as cysteine, methionine, glutamine, certain dipeptides, GSH itself, and water. GSH interacts with γ-glutamyl transpeptidase and is transported across the cell membrane (Ballatori, Krance et al. 2009; Baudouin-Cornu, Lagniel et al. 2012). The cleavage of GSH to produce resulting amino acids takes place externally on the plasma membrane for the regeneration of intracellular GSH (Pompella, Corti et al. 2007). The intra- and extracellular levels of GSH are tightly regulated by the balance of GSH production, consumption, and transportation. The enzymatic activity of GSH metabolism is controlled at the transcriptional, translational, and post-translational levels (Aquilano, Baldelli et al. 2014).

GSH is effectively a scavenger of free radicals and other ROS and RNS (reactive oxygen and nitrogen species, respectively) e.g. superoxide anion, hydroxyl radical, hydrogen peroxide, and lipid peroxyl radical. This scavenging activity can be direct or can occur indirectly via enzymatic reactions (Johnson, Wilson-Delfosse et al. 2012). The thiol moiety of GSH is important due to its antioxidant role in the direct scavenging of radical species. The one-electron reduction with radicals is kinetically driven in the forward direction by the removal of the unstable thiyl radical GS via following reactions with thiolate anion (GS-) and then with oxygen. The first reaction generates GSSG-, GSSG and superoxide is generated when in the presence of oxygen. The antioxidant enzymes superoxide dismutase (SOD) in association with catalase or glutathione peroxidase (GPX) blocks the radical chain reactions which determines the complete free radicals scavenging (Aquilano, Baldelli et al. 2014).

GSH acts as an antioxidant by detoxifying products derived from ROS-promoted oxidation of lipids, such as 4-hydroxy-2-nonenal and malonyl dialdehyde, and also other products from ROS interaction with cellular components (Aquilano, Baldelli et al. 2014). These reactions lead to the formation of thiyl radicals that can combine with different compounds and other thiyl radicals which can lead to the generation of GSSG. Additionally, GSH can conjugate with various highly reactive electrophilic compounds or via the action of GST (Eaton and Bammler 1999; Strange, Jones et al. 2000). GSH can also react with toxic metabolites produced via normal cellular metabolism, such as methylglyoxal derived from the glycolytic pathway (Martins, Cordeiro et al. 2001; Inagi, Kumagai et al. 2010). Methylglyoxal is also involved in the generation of ROS. In methylglyoxal elimination, which involves two glyoxalases, GSH acts as a cofactor (Yadav, Singla-Pareek et al. 2008; Inagi, Kumagai et al. 2010). GSH also have other functions including: storage of cysteine reserves; signal transduction from the environment to the cell transcription machinery; maintaining essential thiol status of cysteine residues on proteins; metabolism of oestrogens, prostaglandins and leukotrienes; participation in iron-sulfur maturation in proteins; and involvement in deoxyribonucleotide production (Dickinson and Forman 2002).

The intracellular levels of GSH can determine the ability of cells to undergo apoptosis via the ROS-mediated intrinsic pathway. This signifies the GSH-related aspects with regards to: (i) indication of S-glutathionylation in apoptotic initiation and protein modulation; (ii) decreased GSH/GSSG ratio leading to changes in cellular GSH redox homeostasis due to either oxidation or export of GSH in association with the initiation or execution of the apoptotic cascade (Ghibelli, Coppola et al. 1995). Many pro-apoptotic stimuli can induce an early GSH extrusion from the cells, which would lead to mitochondrial damage and release of cytochrome c into the cytosol. The GSH extrusion is a result of a process by carriers to efficiently achieve the apoptotic programme (Ghibelli, Fanelli et al. 1998).

The glutathione redox couple GSH/GSSG is very important for cells, along with other redox couples, such as NADPH/NADP+, to maintain and regulate the appropriate cellular redox status. The *in vivo* redox potential for GSH/GSSG has been estimated to range from -260 mV to – 150 mV, depending on the cellular conditions (Jones 2002). Therefore, changes in the ratio of GSH/GSSG are vital for signal transduction (Schafer and Buettner 2001). Cellular conditions caused by an increase in ROS levels would require enhanced GSH activity to maintain redox status and an increase in precursors and energy supply to

increase GSH content and/ or GSH transport to required places. However, when cellular systems are unable to counteract prolonged oxidative stress, GSH decreases, which results in irreversible cell degeneration and cell death (Zhang and Forman 2012).

Other roles that have been discovered for the antioxidant function of GSH in signal transduction include: (i) involvement in the process of protein S-glutathionylation; and (ii) interaction with NO or RNS. Protein S-glutathionylation is a post-translational modification which provides protection for cysteine residues from irreversible oxidation and facilitates redox signal transduction by changing the structure/function of the target protein. This is detected under a large increase in radical species or under physiological flux of ROS. For most proteins, this process of S-glutathionylation occurs only under non-physiological GSH/GSSG ratio (i.e. 1:1) (Klatt, Molina et al. 1999). From this reaction, protein-sulfenic and glutathione sulfenic acids are generated with endogenously produced ROS or RNS. These species are usually rapidly transformed into prot-SSG (GSSR) or GSSG as more stable derivatives (Chen, Lu et al. 2014). De-glutathionylation, the reverse reaction, is mediated by glutaredoxin (GRX) which operated via a nucleophilic ping-pong mechanism and is highly specific for proteins that have been S-glutathionylated (Beer, Taylor et al. 2004). Additionally, the S-glutathionylation can be catalysed by glutathione-S-transferase P (GSTP) (Manevich, Feinstein et al. 2004).

GSH is involved in the generation of reactive oxygen species (ROS) when reacting with diallyl sulfides via reduction. The product of this reaction, perthiol, can react with oxidants, such as oxyhaemoglobin, to form superoxide radical anion and hydrogen peroxide (figure 1.13) (Munday, Munday et al. 2003; Chatterji, Keerthi et al. 2005). This reaction can also produce a perthiyl radical which may dimerize to generate a polysulfide (pathway 1). The perthiyl radical can also react with GSH to generate a radical anion RS_XSG^-, this can reduce oxygen to produce superoxide whilst producing a disulfide. The regenerated polysulfides are able to undergo a pseudo-catalytic cycle (pathway 2), where the polysulfide, perthiol, and perthiyl radical combine to produce ROS from oxygen (Munchberg, Anwar et al. 2007).

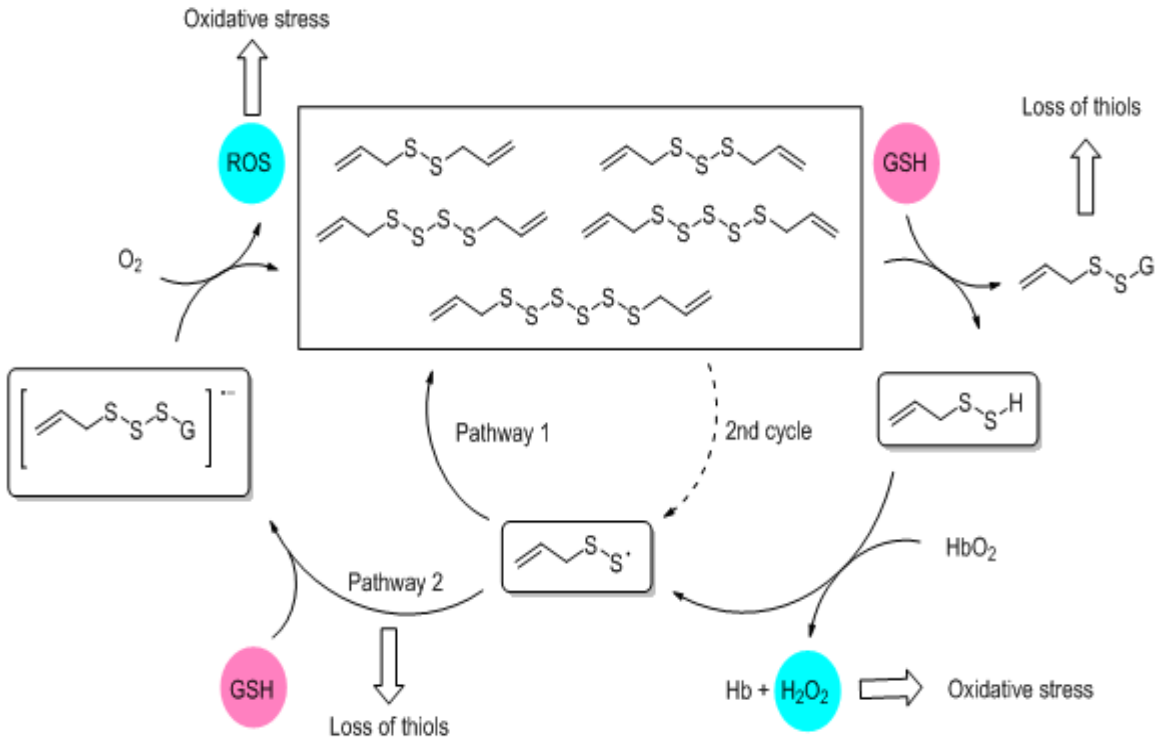


Figure 1.13. The polysulfide reactions with GSH and oxygen represented with diallyl trisulfide. Polysulfides are reduced by GSH via a thiol-trisulfide exchange resulting in disulphide and perthiol. Perthiol reacts with oxygen that is bound to haemoglobin to generate a perthiyl radical and hydrogen peroxide. Hydrogen peroxide can react further and cause oxidative stress. The perthiyl radical can dimerize (pathway 1) to regenerate a polysulfide or it can react with the high abundance of GSH in the cell (pathway 2) to generate a polysulfide radical anion that can reduce oxygen to produce a reactive oxygen species and a polysulfide regenerate. The regenerated polysulfides can enter a pseudo-catalytic cycle (second cycle), which is very damaging to cells (Munchberg, Anwar et al. 2007).

Both the generation of ROS and consumption of thiols cause extensive damage to living cells due to oxidative stress. Hydrogen peroxide and superoxide can damage membranes, proteins and peptides. ROS can also be converted to hydroxyl radicals, in particular hydrogen peroxide, when in the presence of iron or copper. Hydroxyl radicals are extremely aggressive that can attack membranes, proteins, and DNA. The toxicity of perthiol-generating tri- and tetrasulfides, which are found in garlic, could be due to the ROS generating catalytic cycle that concurrently lowers thiol content. This may explain cancer cell selectivity, since the level of ROS in some cancer cell types is close to the threshold for cell death in comparison to normal cells(Munchberg, Anwar et al. 2007).

Many researchers consider that the main explanation for polysulfide toxicity is due to this catalytic cycle (Munday, Munday et al. 2003; Chatterji, Keerthi et al. 2005). The generation of ROS by this route could also explain the increase in oxidative stress in cancer cells when they are killed by diallyl trisulfide (Xiao, Herman-Antosiewicz et al. 2005). Munday and colleagues studied diallyl sulfide redox behaviour with GSH and oxidants, including methaemoglobin and oxyhaemaglobin. They used dioxygen consumption as the measurement and catalase and superoxide dismutase as the interceptors. When GSH and oxy-/methaemoglobin was in the presence of diallyl trisulfide and diallyl tetrasulfide, dioxygen was converted to hydrogen peroxide. In the absence of haemoglobin, dioxygen is not sufficient to oxidise allylperthiol, which leads to a lack of superoxide production (Munday, Munday et al. 2003).

1.5.1 GSH cell survival and cell death

The intracellular levels of GSH can determine the ability of cells to undergo apoptosis via the ROS-mediated intrinsic pathway. This signifies the GSH-related processes with regards to: (i) indication of S-glutathionylation in apoptotic initiation and protein modulation; and (ii) decreased GSH/GSSG ratio leading to changes in cellular GSH redox homeostasis due to either oxidation or export of GSH in association with the initiation or execution of the apoptotic cascade (Ghibelli, Coppola et al. 1995). Many pro-apoptotic stimuli can induce an early GSH extrusion from the cells which would lead to mitochondrial damage and release of cytochrome c into the cytosol. The GSH extrusion is a result of a process by carriers to efficiently achieve the apoptotic program (Ghibelli, Fanelli et al. 1998).

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Protein S-glutathionylation is a post-translational modification which provides protection for cysteine residues from irreversible oxidation and facilitates redox signal transduction by changing the structure/function of the target protein. This is detected under a large increase in radical species or under physiological flux of ROS. For most proteins, this process of S-glutathionylation occurs only under non-physiological GSH/GSSG ratio (i.e. 1:1) (Klatt, Molina et al. 1999). From the reaction, protein-sulfenic and glutathione sulfenic acids are generated with endogenously produced ROS or RNS. These species are usually rapidly transformed into prot-SSG (GSSR) or GSSG as more stable derivatives (Chen, Lu et al. 2014). De-glutathionylation, the reverse reaction, is mediated by GRX which operated via a nucleophilic ping-pong mechanism and is highly specific for proteins that have been S-glutathionylated (Beer, Taylor et al. 2004). Additionally, the S-glutathionylation can be catalysed by glutathione-S-transferase P (GSTP) (Manevich, Feinstein et al. 2004).

The GSH/GSSG ratio is the main redox system protecting mitochondrial proteins and DNA from ROS activity and is involved in maintaining redox homeostasis of the mitochondrial matrix. Selective decreases in mGSH levels result in decreased activity of the respiratory chain complexes, a decrease in transmembrane potential ($\Delta\Psi$), increase in ROS production and release of apoptogenic factors from the mitochondria (Armstrong, Steinauer et al. 2002; Ghosh, Pulinilkunnil et al. 2005). A decrease in mGSH levels can cause changes in the permeability of the mitochondrial matrix which is caused by redox modulation of adenine nucleotide translocase and subsequent release of apoptogenic factors from the mitochondria to the cytoplasm (Chernyak and Bernardi 1996; Costantini, Chernyak et al. 1996). A drop in the mGSH/GSSG ratio to 20:1 from 300:1 leads to anion channel opening in the inner mitochondrial membrane and mitochondrial pore. If the mGSH/GSSG ratio lies between 150:1 and 100:1, the $\Delta\Psi$ value is unstable. When the mGSH/GSSG ratio is less than 50:1, when there is prominent oxidation, the mitochondrial membrane is irreversibly depolarized, along with channel opening and the breakdown of the mitochondria (Aon, Cortassa et al. 2007). The elevated transport of glutathione into the mitochondria suppresses menadione-induced increase of mGSSG levels. This prevents the decrease in ATP levels, increase in the $\Delta\Psi$ value, release of cytochrome *c* from the mitochondria into the cytoplasm and the activation of caspase-3 and caspase-9, consequently leading to apoptosis (Circu, Rodriguez et al. 2008).

It has been demonstrated that diallyl trisulfides (DAS3) can induce apoptosis via the generation of ROS which causes oxidative stress (Xiao, Herman-Antosiewicz et al. 2005; Antosiewicz, Herman-Antosiewicz et al. 2006; Das, Banik et al. 2007; Kim, Xiao et al. 2007). ROS are mostly generated during DAS3 treatment by the mitochondria via the mitochondrial electron transport chain, but can also be generated by other organellar sources (peroxisomal cytochrome P-450) and endogenous enzyme systems (cytoplasmic xanthine oxidase and plasma membrane NADPH oxidase) (Lee, Park et al. 2011).

During mitochondrial respiration, oxygen acts as the terminal electron acceptor by accepting four electrons and is reduced to yield water. However, it is possible to yield superoxide from a one-electron reduction of oxygen. It is proposed that this occurs in the electron transport chain at either complex I (NADH-dehydrogenase) or complex III (ubiquinol:cytochrome c oxidoreductase) (Osellame, Blacker et al. 2012). This superoxide dismutes to hydrogen peroxide and oxygen, catalysed by manganese superoxide dismutase (MnSOD). Glutathione peroxidase detoxifies hydrogen peroxide to water and oxygen in the mitochondria, or, if it diffuses into the cytosol, by catalase in peroxisomes. It is suggested that DAS3-induced increases in intracellular ROS is due to the disruption of mitochondrial electron transport chain activity and/or disruption of downstream glutathione peroxidase/ glutathione reductase system. (Lee, Park et al. 2011). The authors, however, do not suggest a mechanism and, therefore, this would need further investigating. It could be due to disruption of the proton leak which is described by Cheng et al (2017). The increase in proton conductance suppresses ROS production to protect biological systems from oxidative stress. Proton conductance is mediated by uncoupling proteins located in the mitochondrial inner membrane. Disruption of this could alter the positive feedback loop and inhibit the suppression of ROS production.

DAS3 elevates intracellular ROS levels and redox-regulatory proteins, including GRX, which recognize the DAS3-induced oxidative stress(Lee, Park et al. 2011). This activates the ASK1-MEK-JNK-Bim signal transduction pathway which triggers apoptosis via the Bax-dependent mitochondrial apoptotic pathway (Das, Banik et al. 2007; Kim, Xiao et al. 2007). Bim was inhibited as a result of DAS3 application when a specific JNK inhibitor was applied. This suggests that DAS3 activated Bim(Lee, Park et al. 2011).

1.5.2 Glutaredoxin

GRX is an important enzyme involved in disulfide reduction and deglutathionylation. The GRX isoenzymes have low molecular weights of 10-16 kDa and are thermoresistant. They function as GSH-dependent oxidoreductases and have an important role in redox-dependent processes in cells. Some GRX isoenzymes can serve as transfer proteins for iron-sulfur clusters [FeS] using GSH as a ligand (Rouhier 2010). After the reduction of protein disulfides and glutathionylated thiols, the oxidized formed of GRX is formed and is reduced by GSH. However, some GRX isoenzymes are reduced by NADPH- or ferredoxin-dependent thioredoxin reductase, such as human GRX2 (Johansson, Lillig et al. 2004).

There are two catalytic mechanisms used by GRX isoenzymes: monothiol and dithiol, figure 1.14. Dithiol GRXs have high specificity and affinity for GSH. The GSSG is reduced by glutathione reductase and mediated by NADPH. The main activity of GRX is to reduce protein-GSH mixed disulfides (PSSG) i.e. glutathionylation to utilize the monothiol mechanism that only requires the N-terminal active site residue (Holmgren 1989; Holmgren, Johansson et al. 2005). This monothiol mechanism is used for deglutathionylation reactions, where only the catalytic residue (in the active site) contributes to the catalysis. The dithiol mechanism requires an extra cysteine residue, either one that is not the active site cysteine or the second cysteine of the GRX active site. Both GRX1 and GRX2 isoenzymes use the same catalytic mechanism even though they exhibit only 34% structural homology (Gallogly, Starke et al. 2009; Stroher and Millar 2012). GRX2 is approximately 10-fold less active than GRX1 and is more abundant in the mitochondrial intermembrane space, which is likely to compensate for the difference in catalytic activity (Cross and Templeton 2004). The oxidized GRX1 can only be reduced by GSH, whereas GRX2 can be reactivated by glutathione and thioredoxin reductase. This suggests that GRX2 displays properties of GRX and TRX (Johansson, Lillig et al. 2004), and also indicates the connection between metabolic pathways in the mitochondria by GRX and TRX. The reduction of GRX2 by thioredoxin reductase would allow GRX2 to function over a range of GSH/GSSH values and strong oxidative stress (Beer, Taylor et al. 2004).

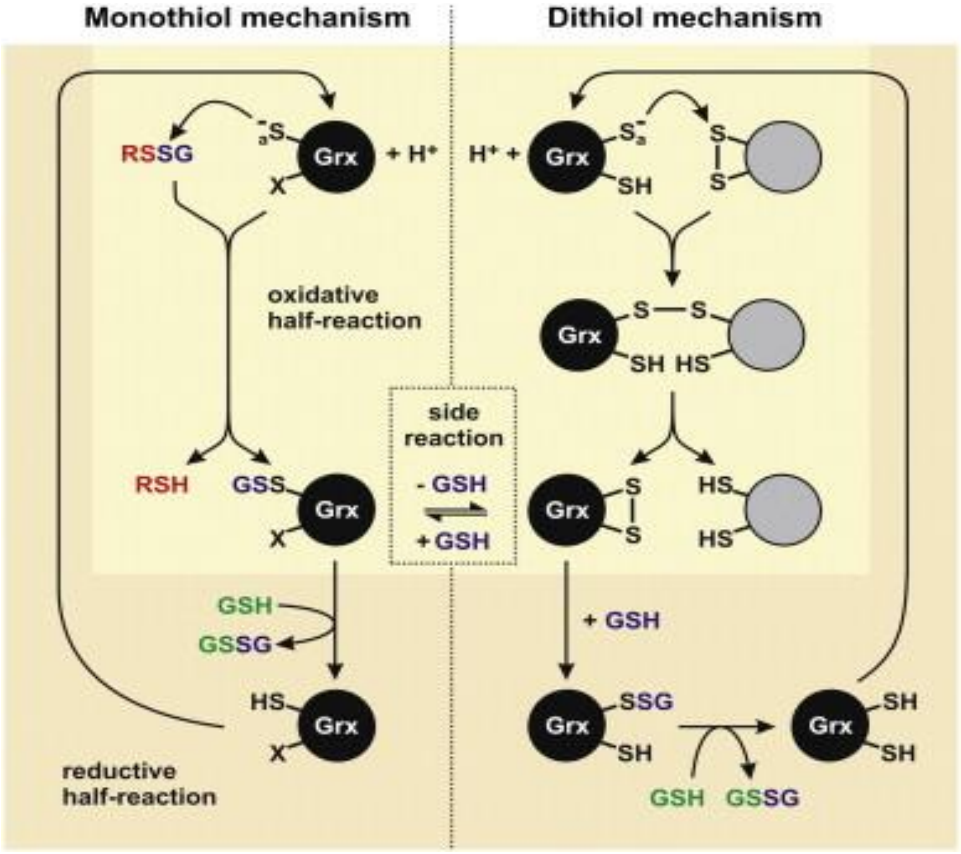


Figure 1.14: Model of GRX catalysis. The monothiol mechanism for glutathionylated substrates is shown on the left and the dithiol mechanism for protein disulfide substrates is shown on the right. A side reaction of the monothiol mechanism for the dithiol GRX is shown in the middle. The second cysteine residue in the monothiol mechanism can be replaced by a serine (X) (Deponte 2013).

GRX1 and GRX2 can catalyse the deglutathionylation and reverse reaction of S-glutathionylation. The GSH/GSSG redox potential is the most important for determining the cellular redox potential which depends on the functional state of the cell. This value is approximately -240 mV during cell proliferation, -200 mV during cell differentiation, and can reach -170 mV during apoptosis (Watson, Chen et al. 2003). GRX acts as the GSSG-dependent oxidase at -170 mV, whereas at -240 mv it acts as the GSH-dependent reductase (Aslund, Berndt et al. 1997).

Under conditions when the action of oxidizing factors decrease the GSH/GSSG value GRX catalyzes S-glutathionylation. Glutathionylation is an oxidative posttranslational modification which takes place on protein cysteines under basal conditions. GRX facilitates S-glutathionylation of proteins via the reaction of the GS· radical with the disulfide bond, producing the intermediate anion radical GRX-SSG·$^{-}$, which gives rise to PSSG (Starke, Chock et al. 2003). The reversion of S-glutathionylation is dependent on the

duration and degree of the initiating stress. When under oxidative stress, GRX catalyzes the deglutathionylation reaction (Ruoppolo, Lundstrom-Ljung et al. 1997; Starke, Chock et al. 2003). The glutaredoxin activity in protein deglutathionylation (reduction of PSSG) is found to be critical in regulation of redox signal transduction and sulfhydryl homeostasis (Dalle-Donne, Milzani et al. 2008; Mieyal, Gallogly et al. 2008). GRX is the main enzyme involved in intracellular deglutathionylation in mammalian cells. Changes in GRX levels have been demonstrated to affect protein glutathionylation status, subsequent downstream signalling events, and regulation of cellular homeostasis in health and disease (Chrestensen, Starke et al. 2000; Clavreul, Adachi et al. 2006; Pimentel, Adachi et al. 2006; Ho, Xiong et al. 2007; Dalle-Donne, Milzani et al. 2008; Mieyal, Gallogly et al. 2008). By regulating the processes of glutathionylation and deglutathionylation, GRX plays an important role in controlling signal transduction.

In *Drosophila* intracellular redox homeostasis is maintained by the thioredoxin reductase (TrxR) system (Kanzok et L, 2001). Genetic impairment of the TrxR biochemical activity does not alter morphological features of fly development, but does cause an unbalanced redox homeostasis, leading to reduced viability of the organism (Kanzok et L, 2001). *Drosophila* do not use glutathione reductase (GR) to maintain high levels of GSH and low levels of GSSG, instead TrxR catalyzes the transfer of reducing equivalents from NADPH to Trx[$NADPH + H^+ + TrxS_2 \rightarrow NADP+ + Trx(SH)_2$] (Amer et al, 1999; Mustacich and Powis et al, 2000; Becker et al, 2000). Because of their tracheal respiratory system, insects are particularly exposed to ROS and depend on high concentrations of intracellular GSH. The [2 GSH]/[GSSG] ratio can serve as an in vivo indicator for oxidative stress response in *Drosophila* as demonstrated by Sohal *et al.* (1990).

1.5.3 ROS production

Mitochondria are ubiquitous organelles in eukaryotic cells that are the primary site of energy production via the form of adenosine-5'-triphosphate (ATP) via the electron transfer chain (ETC) (Attardi et al 1988). The production of ATP provides 90% of the cellular chemical energy required in various biological functions The ETC uses a series of electron flow processes called oxidative phosphorylation (Facucho-Oliveira et al, 2007; Lu et al, 2009). The ETC consists of multi-subunit complexes (complexes I-V) which are encoded by either mitochondrial or nuclear DNA (Huang et al 2007). The ETC is localized in the cristae; the inner mitochondrial membrane and extends the surface (Chada and

Hollenbeck, 2004). The ETC complexes are nicotinamide-adenine dinucleotide (NADH) - ubiquinone oxidoreductase (complex I, EC 1.6.5.3), succinate-ubiquinone oxidoreductase (complex II, EC 1.3.5.1), ubiquinone-cytochrome *c* oxidoreductase (complex III, EC 1.10.2.2), cytochrome *c* oxidase (complex IV, EC 1.9.3.1) and ATP synthase (complex V, EC 3.6.3.14) (figure 1.15). Mitochondria also play a key role in modulating calcium signalling, which is a universal second messenger (Rizzuto et al, 2000; Babcock and Hille, 1998).

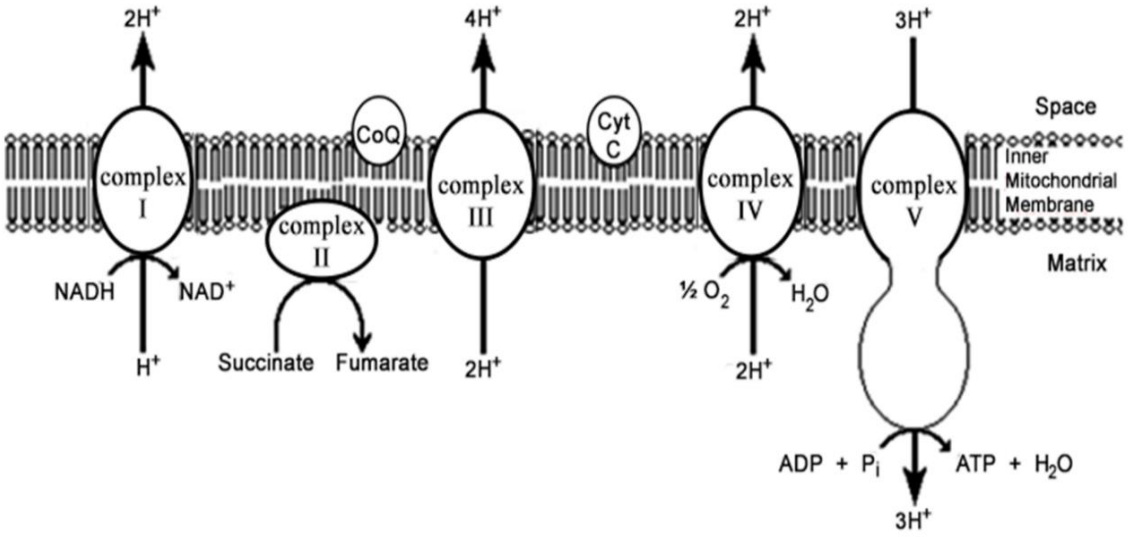


Figure 1.15: The electron transfer chain consisting of complexes I-V embedded in the inner mitochondrial membrane (Image by PrepGenie, 2018).

The complexes of the ETC are where mitochondrial proton pumps (complexes I, III, and IV) transport protons to generate a proton gradient (Kadenbach et al, 2010). Complex 1 catalyzes the oxidation of NADH which generates an oxidized form of NADH for the tricarboxylic acid cycle and fatty acid oxidation. It also reduced coenzyme Q_{10} to ubiquinol (Hirst, 2009). Four electrons are pumped into the intermembrane space from the matrix whilst two electrons pass through complex I. Complex II consists of 4 subunits and is a membrane bound lipoprotein which couples the oxidation of succinate to the reduction of coenzyme Q_{10}. It is also directly involved in the tricarboxylic acid cycle (Tomitsuka et al, 2009). Succinate dehydrogenase has a covalently attached flavin adenine dinucleotide cofactor (FAD) which oxidized succinate to fumarate along with the reduction of FAD to the hydroquinone form. Electrons from the oxidation of the reduced FAD are transferred to coenzyme Q_{10}. Coenzyme Q_{10} is responsible for transferring electrons from complexes I

and II to complex III. The cofactor cytochrome *c* transfers electrons from complex III to complex IV by binding to the membrane proteins (Solmaz and Hunte 2008). Both coenzyme Q_{10} and cytochrome *c* modulate energy and the production of free radicals (Mattson et al, 2008; Rodriguez-Hernandez et al, 2008). Electrons are continuously transported to complex III which catalyzes the oxidation of ubiquinol and reduction of two cytochrome *c* molecules. Complex III consists of two centres: $Q_i$centre-facing to the matrix, and $Q_o$centre-oriented to intermembrane space. The reaction mechanism of complex III occurs in two steps and is called the Q cycle where four protons are released into the intermembrane space (Chen et al, 2003; Trumpower, 1990). Complex IV mediates pumping of four protons across the membrane to allow the reduction of O_2 to H_2O and retains all the partially reduced intermediates until full reduction is attained (Turrens, 2003). Complex V consists of two domains: F_1 and F_0. The F_1 domain has three nucleotide binding sites and is localized above the inner side of the membrane. F_O is connected to the F_1 domain. F_O is a proton pore and consists of three subunits, spanning from the inner to the outer side of the membrane (Noji and Yoshida, 2001; Zanotti et al, 2009). The formation of these domains permits the conversion of electrochemical potential energy to chemical energy. The occurs by the rotation of a portion of F_O as protons pass through the membrane, this forces the F_1 to act as a motor to synthesize ATP (Kadenbach, 2003).

The reduction of O_2 to H_2O in the oxidative phosphorylation process is accomplished by reactive intermediate formation as superoxide. This can also occur during ATP formation or by an imbalance in cellular oxidant/antioxidant systems (Storz et al, 2005; Murphy, 2009; Bell et al, 2007). Superoxide is released by complex I into the matrix, and complex III can release superoxide to both sides of the inner membrane of the mitochondria, figure 1.16 (Jezek and Hlavata, 2005; Muller et al, 2004). Superoxide that is released into the matrix side is converted by superoxide dismutase 2 (SOD2), by other matrix enzymes, or by nonenzymic processes to hydrogen peroxide, which can diffuse out of the mitochondria. Superoxide can reduce cytochrome c (in the intermembrane space). Some of the superoxide on the cytosolic side of the membrane is converted to hydrogen peroxide by SOD1, and extracellular superoxide is converted to H_2O_2 by SOD3 which diffuses relatively free or is transported across biological membranes mediated by receptors (Winterbourn and Hampton, 2008). The conversion of superoxide to hydrogen peroxide can occur by spontaneous dismutation, by any intermembrane Cu/ZnSOD or exogenous contaminating SOD activities (St Pierre et al, 2002). This activity of SODs prevents superoxide accumulation which can inactivate and damage proteins that contain iron-sulfur

clusters (Fridovich, 1997). However, there are some proteins that are particularly sensitive to inactivation by superoxide. This can lead to signalling pathways that promote adaptation to high superoxide levels, or this can initiate cell death (Chen et al, 2009). Another ROS is Hydroxyl radicals which are generated from H_2O_2 and are very reactive indiscriminately with proteins, lipids, and DNA. If the ROS are not adequately reduced, they can cause damage by reacting with macromolecules of biological importance, including lipids, proteins, nucleic acids, and carbohydrates, which would eventually lead to cell death (Dizdaroglu and Jaruga, 2012).

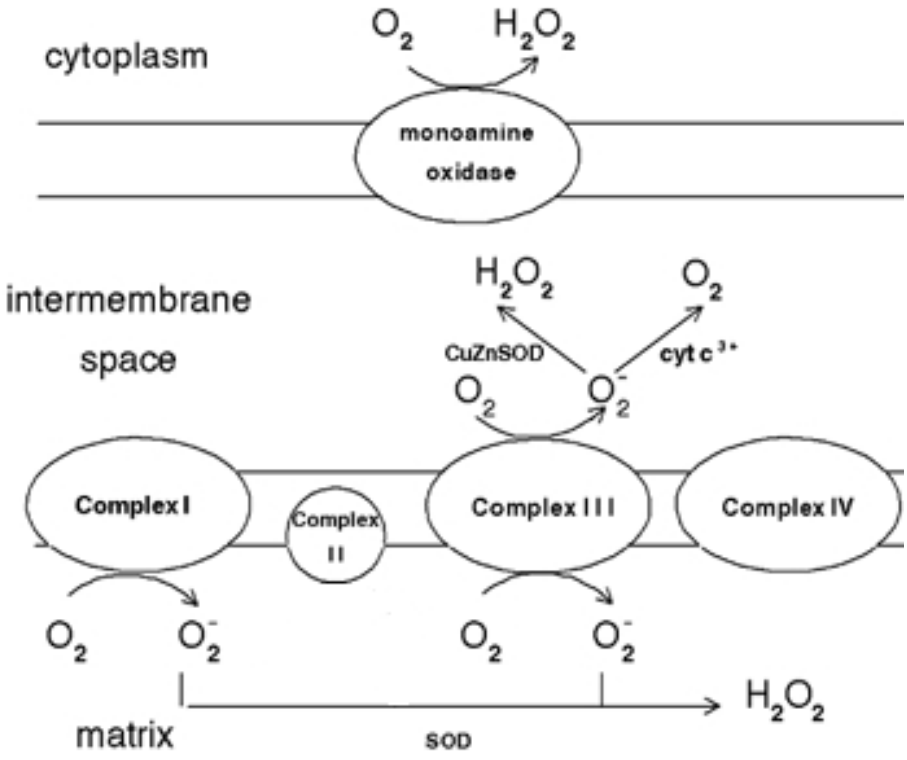


Figure 1.16: Sites of superoxide formation in the ERC. Complex I and complex III can leak electrons to oxygen to produce superoxide (Turrens et al, 2003).

1.5.4 Peroxiredoxins, Peroxidase and hydrogen peroxide

Hydrogen peroxide is a by-product of aerobic metabolism and is the most abundant ROS (Zamocky, Furtmuller et al. 2008). It is produced by a two-electron reduction of dioxygen by oxidoreductases, or by superoxide dismutation. H_2O_2 is involved in signalling pathways in eukaryotic cells, or can act as a defence in the innate immune system as an antimicrobial (Peus, Meves et al. 1999; Zamocky, Furtmuller et al. 2008). H_2O_2 can initiate cellular effects such as changes in cell shape, the formation of actomyosin structures, and immune cell recruitment (Zamocky, Furtmuller et al. 2008). Levels of H_2O_2 in cells are controlled

by continuous formation and degradation reactions. The degradation of H_2O_2 is mediated by thiol-containing peroxiredoxins or metal-containing peroxidises by the reduction to water (Brioukhanov and Netrusov 2004).

H_2O_2 can react with low-molecular weight thiols, such as cysteine and GSH. The reaction with thiols with H_2O_2 involves a nucleophilic attack form the thiolate on the H_2O_2. The electrostatic environment around the –SH group of cysteine residues may increase their acidity and, therefore, increase reactivity towards H_2O_2, resulting in a higher fraction of the thiolate form. Lower stability of the thiolate in cysteine residues increases nucleophilicity towards H_2O_2 of the thiolate (Ferrer-Sueta, Manta et al. 2011).

An increase in H_2O_2 concentrations leads to specific oxidation of signalling proteins such as phosphatases, kinases, and transcription factors that contain cysteine residues, which results in a cascade of molecular events. The reactivity of H_2O_2 with these signalling proteins is much lower than the reactivity of peroxiredoxins (Prxs), selenocysteine residues in GSH peroxidases (GPx), or the heme in catalase. Additionally, the abundance of these antioxidant enzymes such as Prxs, GPx, and catalase is much higher than signalling proteins such as phosphatases, kinases, and transcription factors. These signalling proteins cannot compete with protein antioxidant systems that remove H_2O_2 (Rhee, Woo et al. 2012; Brigelius-Flohe and Maiorino 2013).

Prxs can mediate H_2O_2 signalling via three mechanisms: H_2O_2 directly reacts with target signalling proteins and PRXs regulate this process by controlling H_2O_2 levels in the cell; H_2O_2 reacts with Prx which is then oxidized and relays the oxidation to target signalling proteins (such as phosphatases and transcription factors); H_2O_2 reacts with Prx, which becomes oxidized, then Prx is reduced back by thioredoxin (Trx) and the oxidized Trx relays the oxidation to target signalling proteins. Prxs can become hyperoxidized giving rise to the so-called ‘floodgate hypothesis’(Wood, Poole et al. 2003; Rhee and Woo 2011).

The ‘floodgate hypothesis’ occurs when the active site cysteine of Prx is overoxidized to cysteine-sulfenic acid causing inactivation of Prx. Higher oxidation products are often associated with loss of biological activity. This then allows H_2O_2 to then react with target signalling proteins. Prx reacts with H_2O_2 very rapidly and can react a second time to produce sulfenic acid, which can occur during oxidative stress. It has been proposed that this second reaction occurs during physiological signalling and acts as a floodgate to regulate redox signalling (Wood, Poole et al. 2003). The thiolate of the Prx active site can

be restored, catalyzed by sulfiredoxin (Biteau, Labarre et al. 2003; Chang, Jeong et al. 2004) and allow Prx to once again inhibit H_2O_2-dependent signalling. It has been shown that exogenous H_2O_2 can cause overoxidation of Prx-2 leading to cell cycle arrest (Phalen, Weirather et al. 2006).

There are three problems with the floodgate hypothesis. The first is that, if not supplemented, cells can have severe depletion of selenium and low levels of cytosolic GPx(Maiorino, Chu et al. 1991). *In vivo*, where depletion of selenium is rare, overoxidation of Prxs would still lead to significant GPx activity that would prevent the flood. The second problem is the requirement of the second reaction of Prx to eliminate activity before H_2O_2-dependent signalling occurs. Inactivation of Prx has a very slow rate of 0.072 % during each round of catalysis; therefore, it is questionable whether the inactivation is sufficient to allow the floodgate hypothesis to account for H_2O_2-dependent signalling (Choi, Lee et al. 2005). The third problem is that the cytosolic micromolar concentrations of GS^- would out-compete the nanomolar concentrations of target signalling proteins to react with H_2O_2, even in the absence of both Prx and GPx activities. This floodgate hypothesis also does not take into account the mechanism of H_2O_2 signalling(Forman, Maiorino et al. 2010).

1.5.5 Measuring glutathione and hydrogen peroxide

There is a significant body of research relating to glutathione homeostasis; however, many details remain unclear. A conservative measurement of glutathione can be performed using techniques such as fluorescence-based assays and high throughput chromatography (HPLC) (Vandeputte, Guizon et al. 1994; Rahman, Kode et al. 2006). These techniques can measure the total reduced (GSH) and oxidized (GSSG) glutathione concentrations. They can also achieve high degrees of redox couple sensitivity, specificity and reproducibility. However, they are unable to measure rapid, dynamic processes. Whole-cell measurements using these assays can lead to inaccuracy of subcellular compartment-specific information regarding the glutathione redox state in the cytosol. This could be mixed with the separate compartmental glutathione pools, leading to invalid results (Morgan, Sobotta et al. 2011).

Green fluorescent proteins (GFPs) are responsible for the bioluminence in jellyfish(*Cnidaria*) and emit green light via energy transfer from luciferases or

photoproteins(Morin and Hastings 1971). The optical properties of GFPs and GFP-like proteins are genetically encoded and the chromophore is assembled post-translationally inside the protein shell (Lukyanov, Chudakov et al. 2005; Giepmans, Adams et al. 2006; Shaner, Patterson et al. 2007). Figure 1.17 shows the wildtype GFP structure consists of an 11-stranded β-barrel with a conserved chromogenic Ser-Tyr-Gly amino acid sequence formed by an autocatalytic cyclization that does not require a cofactor (Pakhomov and Martynov 2008; Ong, Alvarez et al. 2011). GFP is able to fluoresce without the presence of other proteins, cofactors, or substrates. Additionally, the function of GFP is not altered when fused to other proteins (Ong, Alvarez et al. 2011). The main functions of GFP include efficient fluorescence, protease resistance, and stability within a range of pH and solvent conditions. GFP can also be targeted to specific organelles, the monitoring of localised areas(Hanson, Aggeler et al. 2004).

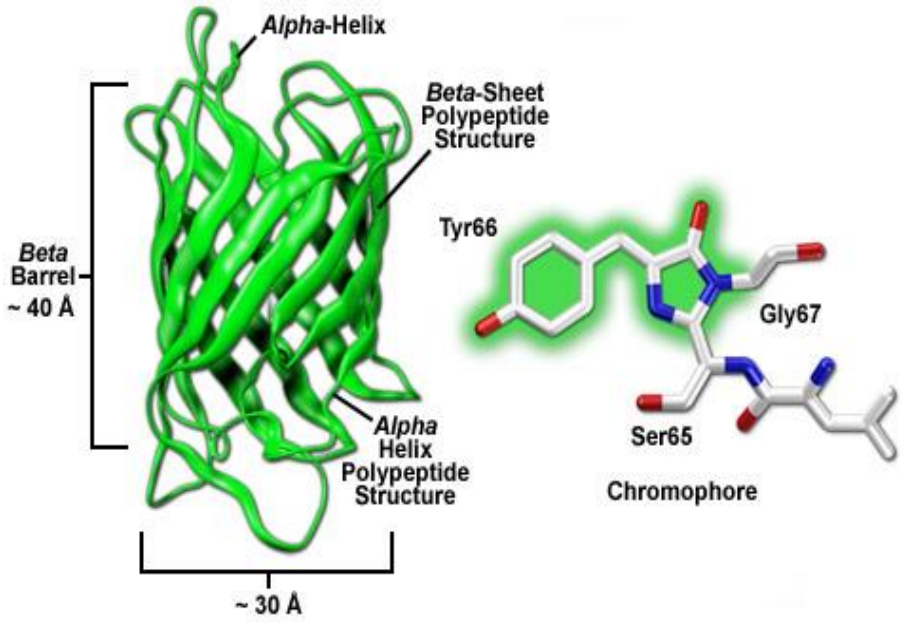


Figure 1.17: Structure of GFP. The cyclic tripeptide chromophore is buried in the centre of a cylinder produced by an interwoven eleven-stranded "beta-barrel" structure (Piston, Campbell et al. 2018).

GFP is mostly used as a fluorescent indicator of protein localization and gene expression. There are also variants of GFP to detect intracellular ion concentrations, such as H^+, Ca^{2+} and halides (Kneen, Farinas et al. 1998; Miesenbock, De Angelis et al. 1998; Nagai, Sawano et al. 2001; Hanson, McAnaney et al. 2002). GFP variants have also been engineered for the measurement of redox status, reactive oxygen species (ROS), and pH (Choi, Swanson et al. 2012). GFP can be used as an indicator for redox status is

constructed by introducing redox-reactive groups into proteins, localized to an area where the fluorescent properties of GFP would be modified by the change in oxidation state of the introduced group(s) (figure 1.18) (Hanson, Aggeler et al. 2004).

A)

GSSG

B)

H_2O_2

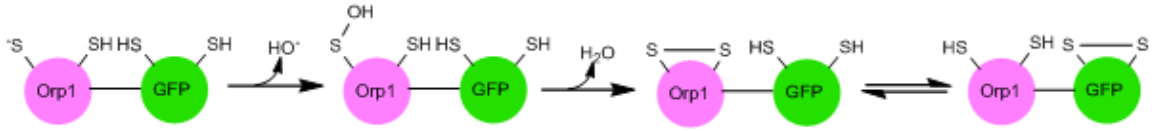


Figure 1.18: Molecular mechanism of roGFP probes. A) Sensing of GSSG with Grx1-roGFP2 flies. B) Sensing of H_2O_2 with Orp1-GFP2. This figure was reproduced from the original(Albrecht, Barata et al. 2011).

Redox-sensitive GFP (roGFP) has been developed to image thiol redox potentials in independent compartments (Hanson, Aggeler et al. 2004; Jiang, Schwarzer et al. 2006; Meyer 2008; Schwarzlander, Fricker et al. 2008). Several different roGFPs have been produced, each with different midpoint potentials. These roGFPs are ratiometric by excitation and are unaffected by errors such as probe concentration, tissue thickness, and photobleaching (Hanson, Aggeler et al. 2004). RoGFPs are generated by cysteine substitutions of the wildtype GFP. The roGFP1 is generated by three point mutations: C48S, S147C, and Q204C. The roGFP2 is generated with an additional S65T mutation (Dooley, Dore et al. 2004; Hanson, Aggeler et al. 2004). In an oxidizing environment roGFPs undergo conformational changes by forming disulfide bridges between C147 and C204. These two introduced cysteine residues are located close to the chromophore, thus, the conformational change leads to a shift in the excitation peak of GFP from 400 to

490nm. Consequently, roGFP1 has a decreasing emission peak with excitation at 400nm and an increasing emission signal with the 490nm excitation. Therefore, roGFPs can be used to study spatio-temporal intracellular oxidation status via ratiometric analysis ($490_{emission}/400_{excitation}$). These roGFPs can be modified to target different subcellular organelles, such as mt-roGFP1 and mt-roGFP2 are used for signal localization to the mitochondria by fusing with a mitochondrial localization signal peptide from the tobacco β-ATPase to the C-termini (Jiang, Schwarzer et al. 2006; Meyer 2008; Schwarzlander, Fricker et al. 2008; Rosenwasser, Rot et al. 2010).

T. Dick and colleagues generated transgenic *D. melanogaster* using four different redox probes in order to measure E_{GSH} in the cytosol (cyto-Grx1-roGFP2) and in the mitochondria (mito-Grx1-roGFP2), and to measure H_2O_2 in the cytosol (cyto-roGFP2-Orp1) and mitochondria (mito-roGFP2-Orp1). Constructs for the in vivo inducible expression of redox biosensors were generated by cloning cyto- Grx1-roGFP2, mito-roGFP2-Grx1, cyto-roGFP2-Orp and mito-roGFP2-Orp into pUAST. For stable ubiquitous expression of these biosensors, the open reading frames were cloned into the pCasPeR4 vector containing an alphaTub84B promoter and SV40 polyA ('pCasPeR4:Tub'). These probes were expressed ubiquitously through the tubulin promotor, using the GAL4-UAS system (Figure 1.19) (Albrecht, Barata et al. 2011).

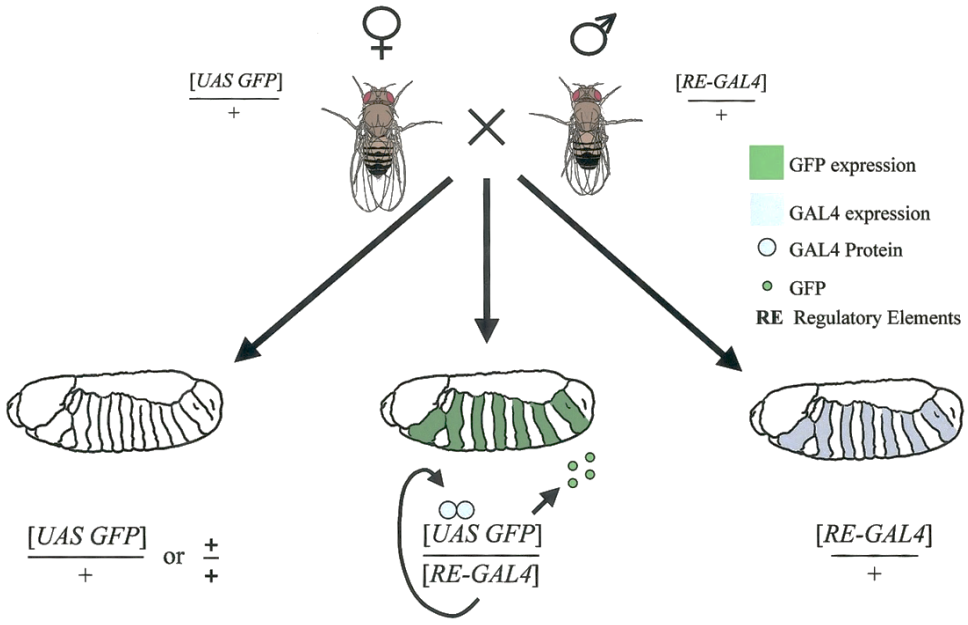


Figure 1.19: The UAS/GAL4 system in Drosophila. Mating of females carrying the UAS responder (UAS-GFP) with males carrying the gal4 driver produce progeny with both elements of the system. The presence of GAL4 drives the expression of the UAS responder gene (Duffy, 2002)

D. melanogaster larvae have a transparent cuticle which makes it easier for direct live imaging. However, in the adults, most tissues are optically inaccessible for viewing GFP tags. Therefore, the adult flies need to be dissected for microscopic examination of their organs. There is concern that the dissection and tissue preparation could damage the probe redox state which would lead to artefacts. This problem can be overcome by adding the alkylating agent, N-ethyl maleimide (NEM), during dissection to trap the redox state of the sensor (Barata and Dick 2013).

The distribution of the probe redox state in either the intact larvae or dissected adult tissue can be determined by confocal microscopy. The specimen is excited at 405 nm (oxidised) and then 488 nm (reduced) wavelengths (figure 1.20). The emission fluorescence intensity for both wavelengths is recorded between 500 and 530 nm. The ratio of the fluorescence intensities represents the redox state of the probe; roGFP2-Orp1 or roGFP2-Grx1 (Barata and Dick 2013). Thedetails of the relationship between fluorescence ratios and redox state can be determined using the Nernst equation (Meyer and Dick 2010).

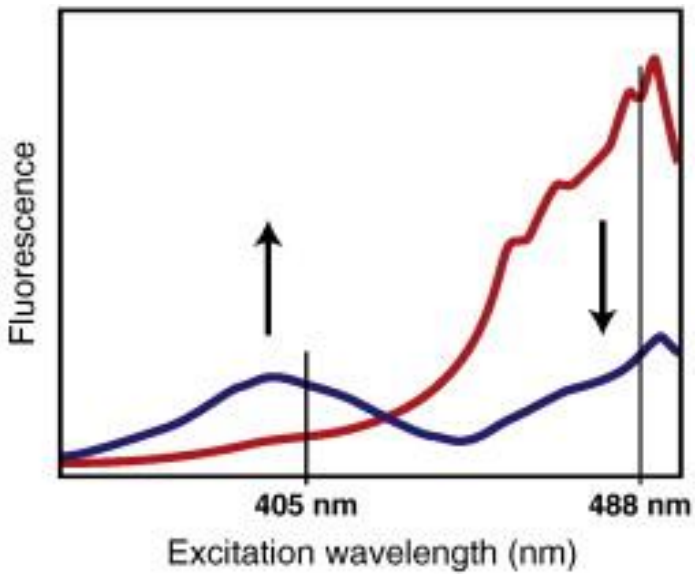


Figure 1.20. Fluorescence excitation spectra of roGFP2 in the fully reduced (red line) or fully oxidized (blue line) state. Excitation peaks at 405nm and 488nm. Emission is at 510 nm (Morgan, Sobotta et al. 2011)

1.5.5.1 Measuring E_{GSH}

It is known that endogenous cellular glutaredoxins play a role in regulating the equilibrium of roGFPs with the glutathione (GSH) pool (Meyer, Brach et al. 2007). It has also been shown that roGFP2 can be directly fused with glutaredoxin (Grx) (Albrecht, Barata et al. 2011). This enhances the equilibrium of roGFP and GSH redox couples, and allows measurement of the GSH redox potential (E_{GSH}) (Gutscher, Pauleau et al. 2008). Fusion of Grx increases the specificity of roGFP2 for the GSH/GSSG redox couple, overcoming kinetic limitations caused by the dependency of the biosensor on endogenous Grx. This is due to local concentration of Grx that is available to interact with the attached roGFP2, which increases by at least 1000-fold compared to the endogenous redox enzyme concentration. Additionally, this facilitates measurements of E_{GSH} with high temporal resolution (Gutscher, Pauleau et al. 2008; Meyer and Dick 2010).

The midpoint potentials for roGFP1 and roGFP2 are -288 to -272 mV, and -287 mV, respectively. This can be determined by titration with DTT, lipoic acid, or bis(2-mercaptoethyl) sulfone (BMES)(Dooley, Dore et al. 2004). There are also differences with roGFP1 and roGFP2 in their maximum dynamic range for fluorescent changes between fully reduced and oxidized probes. If the roGFPs were to be used as a single wavelength probe, they would show fluorescence with an approximate 3-fold change. However, roGFPs have a significantly increased dynamic range due to being ratiometric(Meyer, Brach et al. 2007).

The redox behaviour of roGFPs can be predicted by the Nernst equation, based on the concept that roGFPs equilibrates with the 2GSH/GSSG redox couple. The Nernst equilibrium $E_{GSH}=E_{roGFP2}$ can describe the Grx-mediated electron exchange between GSH and roGFP2 (Meyer and Dick 2010). The following equation shows the Nernst equation, specifically:

Equation 1

$$\begin{aligned} E_{GSH} &= E^{o\prime}_{GSH} - \frac{RT}{2F}\ln\left(\frac{[GSH]^2}{[GSSG]}\right) \\ &= E^{o\prime}_{roGFP2} - \frac{RT}{2F}\ln\left(\frac{[roGFP2_{red}]}{[roGFP2_{ox}]}\right) \\ &= E_{roGFP2} \end{aligned}$$

In this equation, R is the gas constant (8.315 $JK^{-1}mol^{-1}$), T is the absolute temperature (298.15 K), and F is the Faraday constant (96485 C mol^{-1}). $E°'_{GSH}$ is -240 mV and $E°'_{roGFP2}$ is -280 mV (Schafer and Buettner 2001; Dooley, Dore et al. 2004).

Practically, it is beneficial to define the degree of oxidation (OxD) of the two redox pairs, which is shown in the equation below. The GSH_{total} refers to the equivalents of GSH; GSH_{total} = [GSH] + 2[GSSG]. The OxD_{GSH} is the portion of the GSH_{total} that occurs as [GSSG]. For example, if OxD_{GSH} = 0.5, this would mean that 50% of all GSH equivalents exists as GSSG. This would mean that GSSG and GSH are in a 1:2 molar ratio (Meyer and Dick 2010).

Equation 2

$$OxD_{GSH} = \frac{2[GSSG]}{[GSH] + 2[GSSG]} = \frac{2[GSSG]}{GSH_{total}}$$

$$OxD_{roGFP2} = \frac{[roGFP2_{ox}]}{[roGFP2_{ox}] + [roGFP2_{red}]}$$

Equation 3

$$E_{GSH} = E^{\circ\prime}_{GSH} - \frac{RT}{2F}\ln\left(\frac{2GSH_{total}(1 - OxD_{GSH})^2}{OxD_{GSH}}\right)$$

$$= E^{\circ\prime}_{roGFP2} - \frac{RT}{2F}\ln\left(\frac{1 - OxD_{roGFP2}}{OxD_{roGFP2}}\right) = E_{roGFP2}$$

It is useful to express the Nernst equilibrium relationship as a function of OxD_{GSH}, GSH_{total}, and OxD_{roGFP2}, since GSH_{total} is an important biological variable. This allows to make several predictions about the behaviour of the roGFP2-based biosensor system (Meyer and Dick 2010).

1.5.5.2 Measuring H_2O_2

It is well known that hydrogen peroxide (H_2O_2) acts as a signalling molecule in various physiological activities. It is generated in a regulated manner and causes selective posttranslational modification of cysteine residues on target proteins. As discussed in section 1.5.3, it can also be generated in an unregulated manner and cause oxidative stress. The functional properties of these affected proteins can be altered by thiol oxidation, particularly the formation of disulfides. H_2O_2 oxidizes its target proteins indirectly by a proposed mechanism where a peroxidase acts as the primary oxidant acceptor and passes the oxidation to the target protein. Oxidative redox relay could explain the selectivity in redox signalling of H_2O_2. The most well-known peroxidase-based redox relay is Orp1-Yap1(Toledano, Delaunay et al. 2004; Gutscher, Pauleau et al. 2008).

The development of Grx1-roGFP2 led to the possibility of using roGFP2 for peroxidase (Orp1) to explore H_2O_2 redox activities. Orp1 is a seleno-independent member of the glutathione peroxidase family and is thioredoxin-dependent, therefore is functionally classified as a peroxiredoxin (Maiorino, Ursini et al. 2007). When roGFP2 is fused to peroxidase, it acts as a reducing substrate, and H_2O_2 is a second substrate. This H_2O_2 probe is ratiometric pH-stable and is able to report submicromolar H_2O_2(Gutscher, Pauleau et al. 2008). Cysteines react with H_2O_2 very slowly compared to abundant peroxiredoxins, even those that are redox-reactive (Winterbourn 2008). It is suggested that cysteines react with H_2O_2 via oxidation mediated by peroxidases (Delaunay, Pflieger et al. 2002). When fused with roGFP2, Orp1 catalyses the stoichiometric conversion of H_2O_2 to disulfides in roGFP2. When Orp1 encounters H_2O_2 it forms a sulfenic acid (Cys-SOH) at its peroxadic cysteine (Cys^{36}). Cys^{36}-SOH condenses with the resolving cysteine of Orp1 (Cys^{82}) and generates an intramolecular disulfide bond (Ma, Takanishi et al. 2007; Gutscher, Pauleau et al. 2008).

Albrecht *et al* (2011) used the roGFP transgenic flies to study the *in vivo* status of GSH/GSSG redox couple and H_2O_2, respectively, in the cytosol and mitochondria. From their studies, the authors established the followings: There are natural redox differences between different cells and tissues in the larvae, and redox changes occur during physiological transitions. The *in vivo* redox state of the probes can be chemically trapped and conserved which allows the animal to be dissected to image otherwise optically inaccessible tissues. The midgut enterocytes are the main sites of cytosolic H_2O_2 accumulation during ageing with an increased in oxidant formation. GSSG and H_2O_2 can

change independently of each other, therefore, should be measured separately. Lastly, redox imaging allows analysis of the effect of individual gene products and pharmacological agents (Albrecht, Barata et al. 2011).

1.5.6 Cysteine sulfhydration

Polysulfides have the ability to bind to protein cysteine via sulfydration, also known as persulfidation, and cause change in protein function (Filipovic, 2015; Paul and Snyder, 2015). There have been numerous proteins found to be modified by this process, many of which can be activated or inactivated by organosulfur compounds (Paul and Snyder, 2015).

The reduction of diallyl polysulfides by glutathione can release RSS such as inorganic H_2S_X species, including hydrogen sulfide (H_2S) (Cai and Hu, 2017; Block, 1992; Kharma et al, 1019). The interest in H_2S has increased over recent years as it was discovered to be involved in signalling. H_2S was related to be a strong toxin until the first reports of H_2S as a gaseous, signalling molecule (Abe and Kimura, 1996).

Snyder et al (2009) showed that H_2S modifies cysteine residues via sulfhydration of target proteins which induces conformational changes and alters activity of the protein (Mustafa et al, 2009). It was also shown that sulfhydration of cystine generates bounds sulfane sulfur, which is proposed to act as the intracellular storage molecule of H_2S (Ishigami et al, 2009). It is proposed that protein sulfhydration to hydropersulfides, causing reversible oxidation of thiol -SH groups, is the oxidation reaction that is a fundamental process in thiol-based redox regulation (Mustafa et al, 2009; Ono et al, 2014; Kabil et al, 2014).

H_2S is an abundant gaseous signalling molecule and has been investigated for many decades (Giuffre and Vincente, 2018). H_2S is produced endogenously by the enzymes cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), cysteine aminotransferase (CAT), and mercaptopyruvate sulfurtransferase (3-MST). CBS utilizes homocysteine as a substrate which results in the formation of cystathionine. CSE converts the cystathionine to L-cysteine, α-ketobutyrate and ammonia. L-cysteine is used as a substrate by CBS to release H_2S along with the formation of pyruvate and ammonia (figure 1.21), or to form 3-mercaptopyruvate (3-MP). The last enzyme, 3-MST, employs 3MP as its substrate which leads to the formation of pyruvate and H2S. Cysteinyl-tRNA synthetases have been discovered to utilize cysteine as a substrate to catalyse the formation of cysteine persulfide (CysSSH) and polysulfides (CysSxSH) which acts as another important precursor of H_2S_X.

Subseqently, cysteine persulfides interact with thiol-SH groups on proteins, causing polysulfuration (Liu et al, 2018).

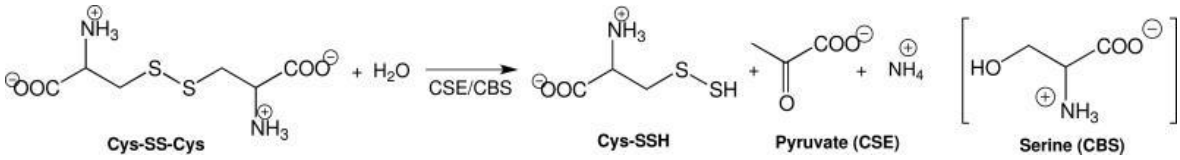


Figure 1.21: Cys-SSH generation from Cys-SS-Cys. (Ono et al, 2014).

1.6 *DROSOPHILA MELANOGASTER* AS A MODEL ORGANISM

In this thesis I use the fruit fly *Drosophila melanogaster* model organism as a model for the pest species *D. radicum* and *D. suzukii*. *D. melanogaster* has been used as a model species for over 100 years, particularly for genetic and developmental research. The fly genome was sequenced in 2001 and there is a large volume of data on fly biology, including established methods of handling in the laboratory. The fruit fly also has a simple and relatively short life cycle (figure 1.22). *Drosophila* is not typically considered a pest, however, it is suitable to use for insecticide screening due to its biochemical, physiological and genetic similarities to *D. radicum*, *D. suzukii* and other pest species. *D. melanogaster* is an excellent model system to assess the effects of garlic and its constituents as a potential insecticide for controlling and managing *D. radicum*, *D. suzukii* and other pests (Scharf, Nguyen et al. 2006; Zolfaghari Emameh, Syrjanen et al. 2015).

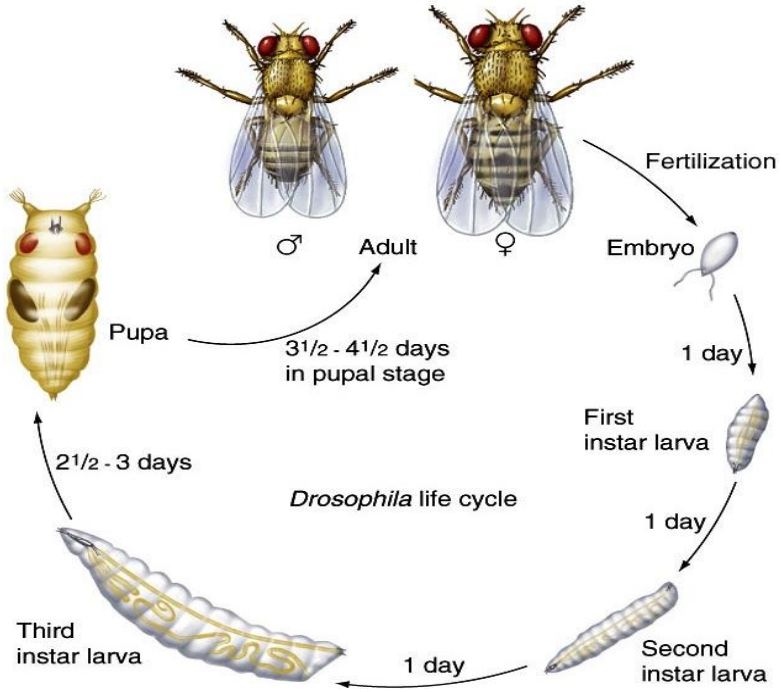


Figure 1.22: *Drosophila melanogaster* life cycle. One day after fertilization, the embryo develops and hatches into a larvae. The larva continuously grow and eat, moulting at one (first instar), two (second instar) and four days (third instar) after hatching. The third instar larva moults into an immobile pupa after two-three days. The pupa takes four days to hatch into the adult fly, within which the body is completely remodelled and is fertile within 12 hours of being an adult. This timing described is when kept at 25°C; at 18°, development takes twice as long(Ong, Yung et al. 2015).

There are many features that make *D. melanogaster* an attractive model to study. As mentioned previously, the genome (~14,000 genes located on four chromosomes) has been sequenced and is well annotated (Reiter, Potocki et al. 2001; Lloyd and Taylor 2010). *D. melanogaster* has a rapid life cycle and a single mating pair can produce hundreds of offspring within 10 days at 25°C. The embryo is often used for developmental studies to explore organogenesis, pattern formation, cell fate determination, and neuronal development. The larva, in particular the third instar larva, is used to study developmental and physiological processes in addition to simple behavioural studies. The larva contains imaginal discs of the future adult structures, which are mostly composed of undifferentiated epithelium. These structures undergo massive morphological changes from late third instar larval stage to pupal stage. The brain of the adult contains more than 100,000 neurons that form discrete circuits and neurons that mediate behaviours such as sleep, learning, circadian rhythms, memory, courtship, aggression, grooming and flight navigation(McClung and Hirsh 1998; Moore, DeZazzo et al. 1998; Bainton, Tsai et al. 2000; Nichols and Sanders-Bush 2002; Rothenfluh and Heberlein 2002; Satta, Dimitrijevic et al. 2003; Wolf and Heberlein 2003; Andretic, Kim et al. 2008).

The use of *D. melanogaster* is not limited to genetic and developmental research. It has also been used for pharmacological studies. Many drugs used in mammals were first detected and validated in *D. melanogaster* (Heberlein, Tsai et al. 2009; Kaun, Devineni et al. 2012). *D. melanogaster* is an efficient model system for human disease and to dissect host interactions with known insect pathogens (Dionne and Schneider 2008; Chhabra, Kolli et al. 2013; Li, Qian et al. 2013). It is important to note that *D. melanogaster* is not a perfect representative model; however, some conserved biology between *D. melanogaster* and humans allows it to be exploited in the drug discovery processes. Significant discrepancies in drug levels and tissue distribution profiles between *D. melanogaster* and humans are apparent due to potential differences in the pharmacokinetics and pharmacodynamics of small molecules (Pandey and Nichols 2011). Due to metabolic differences some drugs that may be toxic in *D. melanogaster* but not in humans and *vice versa*(Rand 2010). Therefore, *D. melanogaster* should only be used as a screening platform for small-molecule screening, target discovery, or post-screening validation to shorten a large pool of potential drug candidates that will be validated using traditional mammalian model systems (Pandey and Nichols 2011). Despite certain limitations, *D. melanogaster* is a good model system for drugs, target discovery processes, and pesticides. *D.*

melanogaster was used as the model here due to its similarities to pest organisms and the tools and techniques available (chapter 3).

Utilizing *D. melanogaster* as a model provides a rapid, high-throughput *in vivo* platform to study effects of insecticides. *D. melanogaster*, as a model organism, will have similar behaviours, molecular biology, and metabolism to pest insects. *D. melanogaster* are also highly tractable with short generation time and lifespan which allows for high-throughput screening of lethal and sub-lethal effects of insecticides. This makes them ideal for testing new insecticides and drug development (Jones et al, 2007; Tasman et al, 2021; Martelli et al, 2020; Harrop et al, 2018). Since D. melanogaster are small, they are often used by the EU for testing pesticide safety as many flies can be tested, mutants can be generated, they are easily stored, and are relatively cheap (Tasman et al, 2021).

The complete genome sequence of *D. melanogaster* was revealed in 2000 (Adams et al, 2000). Due to the vast annotation of its 14,000 genes, many tools are available to influence gene expression using mutations such as null (knockout) alleles, knockdown or over-expression of transgenes using *RNAi* (RNA interference) of endogenous genes. The two most used gene tools of *D. melanogaster* are RNAi and the GAL4-UAS system. These tools can be combined to allow sequencing of genes in a spatio-temporal pattern resulting in the detailed mapping of genetic phenotypes (Duffy, 2002; Heigwer et al, 2018). The genetic tools have been used to study insecticide resistance is *D. melanogaster*. Gnese have been identified that grants resistance to pesticides such as DDT, organophosphates, neonicotinoids, Spinosad, and pyrethroids (Soderlund and Knipple, 2003; Perrt and Batterham, 2018; Ffrench-Constant et al, 2004; Bass, 2016). These examples demonstrate the use of D. melanogaster as a model pest and is suitable to use for the exploration of pesticide effects.

1.7 AIMS AND OBJECTIVES

The insecticide trade in the U.K. is a multimillion-pound industry; however, due to changes in legislation outlawing commonly exploited pesticides due to the toxicity problems that are associated with the use of many current insecticides, it is declining. This provides an opportunity for the development of a new suite of control products that conform to U.K. government requirements for crop production (Prowse, Galloway et al. 2006). This in combination of insect pests, such as the cabbage root fly, which continues to be a significant problem for crops with the genus *brassica*, can cause worldwide horticultural problems demonstrate the demand for new insecticides, those of which are more natural and less harmful to the environment but are just as effective.

Garlic has bactericidal and fungicidal effects due to the presence of organosulfur compounds that are produced when the garlic is crushed. Previous research from bacterial studies shows that diallyl polysulfides can react with glutathione, leading to the generation of reaction oxygen species (ROS) such as hydrogen peroxide. However, it has not previously been tested whether these same biochemical activities would occur in insects. The known ROS-generating properties of garlic constituent compounds suggested that garlic products could have insecticidal effects, and hence could be used to control serious insect pests. However, the details of insecticidal action in Diptera has not been investigated. The research is important because of the ever-demanding need for new and environmentally benign insecticides for the control of serious UK agricultural pests such as the cabbage root fly, spotted wing fruit fly and vine weevils.

This thesis focused on two main areas: a) the insecticidal effect of garlic solutions against *Drosophila melanogaster* using different application methods; and b) the biochemical effect of garlic-derived compounds regarding oxidative stress.

In this thesis we investigated the insecticidal effects of diallyl polysulfides on the different life stages of the model species *D. melanogaster*. Eggs, larvae, pupae and adult *D. melanogaster*were initially used to compare effects of diallyl polysulfides on mortality, pupariation rate and timing of adult emergence. Different methods of application of the diallyl polysulfides were used, including direct contact and fumigation. Individual polysulfides from synthetic DAS2-6 mixture were purified and used to treat *D. melanogaster* to identify the most effective diallyl polysulfide. This revealed which life stages are most sensitive to diallyl polysulfides giving both quantitative (dose) and qualitative (delivery formulation).

Analytical methods were used to develop a way of probing how these insecticidal effects relate to the biochemical level. In this way we investigated how diallyl polysulfides reacted with glutathione to cause the production of ROS. The content of GSH and thiols was quantified by labelling with a thiol-specific fluorescent probe (monobromobimane) and by using HPLC analysis. In parallel, ROS production was analysed to identify whether there was a correlation of diallyl polysulfide-induced phenotypic effects with oxidative stress induction at the molecular level. Transgenic roGFP transgenic flies were used (chapter 3) to determine redox status analysis upon treatment with garlic oil, to locate areas of effect on the organisms and to monitor changes in redox homeostasis.

Using the information from these studies, we were able to determine what garlic-based product dosage, formulation and application strategy would be the most effective in field trials. We then performed field trials in association with ECOspray Ltd using appropriate applications to investigate diallyl polysulfide insecticidal effects in a more realistic environment.

2 INSECTICIDAL EFFECT OF GARLIC ON *DROSOPHILA MELANOGASTER*

2.1 INTRODUCTION

Garlic is known for its high content of organosulfur compounds. In an intact garlic bulb, the sulphur compounds are mostly γ-glutamyl peptides and allylcysteine sulfoxides. When damaged, alliin (S-allyl-L-cysteine sulfoxide) is metabolized to allicin via the activity of the carbon-sulfur lyase enzyme, alliinase. Allicin is very unstable and is converted to more stable compounds including diallyl polysulfides such as diallyl sulphide (DAS1), diallyl disulfide (DAS2), diallyl trisulfide (DAS3) (Berges, Siess et al. 2004; Liang, Wu et al. 2015). These compounds are very volatile, which may facilitate their effectiveness as defence mechanisms used by garlic plants against pests and pathogens (Hile, Shan et al. 2004).

There are four types of garlic preparations available: garlic essential oils, garlic oil macerate, garlic powder and garlic extract. It has been shown that some garlic products contain differing amounts of active compounds, depending on how the product was made(Adaki, Adaki et al. 2014). The compounds can be isolated by steam distillation or oil extraction, sometimes using heat. The typical yields are: DAS 20-240; DAS2 280-900; and DAS3 40-200(µg/g of garlic preparation)(Laakso, Seppanen-Laakso et al. 1989; Lee, Kim et al. 2003; Kimbaris, Siatis et al. 2006).

In this chapter, the insecticidal properties of garlic extract in the form of natural and synthetic garlic oils were tested. *Drosophila melanogaster* was used as a model organism in which to test the insecticidal activity of garlic and the susceptibility of all the different life stages of this insect was investigated. Different application methods and formulations of garlic preparations were also tested. The aim was to compare the insecticidal activity of different diallyl polysulfides and garlic products, analyse the differences between them, study the tolerances of the different life stages to the garlic products and find the most appropriate mode of application and formulation for use of insecticidal garlic preparations in an agricultural setting.

2.2 METHODS

2.2.1 Garlic products tested

The garlic products tested were: garlic extract (Ecospray Ltd) (which has the addition of 2.4% diallyl trisulfide and 3% TWEEN20), Mexican (Sigma-Aldrich), Chinese (Elixens Ltd), and the synthetic garlic oil, R22 (Ecospray Ltd) garlic oil. Manufacturing details of products could not be found or are confidential to Ecospray Ltd. Concentrations used in experiments were 0-5% (v/v). Units of percentage (v/v) were used for all treatments as this is how Ecospray Ltd state their concentrations and to keep units consistent. In the following experiments water was used with garlic extract as the control. For the garlic oils, both ethanol and TWEEN20 (3% v/v) were used and were the controls. Two different carriers were used as ethanol is the least harmful solvent to emulsify the garlic oil and TWEEN20 is used in the formation of Ecospray Ltd products.

2.2.2 *Drosophila melanogaster* egg harvesting and housekeeping

D. melanogaster were maintained in the fruit fly facility (UEA) at constant 25°C under a 12 hour light 12 hour dark cycle and 50% relative humidity. Wildtype *D. melanogaster* Dahomey cultures were derived from laboratory strains established in in the 1970s. Flies were fed a mixture of sucrose, agar, and brewer's yeast (*Saccharomyces cerevisiae*) (15g Agar, 50g sugar, 100g brewer's yeast, 30ml Nipagin (10% w/v solution), 3ml propionic acid per litre of water). To collect eggs, agar plates comprising agar and grape juice with yeast mixture were placed in the *D. melanogaster* population cages for up to 4 hours. Identification of the three larval stages was carried out according to time, changing in morphology such as the anterior spiracles and number of teeth on the dark coloured jaw hooks, and from behaviour of the larvae. One day old adults were used for all adult experiments. All experiments used both male and female as experiments were not gender specific as both are present in natural environments.

2.2.3 Egg pipetting for obtaining standardised egg numbers

A method for collecting large but defined numbers of *D. melanogaster* eggs at a synchronised stage of development was developed from the method of Clancy and Kennington (2001).In this, collections of eggs laid onto a petri dish containing grape juice agar medium (1 L water, 50g agar, 600 ml red grape juice, 42 ml 10% Nipagin® (v/v in ethanol) to make 40 plates) within a defined period of time are washed off the agar plate into a 15 ml Falcon tube and allowed to settle. Various volumes of this solution can then

be dispensed using a standard Gilson pipette into culture media, to generate standardised density treatments for the insecticidal tests. A 100 µl pipette tip was initially cut at 6mm from the end (to allow for easier transit of the eggs) and different volumes of eggs were dispensed, from 5-15 µl. The number of eggs was counted for each volume and each test was replicated three times. This gave defined egg numbers per volume. In further pilot testing, the 100 µl pipette was cut at different lengths (4-10 mm) using a standard volume of 8µl. Again, each test was replicated 3 times. The results of these tests allowed me to as certain the accuracy and repeatability of this procedure and decide upon the optimal protocol for decanting known numbers of eggs rapidly and consistently. For the ovicidal experiments, the optimum protocol was a 6mm cut from the pipette tip and 8µl, to decant approximately 100 eggs per sample.

2.2.4 Dipping/pipetting application methods

For tests of the insecticidal effects of direct contact of garlic products to *D. melanogaster* eggs and larvae, a dipping/pipetting method was used. This was used to represent the scenario of a pest insect coming into contact with the garlic product on a crop. This method was applied to eggs (dipping) and larvae (pipetting). For the pupae and adults, this method would likely have been ineffective and for these stages a spraying application method (below) was used to simulate direct contact application. Concentrations of 0-5 % (v/v) of garlic treatment were used for each experiment and 4 replicates were used for all experiments.

For the dipping method: approximately 100 eggs per condition were pipetted onto metal gauze and dipped into the garlic or control treatments, then placed in petri dishes and scored for viability after 24 hours of incubation at 25°C.For the pipetting method: 200 µl of garlic treatment was pipetted onto a 30 mm grape juice agar plate. The larvae were placed on the top of these plates and then scored for viability after 24 hours incubation at 25°C.

2.2.5 Spraying application method

An additional method for testing the insecticidal effects of direct contact of garlic products was spraying and this was particularly useful for the pupal and adult stages. The insects were sprayed directly with garlic treatments (0-5 % v/v) and then scored for viability after 24 hours incubation at 25°C (or for pupal emergence for the tests of pupae), 4 replicates were used for all experiments. This method is more likely to be applicable to an agricultural setting and it also has the potential to coat the entire insect with the control or

experimental treatments. This method was used on all life stages of *D. melanogaster*. Studies were also performed using concentrations within 0-1 % on the eggs and adult life stages for greater accuracy of LC50 calculation.

The insecticidal experiments using diallyl polysulfides were performed using the spraying method in order to ensure precise dosage was achieved. Insecticidal experiments using DASs (DAS2-5) were performed on *D. melanogaster* eggs and the three larval stages (L1-L3). A 2% (w/v) solution in ethanol of DAS was used for each experiment; final concentrations of the DASs are shown in table 3.

2.2.6 Egg exposure

Wild type *D. melanogaster* eggs were obtained as described above. For each trial there were six different concentrations (0%, 1%, 2%, 3%, 4%, and 5% v/v) of garlic extract or garlic oil. There were four replicates for each concentration. The garlic extract (provided by ECOspray Ltd) solutions were made up with distilled water and mixed thoroughly before use. Two different garlic oils were used; Mexican garlic oil (reference: 9007681; ELIXENS Ltd) and China garlic oil (reference: 9007683; ELIXENS Ltd). The garlic oil solutions were made with ethanol or 3% TWEEN20 and were thoroughly mixed before use. Each treatment consisted of using 5 mL of the garlic extract/oil solution in a 30 mm petri dish. Using the method by described in section 2.2.3, 8 µL of eggs were pipetted onto a small metal gauze and dipped into the garlic solution. The eggs were then transferred onto 30 mm petri dishes of grape juice agar, the total number of eggs were counted. The dishes were incubated for 24 hrs at 25˚C. The number of eggs that had hatched were counted and the percentage of hatching was calculated. Whole experiments were repeated 2-3 times.

2.2.7 Larvae exposure

The same garlic extract and garlic oils were used as with the eggs, with the same concentrations of 0%, 1%, 2%, 3%, 4%, and 5%. There were four replicates for each concentration, sample size was 100 larvae. Prior to adding 100 larvae per 30 mm plate of grape juice agar, 100 µL of the garlic extract/oil solution was added to the plates and allowed to dry. An optical microscope was used to transfer the larvae onto the treated grape juice agar plates. The total amount of larvae per sample was counted and were then incubated for 24 hrs at 25˚C. The amount that survived was counted and percentages were calculated. Whole experiments were repeated 2-3 times.

2.2.8 Pupae exposure

D. melanogaster eggs were collected for 4 hours and incubated at 25°C for 3 days until organisms developed into 3rd instar larvae. The larvae were placed in small vials of SYA (15g Agar, 50g sugar, 100g brewer's yeast, 30ml Nipagin (10% w/v solution), 3ml propionic acid per litre of water) with paper inserted covering the inside of the vials. Larvae were further incubated at 25°C for 4 days until pupal life stage is reached. The paper with the pupae was carefully removed with forceps and taped to the inside of a 9x9x10 cm (810 cm^3) plastic box and enclosed (refer to section 6.4 for images). The box had small air holes on the lid and a 2 inch hole on one of the sides covered with mesh fabric. Garlic oil solution (concentrations of 0-5% (v/v)) was sprayed for 2 seconds (approximately 500 µl total volume) through the hole onto the pupae. Pupae were left for desired number of days and emergence of adults is counted and recorded. There were four replicates for each concentration. Whole experiments were repeated 2-3 times.

2.2.9 Adult exposure

Approximately 100 larvae were placed in SYA vials until adult emergence. Each vial was placed in a 9x9x10 cm (810 cm^3) plastic box, section 6.4. The box had small air holes on the lid and a 2 inch hole on one of the sides covered with mesh fabric. Garlic oil solution (concentrations of 0-5% (v/v)) was sprayed for 2 seconds (approximately 500 µl total volume) through the hole onto the adults. After 24 hours, survived adults were recorded. There were four replicates for each concentration and whole experiments were repeated 2-3 times. The adults were of mixed population of male and female, fertile and were treated 24 hours after emergence.

2.2.10 Non-volatile experiments

An aliquot of garlic treatment (0-5% v/v) was pipetting onto agar in 50 mm petri dishes and left for 10 min to dry. Approximately 100 eggs/larvae were placed on the treated agar and a mesh was placed over the dish to create an open environment. Tests of non-volatile components of garlic were not performed on the pupae and adults as it was not feasible to ensure sufficient direct contact for either life stage. Concentrations of 0-5% (v/v) garlic extract in water were used on eggs, 1st, 2nd, and 3rd instar larvae of *D. melanogaster*, with water as the control. Survival was scored after 24 hours of subsequent incubation at 25˚C. Mexican, China and R22 garlic oil were used at concentrations of 0-5% (v/v) garlic oil in ethanol and 3% (v/v) TWEEN20. There were four replicates for each concentration and whole experiments were repeated 2-3 times.

2.2.11 Fumigation application method

The fumigation method for garlic compound delivery was performed on all life stages of *D. melanogaster*. The organisms were placed in 810 cm^3 tubs (refer to section 6.4 for images) in which a cotton wool wick treated with 1 ml of the R22 garlic preparation solution was placed against an opening. The opening was covered in mesh to prevent direct contact of the organisms with the test garlic solution. Therefore, the organisms would only be exposed to the volatile compounds from the treatments. The concentrations used were 0-5% (v/v) R22 with ethanol, with ethanol carrier used as a control. Insects were treated for 24 hours and the survival of eggs, larvae and adults was then scored. For pupae, the number of adults emerging from the treated pupae was counted after 4 days. There were four replicates for each concentration and whole experiments were repeated 2-3 times.

2.2.12 Garlic oil and copper treatment

The effect of copper in conjunction with R22 garlic oil was tested to see if a synergic insecticidal effect occurred. The R22 garlic oil concentrations used were 1-5% (v/v), the concentrations used for $CuSO_4$ were 0.054, 0.108, 0.168, 0.216, and 0.27 mg/ml $CuSO_4$, respectively. Ethanol was used as the control. These concentrations were based on a nematicidal study performed by a colleague (Dr Ryan Tinson, UEA), which were determined following HPLC analysis to monitor the reaction of R22 with $CuSO_4$and increasing amounts of $CuSO_4$was added to R22 garlic oil until a linear steady state was obtained. The insecticidal studies were performed on the eggs, 1^{st}, 2^{nd}, and 3^{rd} instar larvae of *D. melanogaster*. Treatments were applied using the spraying method as described previously.

2.2.13 Beta-cyclodextrin encasing of garlic oil to reduce odour

Diallyl polysulfides from garlic oil were encased in β-cyclodextrins and this procedure was performed by a colleague (Dr Ryan Tinson, UEA). The inclusion of the garlic oil was confirmed by NMR. The aim was to test whether effective masking of the odour of the diallyl polysulfides (hence minimising any repellent effect) would still retain the insecticidal effect. Four different treatments were used; β-cyclodextrin (control), Mexican garlic oil with β-cyclodextrin, China garlic oil with β-cyclodextrin, and R22 garlic oil β-cyclodextrin. The garlic oils and β-cyclodextrin were in a 1 in 10 ratio. Therefore, concentrations of 10-50 % (w/v) of the test solutions were used, to mimic the 1-5 % (v/v) garlic oil concentrations used in the previous insecticidal tests.

2.2.14 DAS isolation

The diallyl polysulfides (DAS) were isolated via HPLC from the synthetic garlic oil R22 from a 10% (v/v) solution. The DAS compounds were isolated using a HiChrom ACE-AR C18 4.6 x 250 mm, 5 um column, equilibrated at 37℃ and UV detection at 2010 nm. DAS1 to DAS6 eluted at approximately 2.6, 3.0,4.4, 5.8, 8.3, and 11.4 respectively.Isocratic elution of R22 garlic oil was performed at a flow-rate of 1.5 mL/ min using 90% (v/v) MeOH. This provided good separation of DAS1-6 through a C18 column (250 x 4.6 mm) within 17 min. From the isolated diallyl polysulfides, DAS2-5 were used for the insecticidal experiments as the DAS1 and DAS6 did not yield sufficient amounts for the experiments. The DAS products were collected by fractions and extracted in hexane and dried under vacuum at temperatures under 30℃ due to heat instability. Purities of 90-99% were obtained and were stable at -20℃ for several months when stored as dried product.

2.2.15 Field trial

2.2.15.1 Garlic smoke generators

As a method of field application, garlic smoke generators (BioFume™ Greenhouse) produced by Octavious Hunt Ltd were tested against adult *D. melanogaster*. Four different smoke generators were used; garlic extract (clail, Ecospray Ltd) (~3%TPS), garlic extract with 9% total polysulfide (TPS), 3% GO with 1.5% TWEEN, and 9% GO with 4.5% TWEEN.

2.2.15.2 Trial procedure for garlic smoke generators

The trials were conducted outside at the University of East Anglia in a 5m x 2m x2m New Leaf™ Polytunnel (GH2010) (images shown in section 6.5). Approximately 100 eggs, larvae or adults of *D. melanogaster* were placed in 810 cm^3 tubs (section 6.4 for images). The organisms were placed approximately half a meter away from the ignited smoke generator. As control, organisms were left outside of the polytunnel. Organisms were left for a total of 20 min upon ignition of smoke generator. Some samples were placed in the polytunnel after 10 min of ignition. Survival was then recorded straight after treatment and again after 24 hours.

2.2.16 Statistical analysis

Data were analysed using one-way ANOVA (followed by Tukey's post hoc analyses) and t-tests, $P<0.05$ was considered significantly different. Analysis performed using IBM SPSS

Statistics software and Microsoft Excel 2010 (Excel 2010; SPSS 2016). The LC50s were calculated using non-linear regression with GraFit software (GraFit 2016) by plotting concentration data and using the software to calculate the concentration at the 50% interval. There will be some accuracy/validity of LC50 values <1, when only 0 and 1% are measured; therefore some experiments were performed with garlic oil concentrations between 0 and 1 % (v/v).

2.3 RESULTS

2.3.1 HPLC analysis of garlic oils

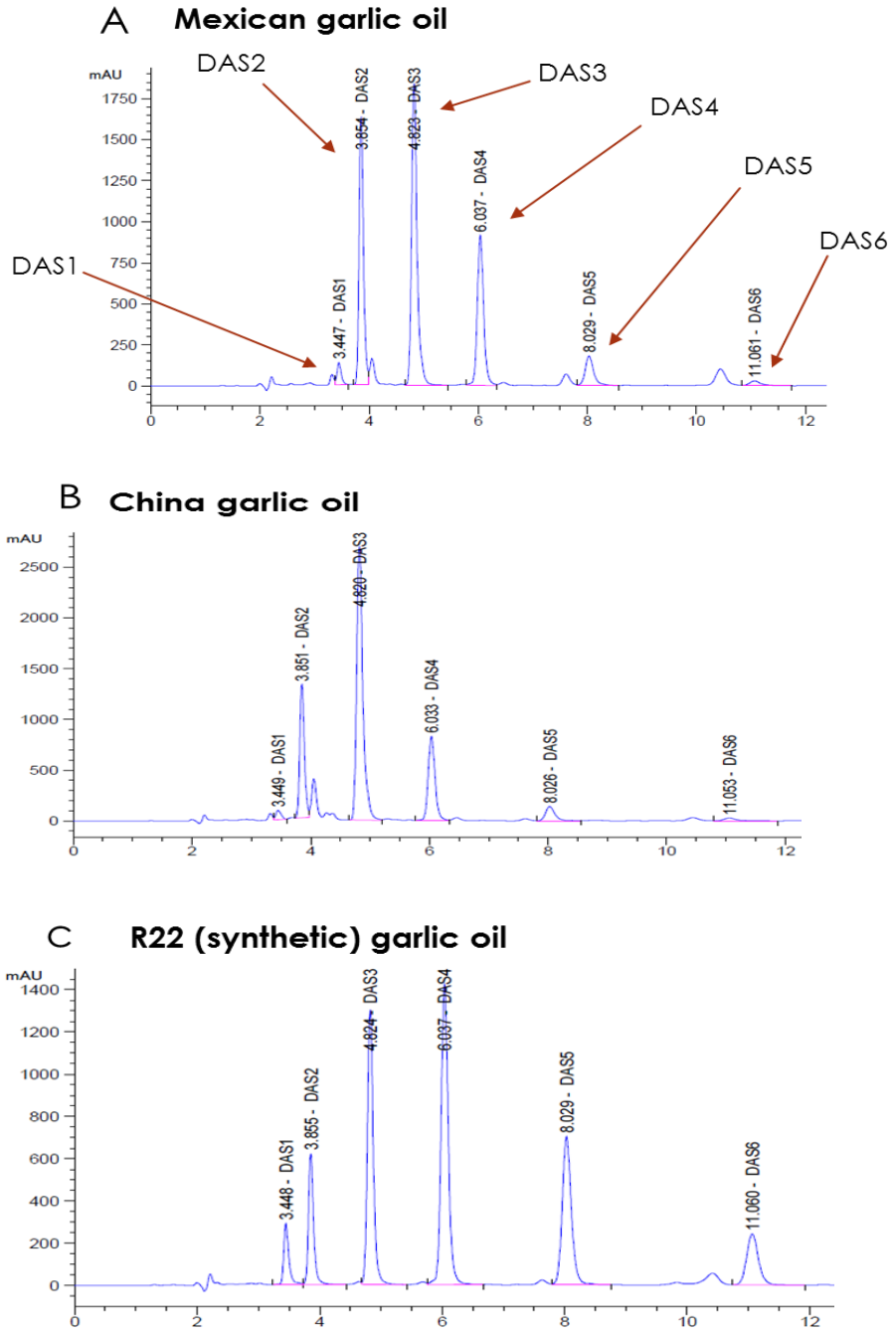


Figure 2.1: Single HPLC traces of diallyl polysulfides (DAS) in Mexican, Chinese, R22 garlic oils. As annotated, peaks represent DAS1-6. The natural garlic oils; Mexican and Chinese (panels A and B), contain more DAS3 compared to the other diallyl polysulfides. The synthetic garlic oil, R22 (panel C), had more DAS3 and DAS4, compared to the other diallyl polysulfides.

HPLC analysis was performed on three different garlic oils in order to determine the different concentrations of the diallyl polysulfides present (figure 2.1 and table 2.1). This was essential for the subsequent insecticidal studies to enable insights into the differing activities of the garlic oils. The differing amounts of diallyl polysulfides in the garlic oils were likely due, at least in part, to different preparation methods, e.g. temperature, as this is known to significantly alter the DAS content (Satyal, Craft et al. 2017). The concentrations of the diallyl polysulfides could affect insecticidal activity of the garlic oils.

Table 2.1: Calculated percentage concentrations of diallyl polysulfide content of Mexican, China and R22 garlic oils.

DAS	**MGO**	**CGO**	**R22**
DAS1	2.26	1.57	4.45
DAS2	28.09	19.82	9.46
DAS3	39.49	54.65	23.03
DAS4	22.46	18.05	30.21
DAS5	6.02	4.37	19.24
DAS6	1.35	1.14	8.60
DAS7	0.33	0.30	3.89

2.3.2 Insecticidal effects of garlic on *Drosophila melanogaster*

Before performing the main insecticidal assays with the garlic products, the effectiveness of the egg collection method used was established as well as an assessment of any potential toxic effects of the TWEEN20 carrier. The results of these two preliminary tests are given below.

Accuracy and repeatability of egg collection: For the ovicidal studies, a method to apply a defined number of eggs was first tested and was used to minimise preparation time for the main ovicidal experiments and to give repeatable egg numbers for the different assays. The experiments to test the consistency and accuracy of the egg collection method showed, for different micropipette tip lengths and different egg solution volumes, high repeatability and a linear relationship between the volume of egg solution dispensed and number of eggs within it (Figure 2.2).

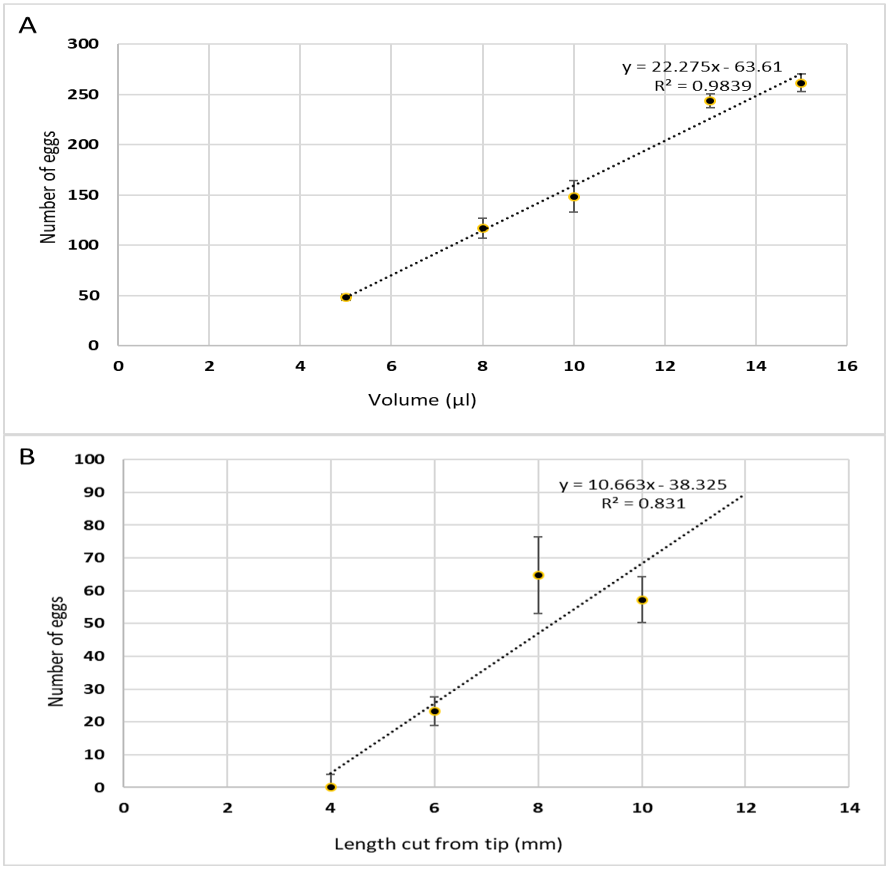


Figure 2.2: Developing the egg pipetting method to achieve consistent egg number delivery.A) testing different volumes of pipetting using 6 mm cut from a 100 µl pipette tip. B) testing different lengths cut from a 100 µl pipette tip using 8 µl. Error bars show better consistency when using different volumes. Error bars show ± SE, n=4.

Potential toxicity of ethanol and TWEEN20 carrier: Garlic oil has low solubility in water (Zheng, Li et al. 2013), DAS4 has a log P (octanol-water partition coefficient) of 3.931 (The Good Scents (Company 2018). Therefore, to emulsify the garlic oil, it was necessary to use ethanol and TWEEN20. However, it was not known whether the desired concentration of ethanol or TWEEN20 would have any insecticidal effect. Therefore, an ovicidal experiment was first performed using concentrations of 0-5% (v/v) or ethanol (figure 2.3) and TWEEN20 (figure 2.4). None of the concentrations of ethanol or TWEEN20 tested any effect on egg hatching. On this basis, a garlic oil emulsion using 3% (v/v) TWEEN20 was selected as safe to use in the insecticidal experiments as this is what Ecospray Ltd use in their products.

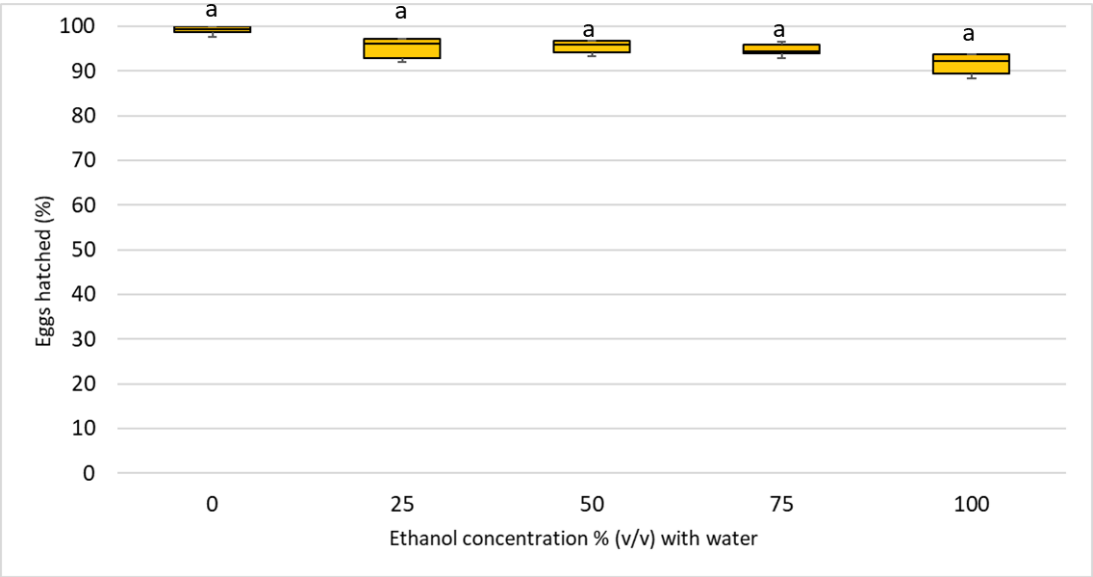


Figure 2.3: Ovicidal experiment ethanol. There was little ovicidal activity. F (5,18)= 0.185, P= 0.926.. Boxes, lower/upper quartile; whiskers, minimum and maximum. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons.There was no significant difference (P < 0.05), n=4.

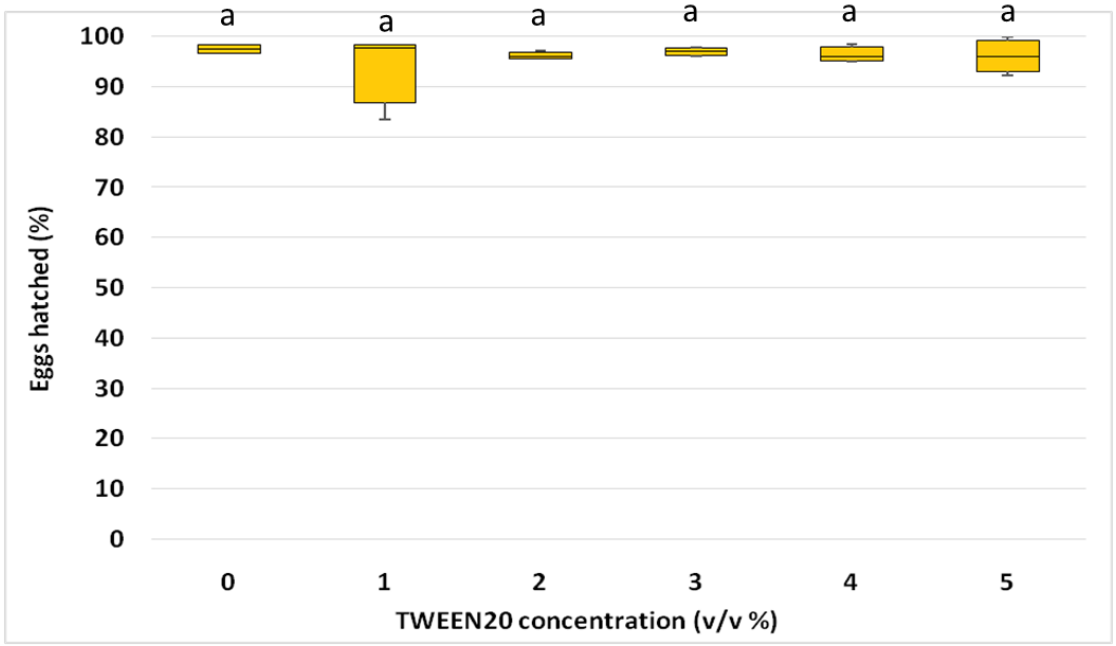


Figure 2.4: Ovicidal experiment of TWEEN20**.** There was no effect of TWEEN20 from 0-5% (v/v) with water on egg viability.TWEEN20 at 3% (v/v) was to be used as this is what is used in products by Ecospray Ltd. F (5,18)= 0.253, P= 0.933..Boxes, lower/upper quartile; whiskers, minimum and maximum. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. There was no significant difference (P < 0.05), n=4.

Insecticidal effects of garlic compounds

To study the activity of garlic, insecticidal experiments were performed using four different garlic products: garlic extract, Mexican garlic oil, Chinese garlic oil, and synthetic garlic oil R22. The garlic extract and R22 are currently used by Ecospray Ltd in field trials to provide protection against pests. Garlic products were tested separately on the different life stages of *D. melanogaster*; eggs, 1st, 2nd, 3rd instar larvae, pupae, and adults. For each experiment, the organisms were subjected to specified garlic treatment for 24 hours and survival was then scored.

Two methods were used: the dipping/pipetting method and the spraying method. The results showed that for all garlic treatments there was a concentration-dependent insecticidal effect on all *D. melanogaster* life stages. The eggs and the adults were the most susceptible and the pupae were the least. The pupae were not affected by any of the garlic treatments up to 5% (v/v). Garlic extract was the least effective as an insecticide and the

synthetic garlic oil, R22, was the most effective. Statistical analyses are shown in section 6.1. The spraying method was more effective and gave more consistent results compared to the dipping/pipetting method. Additionally, a repellent effect was observed for all garlic products where at least 50% of the larvae would try and get as far as possible away, and some attempted to escape out of the petri dish. The detailed results of the tests with each of the different garlic extracts and oils are described below in turn.

2.3.2.1 Insecticidal effect of garlic extract

One of the garlic products Ecospray Ltd employs for field trials is garlic extract (clail, 2.4% garlic oil; Ecospray Ltd). The insecticidal activity of garlic extract was studied on the different life stages of *D. melanogaster*. Figure 2.5 shows the results of the effects of garlic extract along with the calculated LC50s in units of % (v/v). The eggs were the most susceptible to garlic extract, with a LC50 of 0.82%. The 1^{st}, 2^{nd}, and 3^{rd} instar larvae had very similar affects with LC50s of 2.17%, 3.38%, and 2.55%, respectively. The pupae and the adults were the least susceptible with LC50s of >5%, which was the maximum concentration used.

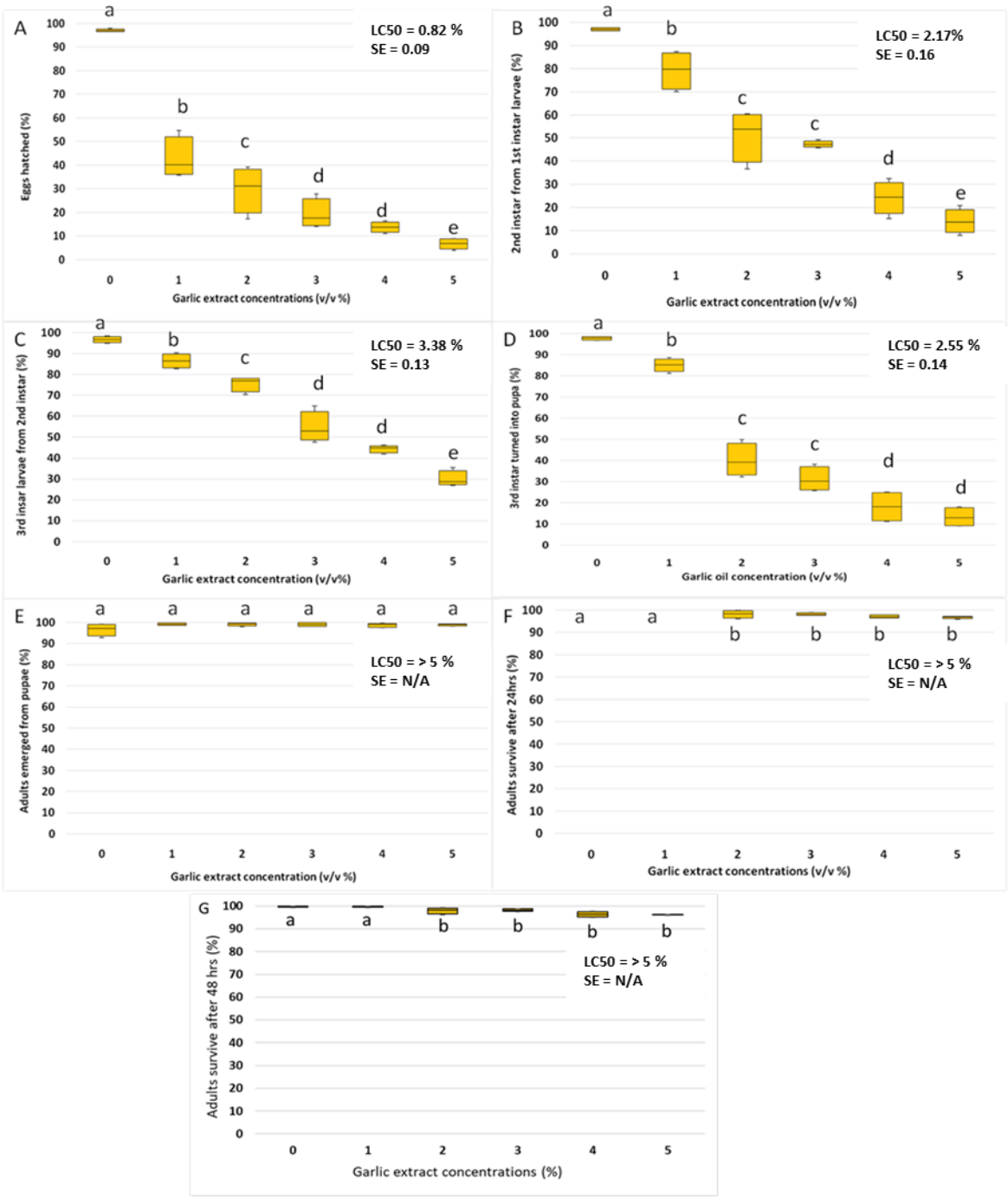


Figure 2.5: Insecticidal effect of garlic extract (clail) with water using spraying application. Insecticidal effect of garlic extract on different stages of the *D. melanogaster* life cycle: A) Eggs, F (5, 18)=136.45, P=0.000016. B) 1st instar larvae, F (5, 18)=92.4, P=0.000043. C) 2nd instar larvae, F (5, 18)=23.53, P=0.0012. D) 3rd instar larvae, F (5, 18)=46.31, P=0.00024. E) Pupae, F (5, 18)=1.44, P=0.37. F) Adults after 24 hours, F (5, 18)=18.57, P=0.0022. G) Adults after 48 hours, F (5, 18)=17.63, P=0.0019 Water was used for control. The LC50 was >5%. Boxes, lower/upper quartile; whiskers, minimum and maximum.SE= Standard error. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another (P < 0.05), n=4.

2.3.2.2 Insecticidal effect of Mexican garlic oil

Natural Mexican garlic oil (Sigma) was used to study insecticidal effects on the different life stages of *D. melanogaster*. Ethanol was used to emulsify the Mexican garlic oil, due to its low solubility in water, and the spraying method described above was used as an application method. Ethanol was used as control Figure 2.6 shows the results of the assays along with the calculated LC50s. The eggs and adults were the most susceptible to garlic oil, with a LC50 of 0.15% and 0.52%, respectively. The 1st, 2nd, and 3rd instar larvae had LC50s of 0.64%, 1.42%, and 2.03%, respectively. The pupae were the least susceptible stage, with LC50 of >5%, which was the maximum concentration used. The results show that different life stage were differentially susceptible to Mexican garlic oil.

In a second assay, the effect of Mexican garlic oil emulsified in TWEEN20 was tested. Such a detergent is much more likely than ethanol to be used in a commercialized garlic product, making this assay a useful complementary test to the study described in the previous section. The control was 3% TWEEN20 (v/v). Figure 2.7 show that *D. melanogaster* eggs and adults were the most susceptible to Mexican garlic oil, with LC50s of 0.57% and 0.49%, respectively. The 1st, 2nd, and 3rd instar larvae had LC50s of 1.48%, 1.75%, and 2.02%, respectively. The least susceptible stage was again the pupae, with an LC50 >5% - the maximum concentration used. When comparing the Mexican garlic oil insecticidal results in ethanol to TWEEN20 there is not much difference demonstrating either carrier can be efficient with the garlic oil.

Figure 2.8 shows the comparison of the survival of adults after 24 hours and 48 hours of treatment. From observing the results, there is little difference, therefore, 24 hours was the time used after treatment to score adult survival.

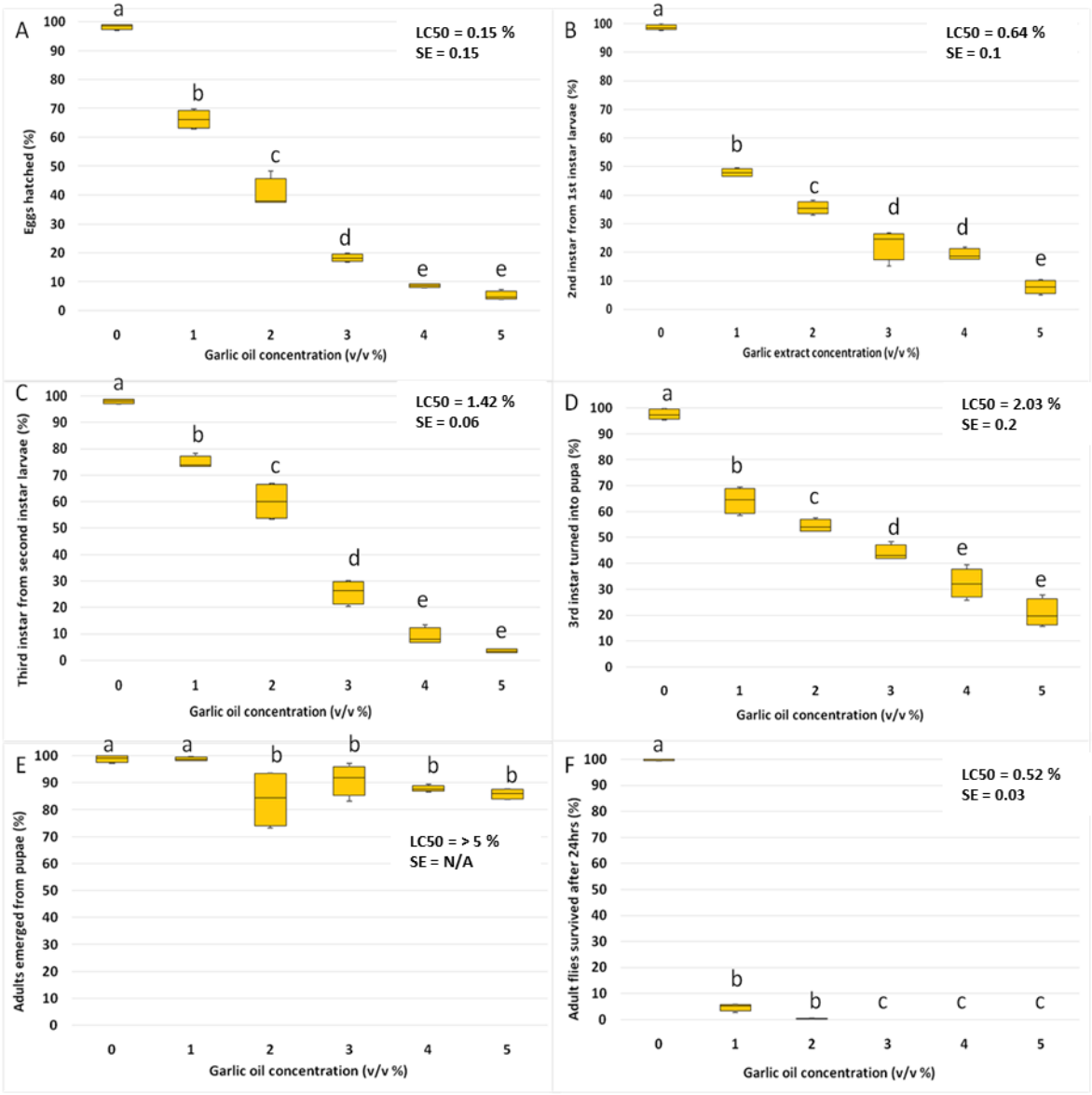


Figure 2.6: Insecticidal effect of Mexican garlic oil emulsified in ethanol and using the spraying application method. Insecticidal effect of Mexican garlic oil with ethanol on different stages of the *D. melanogaster* life cycle: A) Eggs, F (5, 18)=23.59, P=0.0012. B) 1st instar larvae, F (5, 18)=119.67, P=0.000023. C) 2nd instar larvae, F (5, 18)=72.65, P=0.000078. D) 3rd instar larvae, F (5, 18)=127.23, P=0.000019. E) Pupae, F (5, 18)=1.51, P=0.34. F) Adults, F (5, 18)=2340.21, P=0.0. Ethanol was used as control. Boxes lower/upper quartile; whiskers, minimum and maximum. SE= Standard error. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another (P < 0.05), n=4.

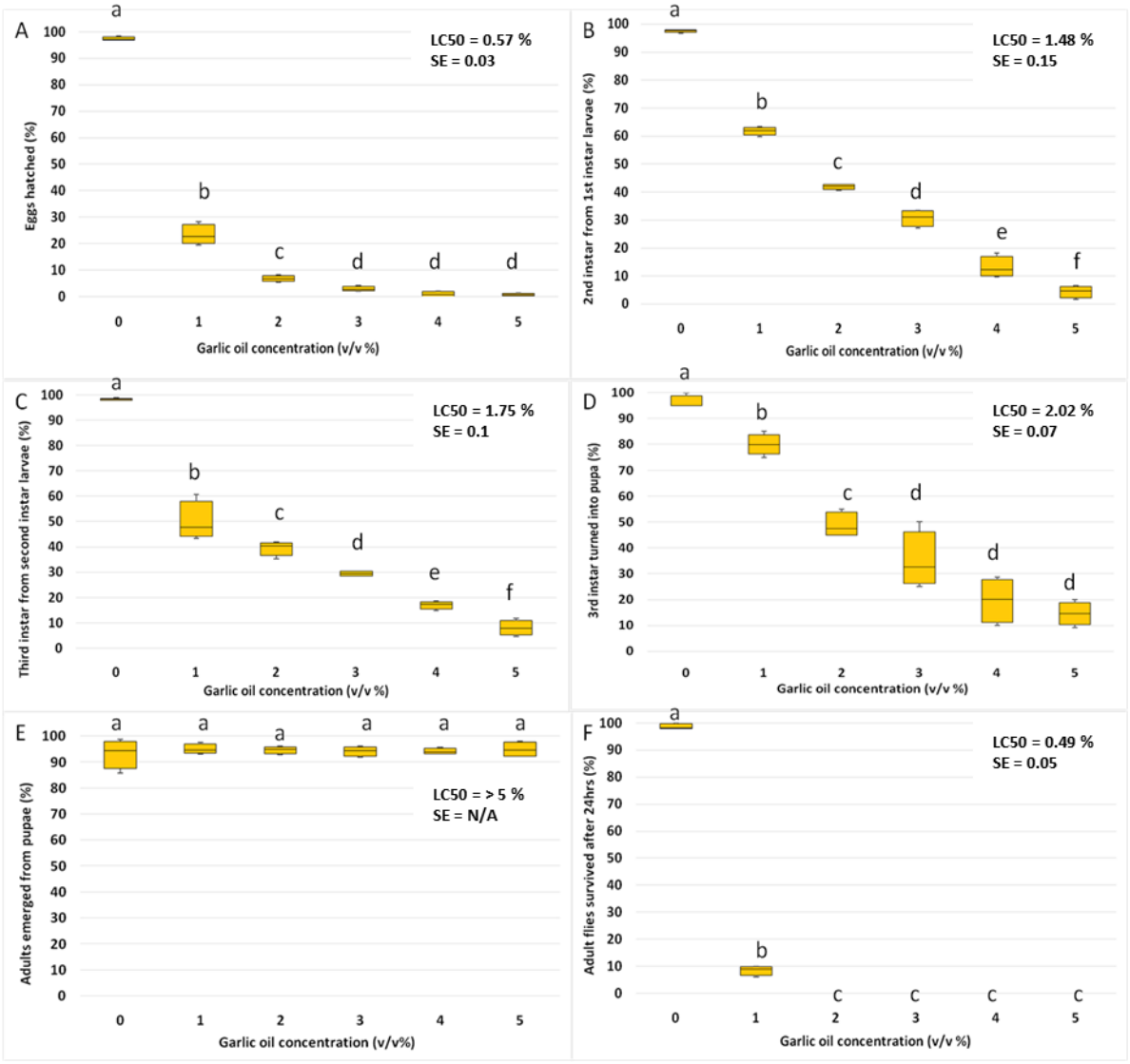


Figure 2.7: Insecticidal effect of Mexican garlic oil emulsified in 3% TWEEN20 and using the spraying application method. Insecticidal effect of Mexican garlic oil with TWEEN20 on different stages of the *D. melanogaster* life cycle: A) Eggs, F (5, 18)=293.12, P=0.0000024. B) 1st instar larvae, F (5, 18)=557.87, P=0.00000049. C) 2nd instar larvae, F (5, 18)=145.62, P=0.00014. D) 3rd instar larvae, F (5, 18)=79.29, P=0.000063. E) Pupae, F (5, 18)=0.125, P=0.98. F) Adults, F (5, 18)=1403.95, P=0.0. The control used was 3% TWEEN20 (v/v). Boxes, lower/upper quartile; whiskers, minimum and maximum. SE= Standard error.The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another (P < 0.05), n=4.

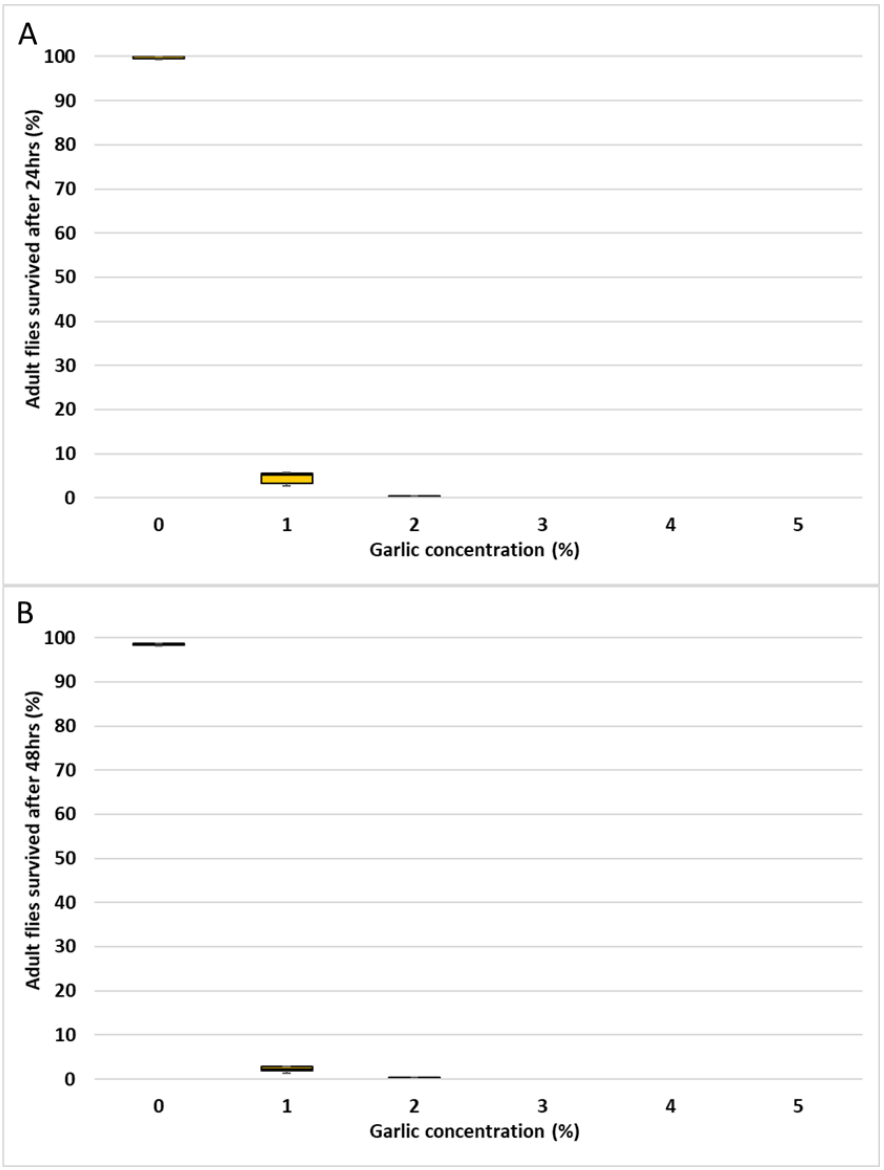


Figure 2.8: Comparison of survival of adults after 24 and 48 hours of treatment with Mexican garlic oil and ethanol. A) Survival after 24 hours. B) Surivial after 48 hours. n=4

2.3.2.3 Insecticidal effect of China garlic oil

Since different garlic oils contain different amounts of diallyl polysulfides depending on how they are manufactured, the effect of natural China garlic oil (Elixens Ltd) was also tested. The same spraying application method as previously described was used and ethanol was used for control. Figure 2.9 shows that eggs and adults were the most susceptible to the effects of China garlic oil, with LC50s of 0.54% and 0.29%, respectively. The 1st, 2nd, and 3rd instar larvae had LC50s of 1.87%, 1.42%, and 2.43%, respectively. The least susceptible life stage was again the pupae with an LC50 >5% - the

maximum concentration used. In general, the results replicated those from the Mexican garlic oil study, above.

As for the Mexican garlic oil, for greater relevance to the commercial market, the effect of Chinese garlic oil emulsified in the detergent TWEEN20 was also tested. The control used was 3% TWEEN20 (v/v). Eggs and adults were again the most susceptible life stages, with LC50s of 0.57% and 0.49%, respectively (figure 2.10). The 1st, 2nd, and 3rd instar larvae had LC50s of 1.48%, 1.75%, and 2.02%, respectively. As before, the least susceptible were the pupae, with an LC50 >5%, the maximum concentration used. The results replicated those of the Mexican garlic oil / TWEEN20 experiment described above.

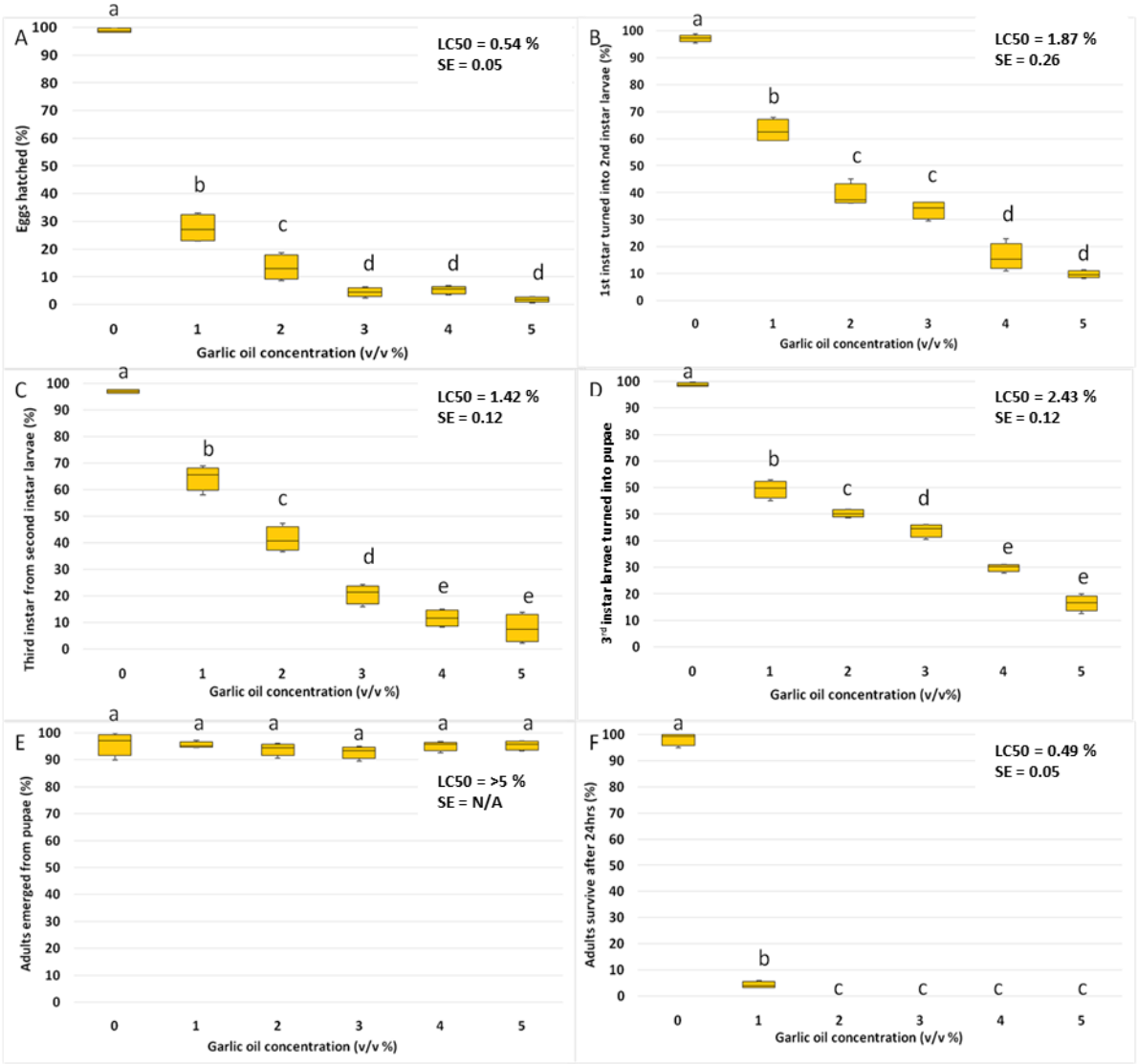


Figure 2.9: Insecticidal effect of China garlic oil with ethanol using spraying application. Insecticidal effect of China garlic oil with ethanol on different stages of the *D. melanogaster* life cycle: A) Eggs, F (5, 18)=399.81, P=<0.00001. B) 1st instar larvae, F (5, 18)=66.03, P=0.000099. C) 2nd instar larvae, F (5, 18)=428.28, P=0.00000095. D) 3rd instar larvae, F (5, 18)=42.57, P=0.00029. E) Pupae, F (5, 18)=1.1,

P=0.5. F) Adults, F (5, 18)=696.35, P=<0.00001.Ethanol was used as control. Boxes, lower/upper quartile; whiskers, minimum and maximum. SE= Standard error. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another (P < 0.05), n=4.

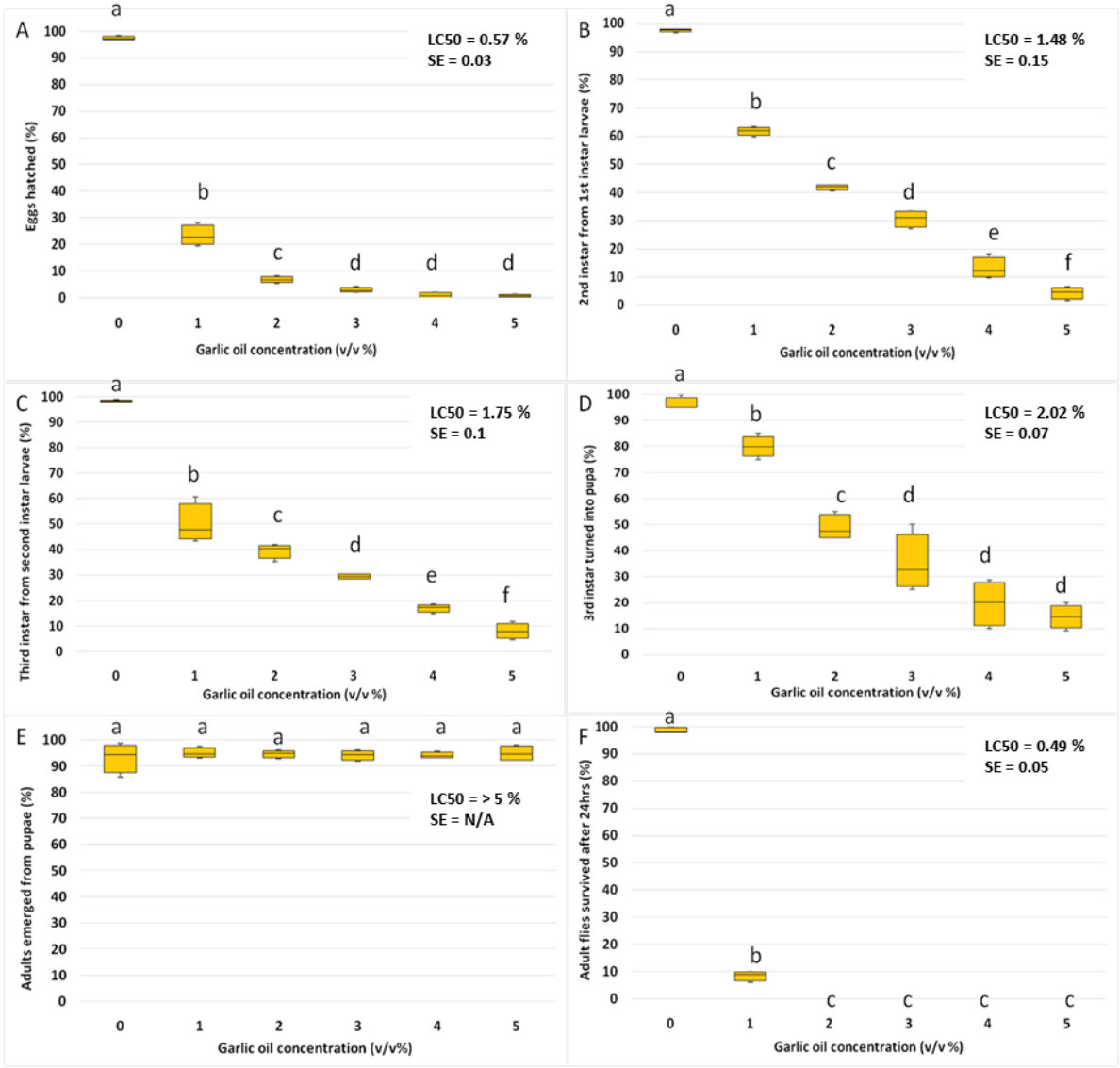


Figure 2.10: Insecticidal effect of China garlic oil with 3% TWEEN20 using spraying application. Insecticidal effect of China garlic oil with TWEEN20 on different stages of the *D. melanogaster* life cycle: A) Eggs, F (5, 18)=45.38, P=0.00025. B) 1st instar larvae, F (5, 18)=80.03, P=0.000061. C) 2nd instar larvae, F (5, 18)=209.13, P=0.0000056. D) 3rd instar larvae, F (5, 18)=78.15, P=0.000065. E) Pupae, F (5, 18)=1.69, P=0.19. F) Adults, F (5, 18)=793.16, P=0.0000002. The control used was 3% TWEEN20 (v/v). Boxes, lower/upper quartile; whiskers, minimum and maximum. SE= Standard error. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another (P < 0.05), n=4.

2.3.2.4 Insecticidal effect of R22 synthetic garlic oil

In the next assays, the insecticidal effect of synthetic garlic oil, R22 (Ecospray Ltd) was compared directly against natural garlic oils, using the spraying method. Eggs and adults were again most susceptible, with LC50s of 0.64% and 0.75%, respectively (figure 2.11). The 1st, 2nd, and 3rd instar larvae had LC50s of 0.98%, 1.31%, and 1.79%, respectively. The least susceptible life stage were the pupae with an LC50 >5%, again, the maximum concentration used. The results show that the synthetic garlic oil R22 had higher insecticidal activity than for Mexican or China garlic oil. The adults had a survival of 90% with the ethanol control, it is expected for some organisms to die of other causes than the garlic treatment. One possible reason for the 90% survival is application method in combination with total amount of adults. The higher number of adults the more likely some will be in close contact to the micropsrayer which could cause harm and lethality.

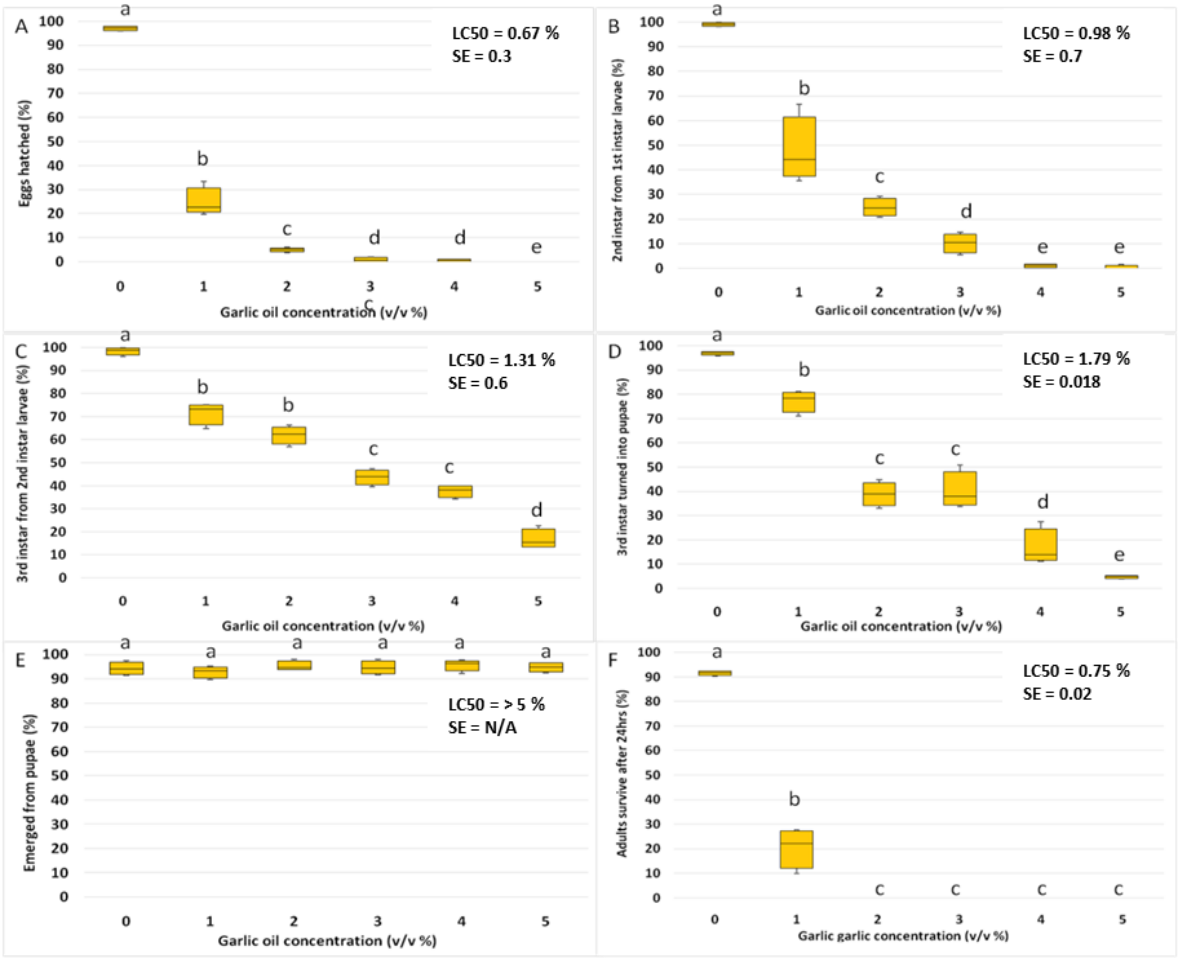


Figure 2.11: Insecticidal effect of R22, synthetic garlic oil, with ethanol. Insecticidal effect of R22 garlic oil with ethanol on different stages of the *D. melanogaster* life cycle: A) Eggs, F (5, 18)=491.52, P=0.0. B) 1st instar larvae, F (5, 18)=197.06, P=0.0000066. C) 2nd instar larvae, F (5, 18)=90.31, P=0.000045. D) 3rdinstar larvae, F (5, 18)=211.41, P=0.0000055. E) Pupae, F (5, 18)=0.62, P=0.79. F) Adults, F (5, 18)=547.53,

P=0.00000051. Ethanol was used as control. Boxes, lower/upper quartile; whiskers, minimum and maximum. SE= Standard error. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another (P < 0.05), n=4.

2.3.2.5 Insecticidal effect of garlic oil at low concentrations on eggs and adults

The previous experiments on *D. melanogaster* eggs and adults resulted in LC50 values below 1 % garlic oil. Due to the range of concentrations used, there could be inaccuracy in the LC50 results. Therefore, further insecticidal studies were performed on the eggs and adults of a concentration range between 0-1 % garlic oil (figure 2.12). China garlic oil and R22 garlic oil were used. Mexican garlic oil was not used as suppliers have ceased availability in the time of performing these experiments. From observing the results, it can be concluded that garlic oil has a greater effect on eggs compared to the adults in all treatments used.

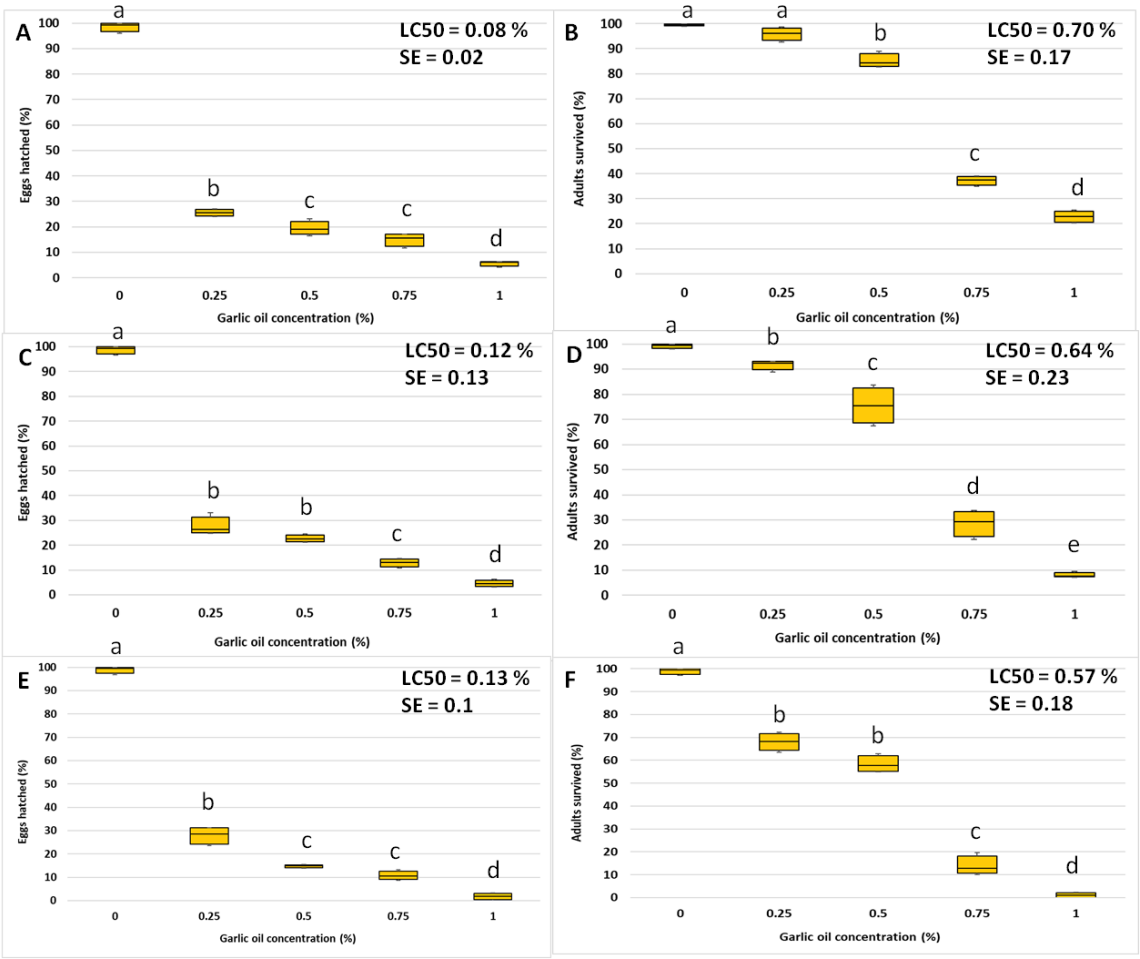


Figure 2.12: Insecticidal effect of low garlic oil low garlic oil concentrations, China garlic oil with 3 % TWEEN20 and R22, synthetic garlic oil, with ethanol. Insecticidal effect of garlic oil with the concentration range of 0-1 % (v/v) on the eggs and adult life stages of D. *melanogaster*: A) Eggs treated with China garlic oil with 3 % TWEEN20, F (5, 18)= 325.24, P=0.00000188. B) Adults treated with China garlic oil with 3 % TWEEN20, F (5, 18)=201.06, P=0.00000623. C) Eggs treated with China garlic oil with ethanol, F (5, 18)= 42.57, P=0.00029. D) Adults treated with China garlic oil with ethanol, F (5, 18)=211.41, P=0.0000055. E) Eggs treated with R22 with ethanol, F (5, 18)= 547.53, P=0.00000051. F) Adults treated with R22 with ethanol, F (5, 18)=247.53, P=0.00000371. Ethanol or 3 % TWEEN20 (V/V) was used as control. Boxes, lower/upper quartile; whiskers, minimum and maximum. SE= Standard error. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another ($P < 0.05$), n=4.

2.3.3 Non-volatile insecticidal effects

All garlic products contain volatile compounds and the extent to which this contributes to their insecticidal effects is not yet known. In the previous experiments garlic treatments were applied in a closed environment (in 30mm petri dishes) which may have maximised the potential for effects of volatiles. The next sets of studies were performed in an open environment to allow release and greater dispersion of volatile compounds, hence a greater contribution of non-volatile effects. The garlic products used were the same as in previous section: namely garlic extract, Mexican garlic oil, China garlic oil and R22. As with the previous insecticidal studies, the spraying method was employed for the application of the garlic extract solutions.

2.3.3.1 Non-volatile insecticidal effects of garlic extracts

The decreased opportunity for volatile effects in this experiment led to the loss of the lethal effect of garlic oil treatments, even at the highest concentration of 5% (v/v) in water (figure 2.13). This suggests that the volatiles in natural garlic extracts make a major contribution to their insecticidal activity. Additionally, garlic extract contains a small amount of diallyl polysulfides, some of which are volatile, that are the main components for the insecticidal activity of garlic. Therefore, the lower the diallyl polysulfide content the lower the insecticidal activity.

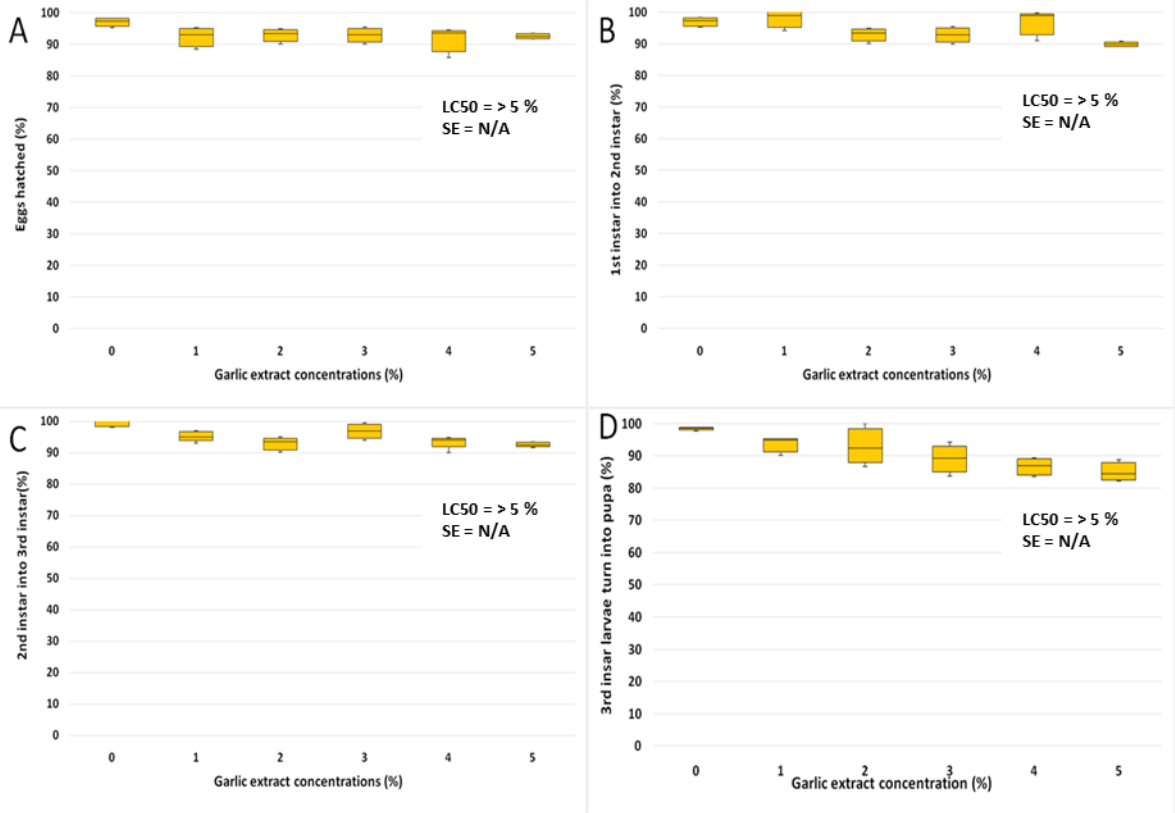


Figure: 2.13: Non-volatile insecticidal effect of garlic extract in water (v/v %). Insecticidal effect of garlic extract on different stages of the *D. melanogaster* life cycle: A) Eggs. B) 1st instar larvae. C) 2nd instar larvae. D) 3rd instar larvae; E) Pupae. F) Adults. Boxes, lower/upper quartile; whiskers, minimum and maximum. Statistical analysis was not performed, as no insecticidal effect was apparent.

2.3.3.2 Non-volatile insecticidal studies of Mexican garlic oil

In contrast to the garlic extract tests in the previous section, similar tests of the Mexican garlic oil in these non-volatile studies had comparable results to the initial insecticidal studies and showed a concentration-dependent effect on viability. Eggs were most sensitive to the Mexican garlic oil emulsified in ethanol and had a LC50 of 1.15% (figure 2.14). The 1st, 2nd, and 3rd instar larvae had LC50s of 1.23%, 1.41%, and 1.95%, respectively. Figure 2.14 shows the corresponding results with the alternative TWEEN20 carrier, in which eggs had a LC50 of 1.89%. The 1st, 2nd, and 3rd instar larvae had LC50s of 1.47%, 1.71%, and 2.1%, respectively.

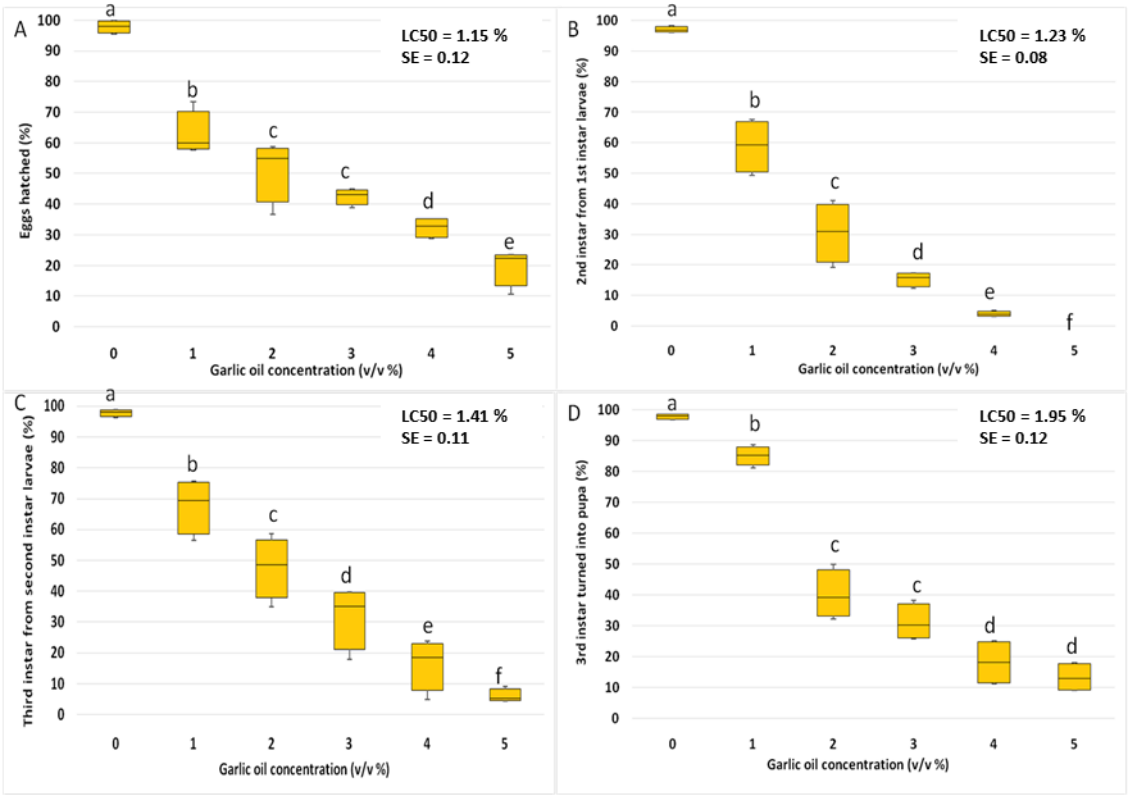


Figure 2.14: Non-volatile insecticidal effect of Mexican garlic oil with ethanol on different stages of the *D. melanogaster* life cycle: A) Eggs, F (5, 18)=76.34, P=0.000069. B) 1st instar larvae, F (5, 18)=275.47, P=0.0000028. C) 2nd instar larvae, F (5, 18)=243.49, P=0.0000039. D) 3rd instar larvae, F (5, 18)=162.22, P=0.000011. Ethanol was used as control. Boxes lower/upper quartile; whiskers, minimum and maximum. SE= Standard error. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another (P < 0.05), n=4.

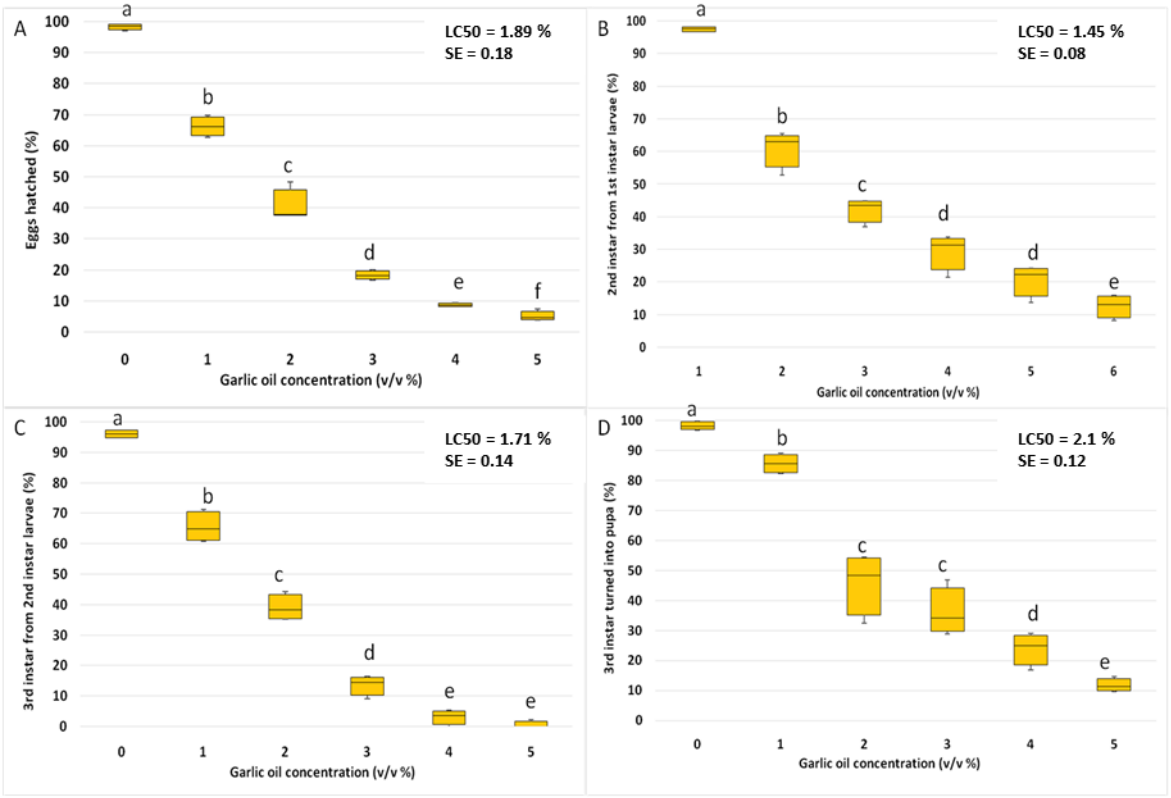


Figure 2.15: Non-volatile insecticidal effect of Mexican garlic oil with 3% TWEEN20 on different stages of the *D. melanogaster* life cycle: A) Eggs, F (5, 18)=84.92, P=0.000053. B) 1st instar larvae, F (5, 18)=215.32, P=0.0000053. C) 2nd instar larvae, F (5, 18)=83.46, P=0.000055. D) 3rd instar larvae, F (5, 18)=139.59, P=0.000015. The control used was 3% TWEEN20 (v/v). Boxes, lower/upper quartile; whiskers, minimum and maximum. SE= Standard error. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another (P < 0.05), n=4.

2.3.3.3 Non-volatile insecticidal effect of China garlic oil

The second natural garlic oil, China garlic oil, was also tested for its non-volatile insecticidal effects. China garlic oil in these non-volatile studies had comparable results to the initial insecticidal studies and showed a concentration-dependent effect on viability. The results were also comparable to the Mexican garlic oil non-volatile studies. The tests with the ethanol carrier showed that eggs had a LC50 of 0.67% and the 1st, 2nd, and 3rd instar larvae had LC50s of 0.74%, 2.15%, and 2.46%, respectively (Figure 2.16). The TWEEN20 carrier tests were similar, with eggs having a LC50 of 0.59% and the 1st, 2nd, and 3rd instar larvae had LC50s of 1.87%, 1.67%, and 2.77%, respectively (Figure 2.17).

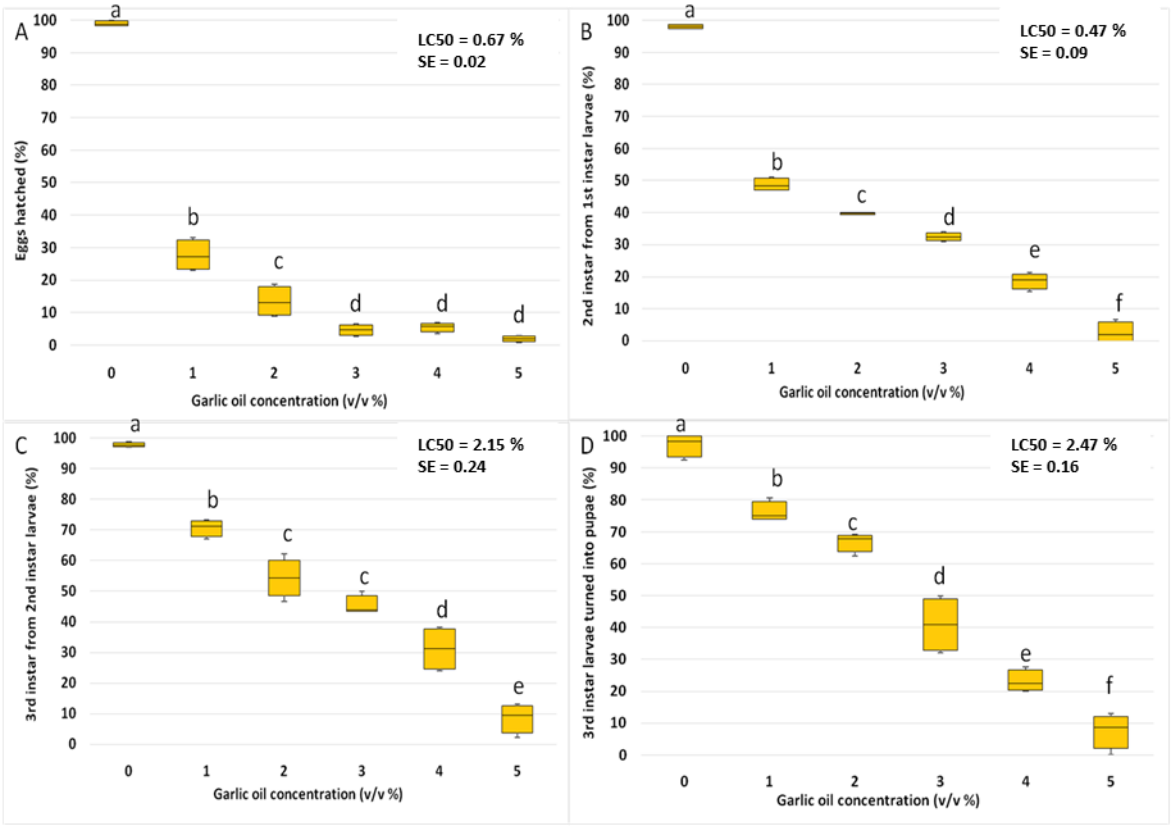


Figure 2.16: Non-volatile insecticidal effect of China garlic oil with ethanol on different stages of the *D. melanogaster* life cycle: A) Eggs, F (5, 18)=677.84, P=0.0000003. B) 1st instar larvae, F (5, 18)=191.5, P=0.000007. C) 2nd instar larvae, F (5, 18)=166.91, P=0.0000099. D) 3rd instar larvae, F (5, 18)=98.5, P=0.000029. Ethanol was used as control. Boxes lower/upper quartile; whiskers, minimum and maximum. SE= Standard error. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another (P < 0.05), n=4.

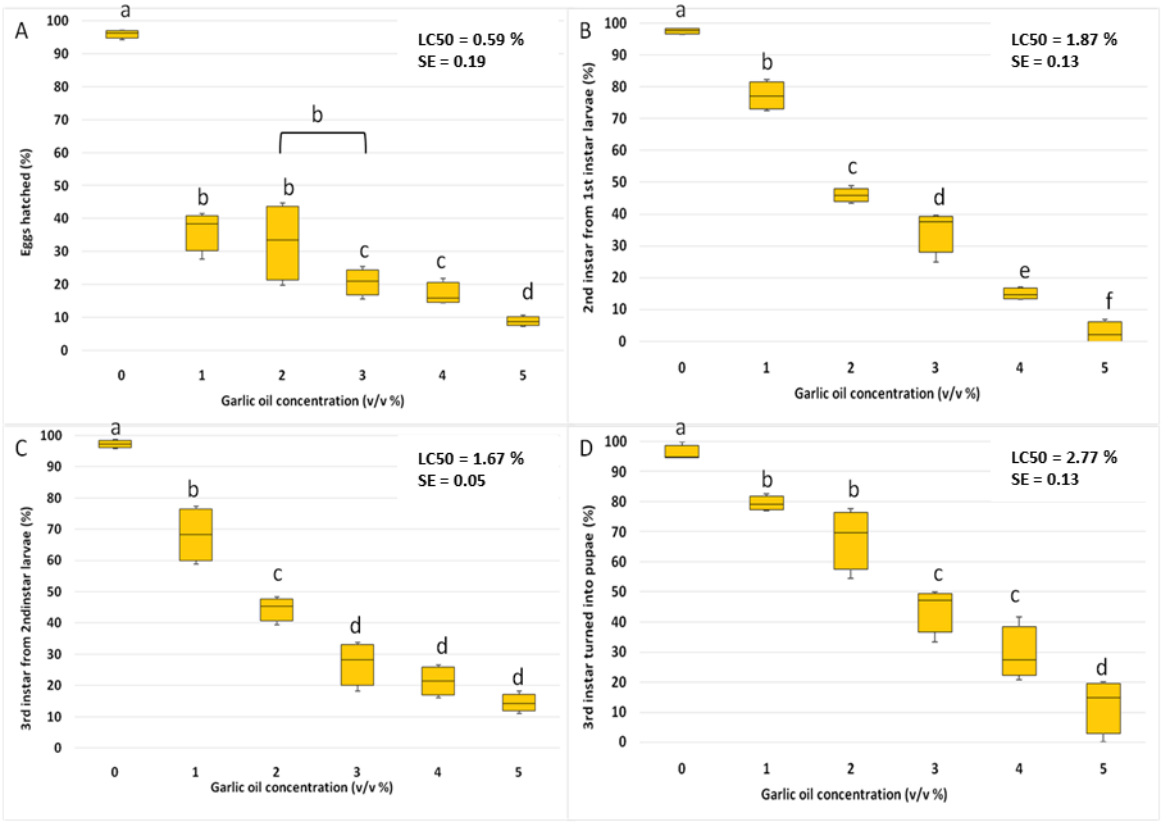


Figure 2.17: Non-volatile insecticidal effect of China garlic oil with 3% TWEEN20 on different stages of the *D. melanogaster* life cycle: A) Eggs, F (5, 18)=130.75, P=0.000018. B) 1st instar larvae, F (5, 18)=183.47, P=0.0000078. C) 2nd instar larvae, F (5, 18)=151.18, P=0.000013. D) 3rd instar larvae, F (5, 18)=60.17, P=0.00033. The control used was 3% TWEEN20 (v/v). Boxes, lower/upper quartile; whiskers, minimum and maximum. SE= Standard error. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another (P < 0.05), n=4.

2.3.3.4 Non-volatile insecticidal effects of synthetic R22 garlic oil

The non-volatile insecticidal effect of the synthetic garlic oil, R22 (Ecospray Ltd), was tested for comparison against natural garlic oils. Figure 2.18 shows the results with the ethanol carrier. Eggs had a LC50 of 1.15%, and the 1st, 2nd, and 3rd instar larvae had LC50s of 1.23%, 1.41%, and 1.95%, respectively. The results showed that even in an open environment, in which volatile effects were minimised, the synthetic garlic oil R22 had higher insecticidal activity than Mexican and Chine garlic oils. The magnitude of effects was similar to the initial insecticidal experiments conducted under closed conditions (see section 2.3.3, above).

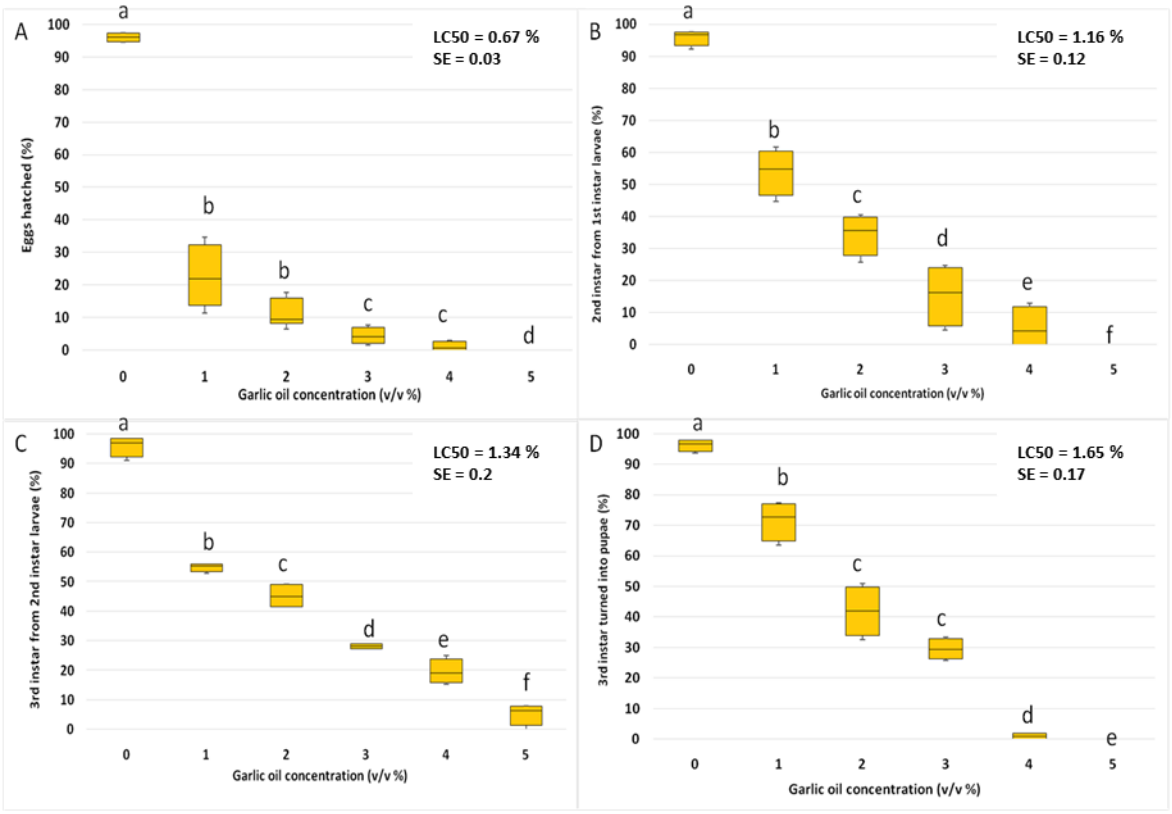


Figure 2.18: Non-volatile insecticidal effect of the synthetic garlic oil, R22, in ethanol carrier, on different life stages of *D. melanogaster*: A) Eggs, F (5, 18)=117.13, P=0.000024. B) 1st instar larvae, F (5, 18)=83.45, P=0.000055. C) 2nd instar larvae, F (5, 18)=127.96, P=0.000012. D) 3rd instar larvae, F (5, 18)=298.9, P=0.00023. Ethanol was used as control. Boxes lower/upper quartile; whiskers, minimum and maximum. SE= Standard error. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another (P < 0.05)., n=4

2.3.4 Insecticidal effect of garlic compounds delivered through fumigation

It was shown in the previous section that garlic products are very effective against *D. melanogaster* when the component volatile compounds are released. In this section it was tested whether the volatile compounds themselves are equally effective. This was done by conducting fumigations studies on all life stages of *D. melanogaster* as described in section 2.2.11, see section 6.4 for figures.

The fumigation test results were very similar to the original insecticidal studies and the non-volatile studies in which there was a concentration-dependent effect (Figure 2.19). This suggests that the volatiles from garlic products alone are sufficient to cause a lethal effect against *D. melanogaster*. The eggs and adults were the most susceptible, with LC50s of 0.46% and 0.49%, respectively. The 1st, 2nd, and 3rd instar larvae had LC50s of 0.89%, 1.69%, and 1.32%, respectively. In common with all the other experiments reported above, the least susceptible life stage was the pupae, with an LC50 >5%, which was the maximum concentration used. Table 2.2 shows the LC50 values obtained from using the fumigation method on *D. melanogaster* using R22 in the ethanol carrier. After performing the fumigation studies; it was also of interest to study the rate of lethality using this method on adult *D. melanogaster*. The fumigation method was applied and number of adults surviving was recorded at 30 minute intervals from the time of application. As expected, an increase in R22 garlic oil concentration caused lethality at a faster rate (Figure 2.20) apart from at the 30 min time point which could have been likely from technical error. Exposure of up to 2 hours was used instead of 24 hours because beyond 2 hours would not have given much more information. The focus was on the rate of which the garlic oil can cause lethality.

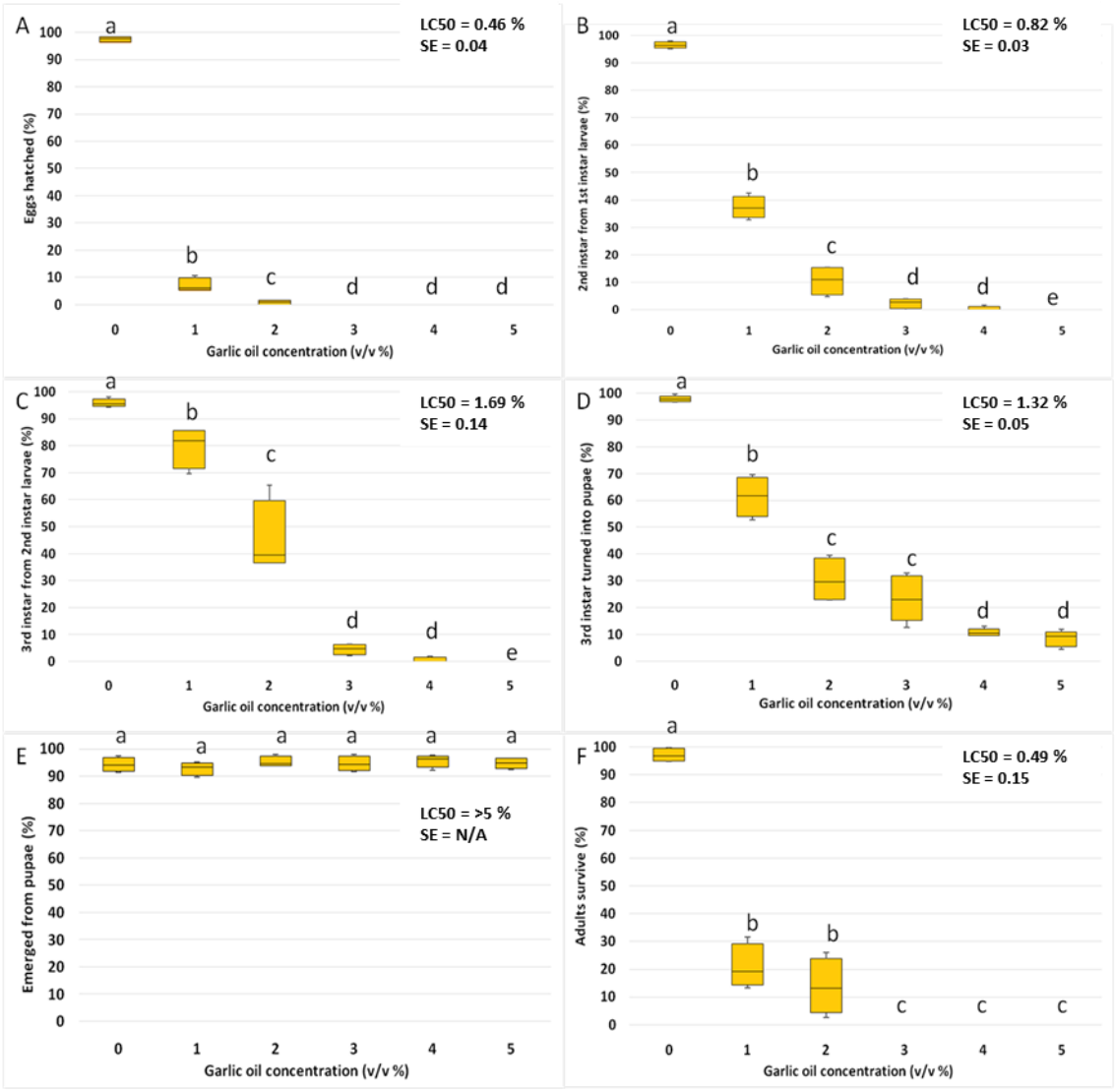


Figure 2.19: Fumigation effect of synthetic garlic oil, R22. Insecticidal effect of R22 garlic oil with ethanol on different stages of the *D. melanogaster* life cycle: A) Eggs, F (5, 18)= 853.33, P= 0.00000017. B) 1st instar larvae, F (5, 18)= 282.8, P= 0.0000027. C) 2nd instar larvae, F (5, 18)=211.81, P= 0.0000055. D) 3rd instar larvae, F (5, 18)= 90.15, P= 0.000046. E) Pupae, F (5, 18)= 0.62, P= 0.68. F) Adults, F (5, 18)=162.1, P= 0.000011. Ethanol was used as control. Boxes lower/upper quartile; whiskers, minimum and maximum. SE= Standard error. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another (P < 0.05), n=4.

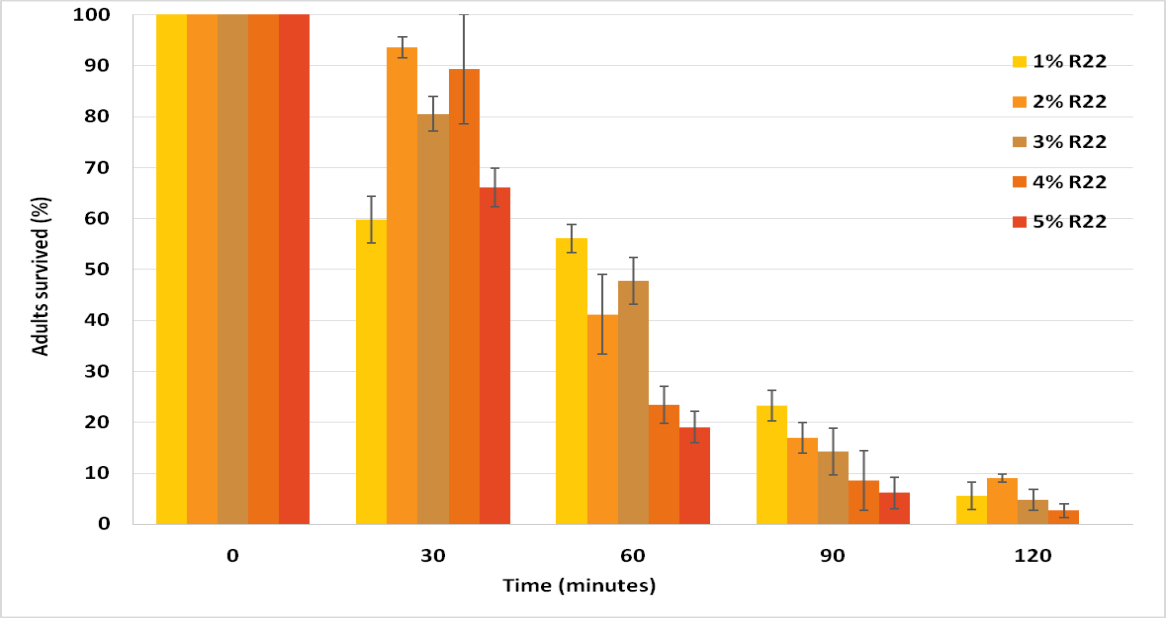


Figure 2.20: Timed fumigation effect on *D. melanogaster* adults. Figure shows that an increase in R22 garlic oil concentration leads to faster lethality. Therefore, the fumigation effect was concentration-dependent. Error bars show ±SE.

2.3.5 Comparison of garlic oil treatments and methods

All life stages of *D. melanogaster* were studied which includes the eggs, 1st, 2nd, 3rd instar larvae, pupae, and the adults. For the initial insecticidal experiments, the organisms were studied in a closed environment and a range of garlic extract/oil concentrations were used, between 0-5% (v/v). The LC50 for each experiment was calculated. Averages of these LC50 values were collected and are presented in Table 2.2 for comparison of values obtained in the different life stages. It is shown from the data that the eggs and adults are the most susceptible and the pupae is the least susceptible to the garlic treatments.

In the tests described above, four different methods were used to study the insecticidal effect of garlic products. All of these methods are analogous to applications in the field, i.e. direct contact with the garlic product in a closed environment, on surface (non-volatile) contact in which the organisms are in direct contact in an open environment so the volatile compounds can be released, and the fumigation delivery method in which only the volatile compounds from the garlic products come into contact with the insects. Table 2.3 shows the LC50s from these studies that used R22, which were highly comparable. This suggests that multiple; equally effective methods can be used for the insecticidal application of garlic products. Hence optimal methods could be adjusted according to differing field requirements.

Table 2.2: LC50s of garlic extract/oil insecticidal experiments. LC50±SE

GraFit LC50s (% ± SE)	Dipping/ pipetting	Spraying	Eggs	1st instar	2nd instar	3rd instar	Pupae	Adults
Garlic Extract	+		0.82 ± 0.09	2.17 ± 0.16	3.38 ± 0.13	2.55 ± 0.14		
		+	0.52 ± 0.17	2.74 ± 0.15	2.8 ± 0.15	3.81 ± 0.18	>5	>5
Mexican garlic oil with ethanol	+		0.15 ± 0.15	0.64 ± 0.1	1.42 ± 0.06	2.03 ± 0.2		
		+	0.23 ± 0.23	0.79 ± 0.01	1.99 ± 0.19	2.34 ± 0.1	>5	0.52 ± 0.03
Mexican garlic oil with 3% TWEEN20	+		0.57 ± 0.03	1.48 ± 0.15	1.75 ± 0.1	2.02 ± 0.07		
		+	0.45 ± 0.26	0.99 ± 0.12	1.13 ± 0.18	1.61 ± 0.17	>5	0.49 ± 0.05
China garlic oil with ethanol	+		0.54 ± 0.05	1.87 ± 0.26	1.42 ± 0.12	2.43 ± 0.21		
		+	0.62 ± 0.05	1.25 ± 0.26	1.72 ± 0.03	1.76 ± 0.25	>5	0.29 ± 0.11
China garlic oil with 3% TWEEN20	+		0.19 ± 0.15	1.12 ± 0.05	1.46 ± 0.08	2.59 ± 0.18		
		+	0.37 ± 0.05	0.56 ± 0.05	1.19 ± 0.17	2.84 ± 0.05	>5	0.26 ± 0.13
R22-TMR3 with 3% Tween		+	0.67 ± 0.3	0.98 ± 0.7	1.31 ± 0.6	1.79 ± 0.18	>5	0.75 ± 0.02

Table 2.3: LC50s of R22 (% v/v) on D. melanogaster life stages to compare the different application methods, showing LC50±SE

	On organism spraying	On-surface spraying	Fumigation**	Non-volatile
Eggs	0.67 ± 0.03	0.67 ± 0.03	0.46 ± 0.04	0.67 ± 0.03
1st instar larvae	0.93 ± 0.07	1.15 ± 0.12	0.82 ± 0.03	1.16 ± 0.12
2nd instar larvae	1.31 ± 0.16	0.71 ± 0.06	1.69 ± 0.14	1.34 ± 0.2
3rd instar larvae	1.79 ± 0.18	1.63 ± 0.17	0.82 ± 0.05	1.64 ± 0.17
Pupae	>5*	>5*	>5*	N/A
Adults	0.75 ± 0.2	N/A	0.49 ± 0.15	N/A

* No kill effect observed at this maximum concentration that was tested

** Equivalent to 11 μL of neat garlic oil in 810 cm^3

2.3.6 Insecticidal effect of R22 with copper

Copper is well known for its antibacterial and antifungal properties (Vincent, Hartemann et al. 2016), and it can also cause toxicity in *D. melanogaster*(Hwang, de Bruyne et al. 2014). Copper can have a synergic effect when used in combination with insecticides (Bindesbol, Holmstrup et al. 2005; Cedergreen 2014; Vaidya, McBain et al. 2017). Hence, it was also of interest to determine whether copper would synergise of the insecticidal effect of R22 garlic oil. Following successful studies performed by Ines Kollo in the Hamilton lab (UEA) of the nematicidal effect of garlic oil, R22 with copper sulfate ($CuSO_4$) it was decided to test this same formulation against *D. melanogaster*, to test whether copper would increase the insecticidal effect of R22. The concentrations used are shown in table 2.4. The concentrations used were based on HPLC experiments by Ines Kollo and Dr Ryan Tinson (UEA) where $CuSO_4$ was continually added to the R22 garlic oil samples until a linear result was obtained. The same concentrations were used in this experiment against D. melanogaster. Dr Tinson also performed microscopy of the R22 and $CuSO_4$ which showed crystallized formations, suggesting a relationship between diallyl polysulfides and $CuSO_4$. There is evidence by Salivon and Shkurenko 2007of the formation of crystals when combining copper with diallyl trisulfide.

Table 2.4: Concentrations of R22 garlic oil and $CuSO_4$used in insecticidal tests.

R22 (v/v%)	$CuSO_4$ (mg/ml)
1	0.054
2	0.108
3	0.168
4	0.216
5	0.27

Before the studies with R22, it first needed to be established if $CuSO_4$ had any insecticidal effect alone (figure 2.21). The concentrations of $CuSO_4$that were to be used with R22 garlic oil were tested against the eggs and the three larval stages of *D. melanogaster*. Figure 2.21 show that $CuSO_4$ at these concentrations did not cause any detectable insecticidal effect against *D. melanogaster*.

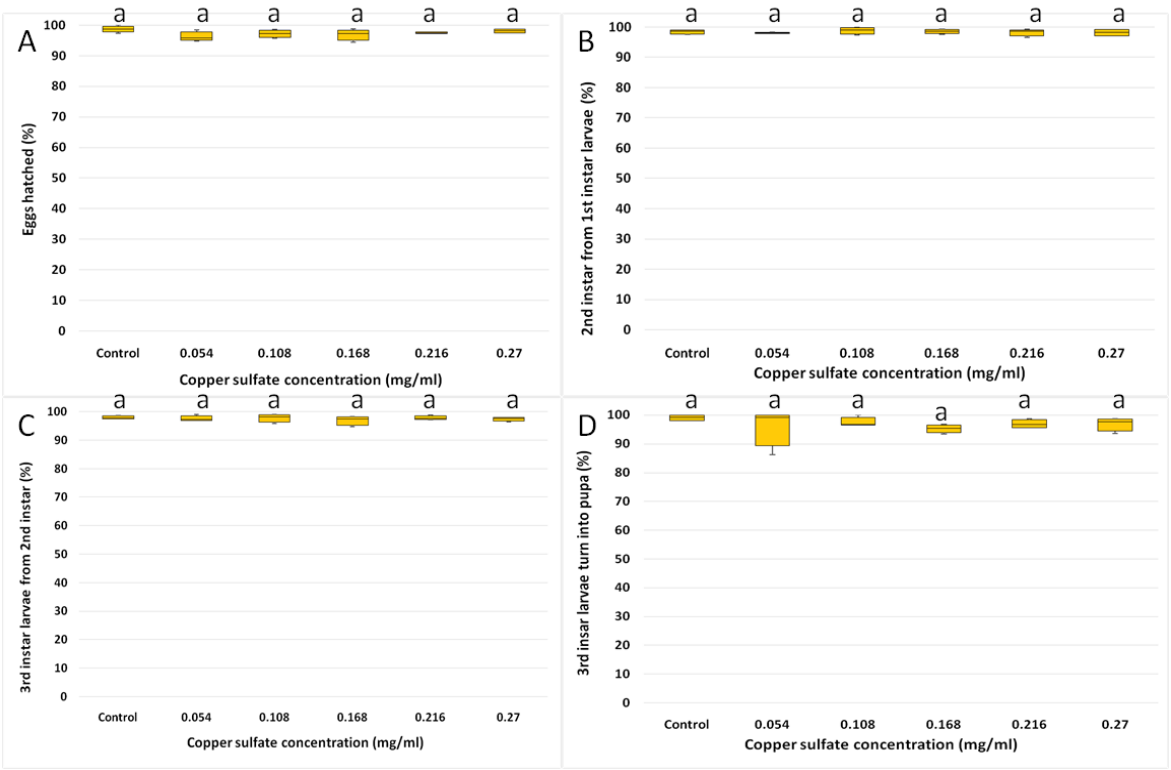


Figure 2.21: Insecticidal effect of $CuSO_4$ against *D. melanogaster*. Concentrations of $CuSO_4$ were 0.054, 0.108, 0.168, 0.216, and 0.27 mg/ml. A) Eggs, F (5, 18)= 2.12, P=0.11. B) 1st instar larvae, F (5, 18)=0.65, P=0.67. C) 2nd instar larvae, F (5, 18)=0.44, P=0.82. D) 3rd instar larvae, F (5, 18)=1.13, P=0.38. Ethanol was used as control. Boxes lower/upper quartile; whiskers, minimum and maximum. SE= Standard error. Differing letters (a, b, c, d, e, f) were significantly different from one another ($P < 0.05$), n=4.

Figure 2.22 shows the results of the R22 and $CuSO_4$ experiments. The R22 garlic oil and $CuSO_4$ had strong insecticidal activity against *D. melanogaster*. The LC50 of R22 against the eggs was 0.50%, 1st instar larvae was 0.71%, 2nd instar larvae was 0.58%, and 3rd instar larvae was 0.57%. Therefore, all life stages of *D. melanogaster* had very similar LC50 results. When comparing these results to the R22 garlic oil assays there was a notable difference - $CuSO_4$ did appear to increase the insecticidal effect of R22 against *D. melanogaster* (table 2.5).

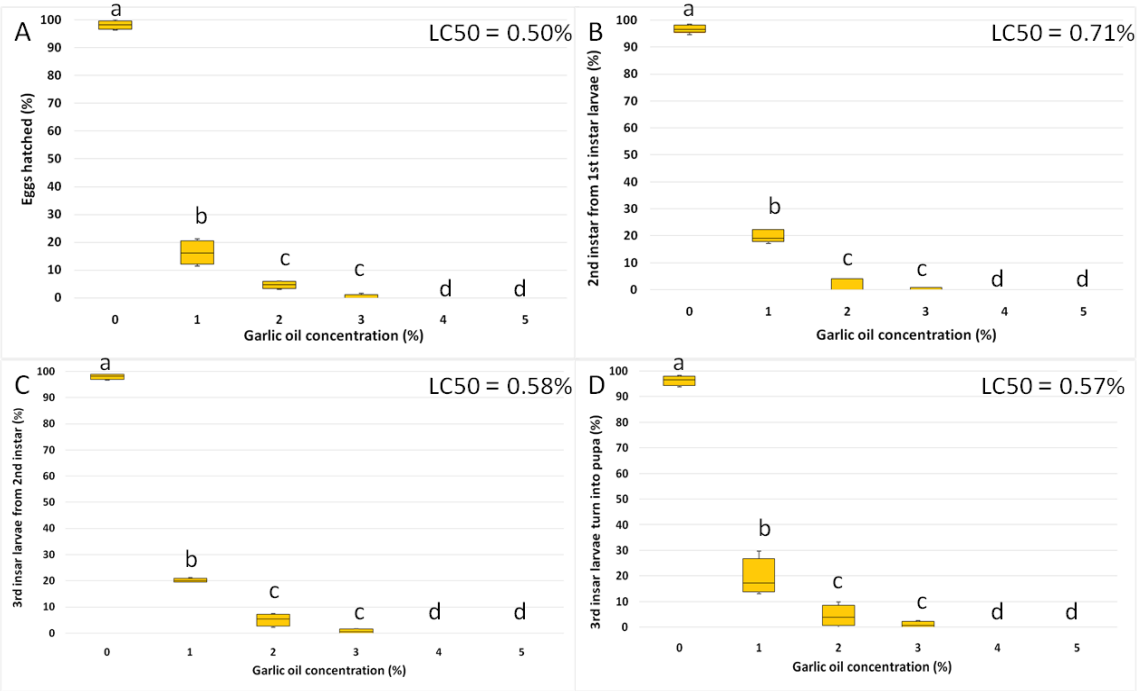


Figure 2.22: Insecticidal effect of R22 garlic oil (v/v %) with $CuSO_4$ against *D. melanogaster*. Insecticidal effect on different stages of the *D. melanogaster* life cycle: A) Eggs, F (5, 18)=503.25, P=0.00000063. B) 1st instar larvae, F (5, 18)=369.55, P=0.0000014. C) 2nd instar larvae, F (5, 18)=772.4, P=0.00000022. D) 3rd instar larvae, F (5, 18)=195.14, P=0.000067. Ethanol was used as control. Boxes lower/upper quartile; whiskers, minimum and maximum. SE= Standard error. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another (P < 0.05), n=4.

Table 2.5: LC50s of R22 and R22 with $CuSO_4$ against *D. melanogaster* ±SE

Life stage	R22 (v/v %)	R22 + $CuSO_4$ (v/v %)
Eggs	0.67 ± 0.03	0.50 ± 0.06
1st instar larvae	0.98 ± 0.12	0.71 ± 0.01
2nd instar larvae	1.31 ± 0.20	0.58 ± 0.05
3rd instar larvae	1.79 ± 0.17	0.57 ± 0.05

2.3.7 Insecticidal effect of garlic oil with beta-cyclodextrins

One problem that makes the use of garlic-derived polysulfides unfavourable to use in agriculture is the potent garlic odour. The odour can be very unfavourable when it comes for crop growers, especially fruit growers and may also reduce the effectiveness of garlic-based insecticides if insects are also repelled. Although thorough washing would relieve some of this problem, the residual diallyl polysulfides that remain on the crops could still impair taste and odour. Therefore, it was tested here whether it was possible to mask the odour of diallyl polysulfides by encasing them in β-cyclodextrins, in a 1 in 10 solution. This step of the experiments was performed by a colleague (Dr Ryan Tinson, Hamilton lab, UEA). Four different treatments were used; β-cyclodextrin (control), Mexican garlic oil with β-cyclodextrin, China garlic oil with β-cyclodextrin, and R22 garlic oil β-cyclodextrin. Since it is a 1 in 10 solution, concentrations of 10-50 % (w/v) of the garlic oil/β-cyclodextrin mixture were used to give the final concentrations of 1-5 % (v/v) of garlic oil, as used in the previous experiments. The results showed that none of the treatments gave any insecticidal effects against the 3rd instar larvae of *D. melanogaster* (Figure 2.23).

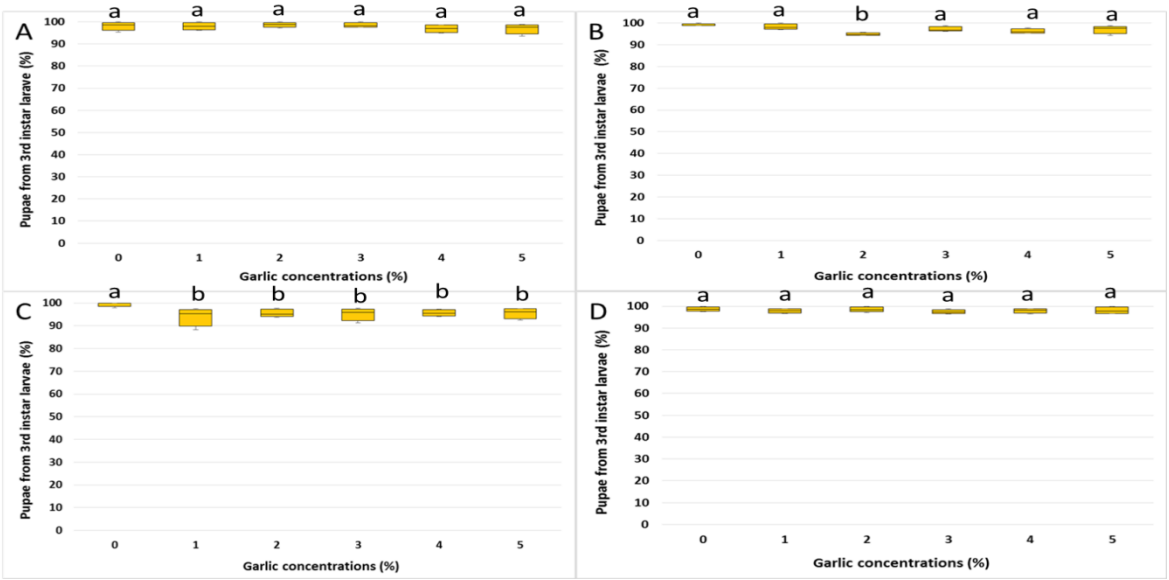


Figure 2.23: Insecticidal effect of polysulfides from garlic oil with β-cyclodextrin. A) shows β-cyclodextrin with garlic oil as a negative control, F (5, 18)=0.71, P= 0.626. B) shows β-cyclodextrin with Mexican garlic oil, F (5, 18)=4.34, P= 0.009. C) shows β-cyclodextrin with China garlic oil, F (5, 18)=5.99, P= 0.002. D) shows R22 with β-cyclodextrin, F (5, 18)=0.63, P= 0.68. All treatments had no insecticidal effect on 3rd instar larvae *D. melanogaster*. Boxes, lower/upper quartile; whiskers, minimum and maximum. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another (P < 0.05), n=4.

2.3.8 Insecticidal effect of diallyl polysulfides

In order to study the insecticidal activity of the individual diallyl polysulfides they were first isolated via HPLC from the synthetic garlic oil R22. A 10% (v/v) was used (figure 2.24) with purities between 90-99%. Isocratic elution at a rate of 1.5 mL/ min using 90% (v/v) methanol provided separation of diallyl polysulfides within 17 min using a C18 column (250 x 4.6 mm). Detection was performed through UV absorption at 210 nm. The diallyl polysulfides were dried and kept at -20°C until use. DAS2-5 were used for the insecticidal experiments, due to poor yield for DAS1 and DAS6. The latter diallyl sulfide is also very unstable and it is difficult to obtain samples with sufficient purity.

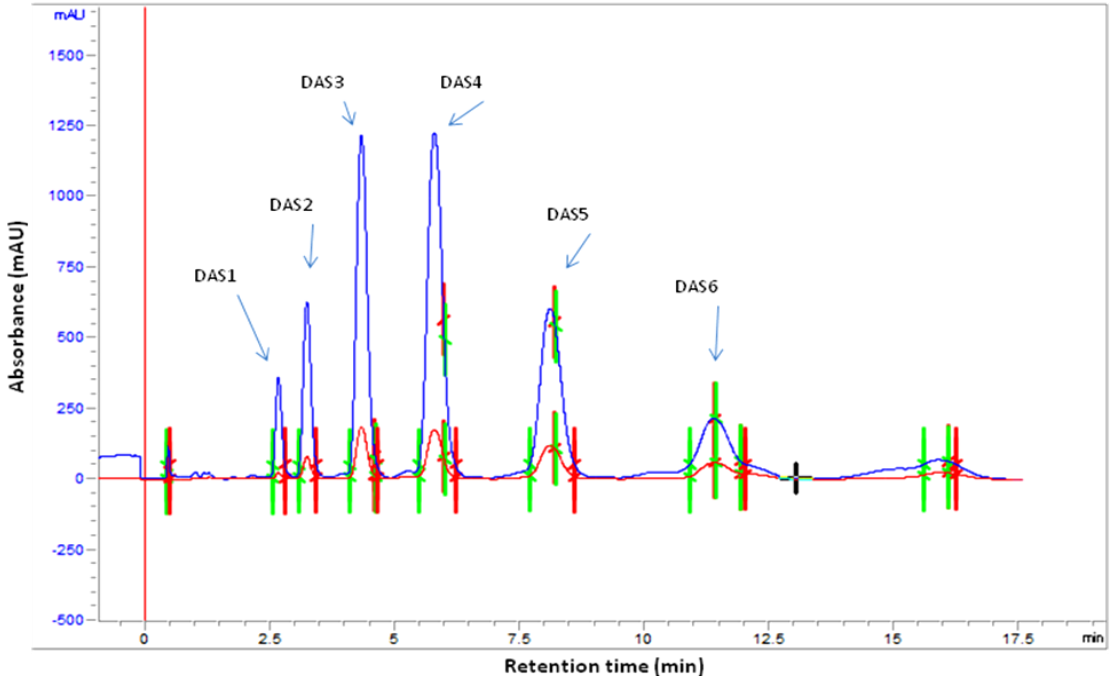


Figure 2.24: HPLC trace for the isolation of DAS1-6 from the synthetic garlic oil R22. A 10% (v/v) R22 solution was used for the extraction. Isocratic elution of R22 garlic oil was performed at a flow-rate of 1.5 mL/ min using 90% (v/v) MeOH. This provided good separation of DAS1-6 through a C18 column (250 x 4.6 mm) within 20 min. From the isolated diallyl polysulfides, DAS2-5 were used for the insecticidal experiments as the DAS1 and DAS6 did not give sufficient experimental amounts. Additionally, DAS6 is very unstable. The green line is the start of a fraction collection, and the red is the end

The experiments to test the insecticidal effects of diallyl polysulfides were performed using the spraying method in order to cover the surface of the insects and to test for surface contact activity. Insecticidal experiments of the DASs (DAS2-5) were performed on *D. melanogaster* eggs and the three larval stages (L1, L2 and L3 larval instars). Ethanol was used as a control. A 2% (w/v) solution in ethanol of DAS was used for each experiment; final concentrations of the DASs are shown in table 2.6. Figure 2.25 shows survival of the organisms 24 hours after spraying. For all life stages tested, DAS4 was the most effective compared to the other DASs and the control. This was unexpected considering the longer the sulfur chain, the more reactive the compounds (Jacob, Kirsch et al. 2014). Therefore, DAS5 was predicted to have the highest insecticidal activity.

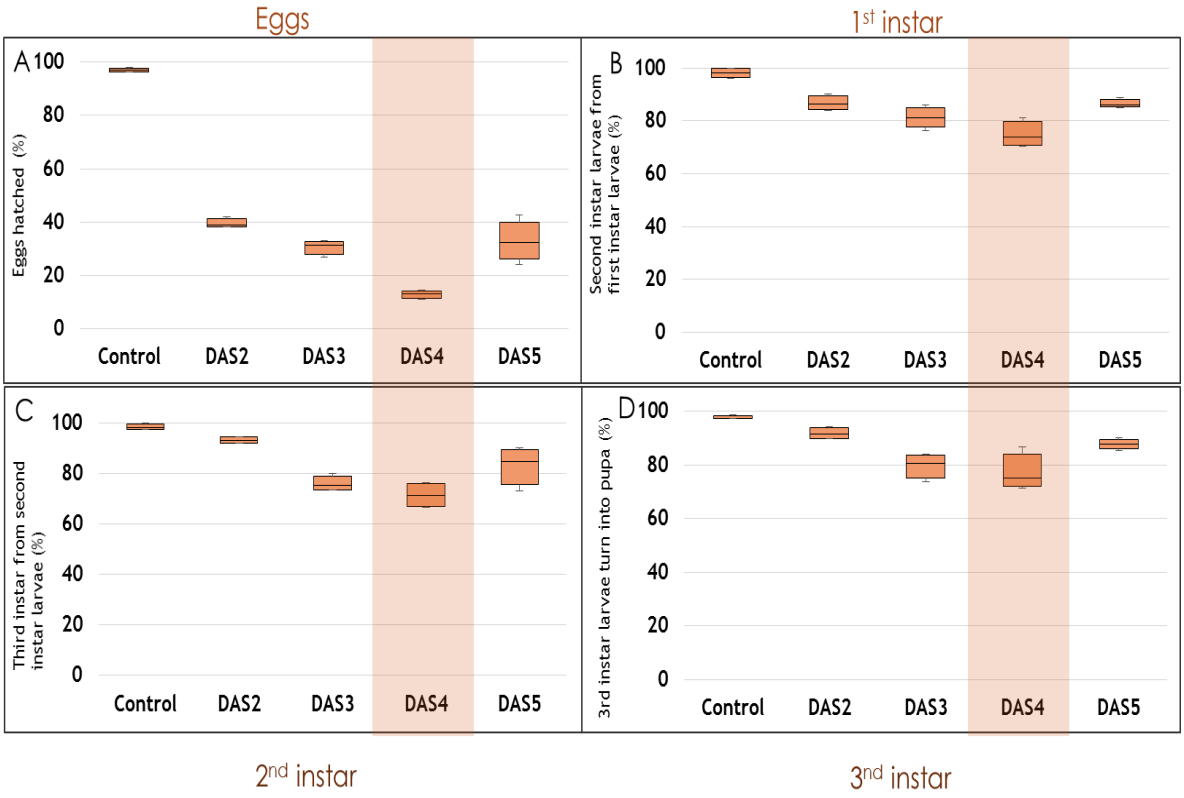


Figure 2.25: The insecticidal effect of DAS2,3,4 and 5 on % viability of *D. melanogaster*. A) eggs, B) 1st instar, C) 2nd instar and C 3rd instar larvae. A 2% (w/v) concentration was used for each DAS and the spraying method was used. Boxes, lower/upper quartile; whiskers, minimum and maximum, n=4.

The concentrations of DAS1-5 were calculated from a 5 ml 2% (w/v) solution as shown in table 2.5. These were calculated after isolated from synthetic R22 garlic oil by using HPLC data of known concentrations of synthetic polysulfides that were performed by Ecospray Ltd. The concentrations differ due to the molecular weight of the compounds, depending on the length of the sulfur chain. This may or may not affect the insecticidal activity of the diallyl polysulfide. Final concentrations can also differ if compounds are left at room temperature as these compounds are very reactive, especially the longer sulfur chained diallyl polysulfides (Jacob, Kirsch et al. 2014). For example, if isolated DAS6 were to be left at room temperature, the amount of DAS6 in the solution would decrease and there would be an increase in DAS1-5 in the same solution (Arbach 2014). The DAS compounds isolated were stored at -20°C.

Table 2.6: Molecular weights (MW) and concentrations (μM) per 5ml 2% w/v working solution of diallyl polysulphides (DAS) 1-6 used in the insecticidal experiments

DAS	MW (g)	Concentration (μM)
DAS1	114.20	175
DAS2	146.28	137
DAS3	178.33	112
DAS4	210.39	95
DAS5	242.47	83
DAS6	274.53	73

Table 2.5

2.4 GARLIC SMOKE GENERATOR APPLICATION

A potential application method of diallyl polysulfides is the use of smoke generators as they can cover a large area and are simple to use. The garlic smoke generators were produced by Octavius Hunt and were conducted in a 5m x 2m x 2m polytunnel as shown in figure 2.26. Outside temperatures were of 19-25℃ and with little cloud cover. The flies were kept at approximately 1 foot away from the smoke generators. Smoke generator samples provided were: B- Blank, G1- Garlic extract (~3% DAS3), G2- Garlic extract with 9% DAS3, G3- Garlic extract with 9% DAS3 with boxed organisms elevated, and G4- Garlic extract with 9% TPS and 4.5% TWEEN. These were tested against adult D. melanogaster in plastic boxes, as shown in section 6.4. Three trials of the smoke generators were conducted. The first was concentrated on the garlic smoke generators (figure 2.27), the second with the blanks that contained the same ingredients but without any garlic products (figure 2.28), and third was a mixture (figure 2.29). Figure 2.28 shows 'controls' and 'blanks'; the controls were completely untreated where they were not to expose to any smoke. The 'blanks' were exposed to garlic-free smoke generators. Many smoke generators were provided by Octavius Hunt; however, many did not ignite, in which we could not use. Therefore, the number of smoke generators that we could use was limited. This led to inconsistencies in the results, with some smoke generators igniting for a short time and others for ~10 min. These trials were conducted over an 8-month span; results could be partially influenced by weather conditions. Due to the limited amount of usable smoke generators, the same samples could not be used for each trial; apart from G1 which contained garlic extract with 9% DAS3 were tested during each trial. GI was effective during the first trial; and was one of the very few samples that would ignite.

Figure 2.26: Images of the garlic smoke generator trail inside the polytunnel. Images show samples placed at approximately 1 foot away from smoke generator.

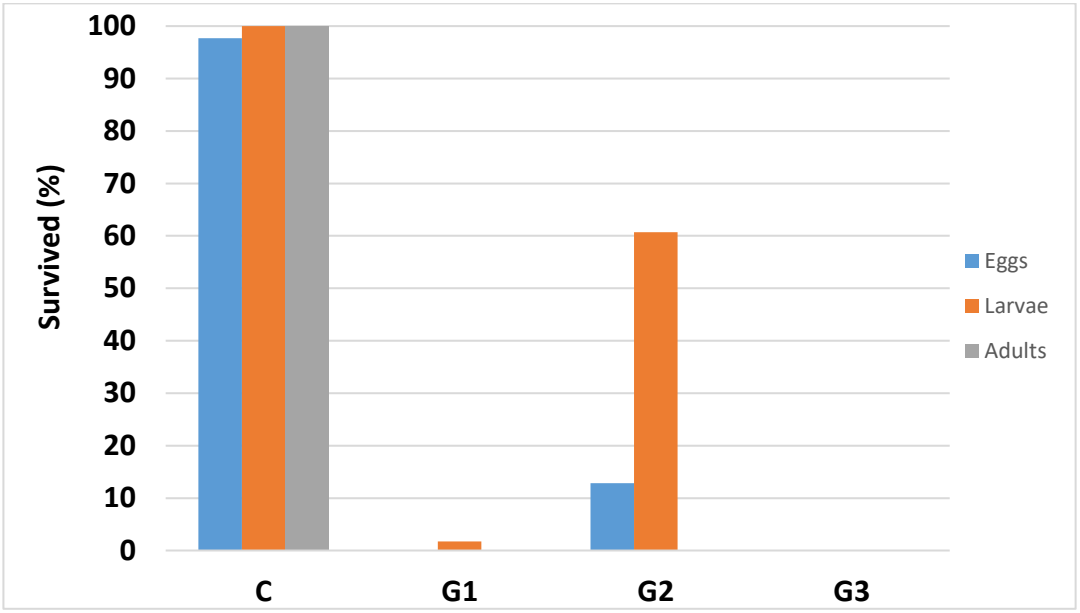


Figure 2.27: First trial of the insecticidal effect of garlic smoke generators against eggs, larvae and adult *D. melanogaster*. C- Control, G1- Garlic extract with 9% DAS3, G2- garlic extract with 9% TPS after 10 min, G3- Garlic extract (~3% DAS3)

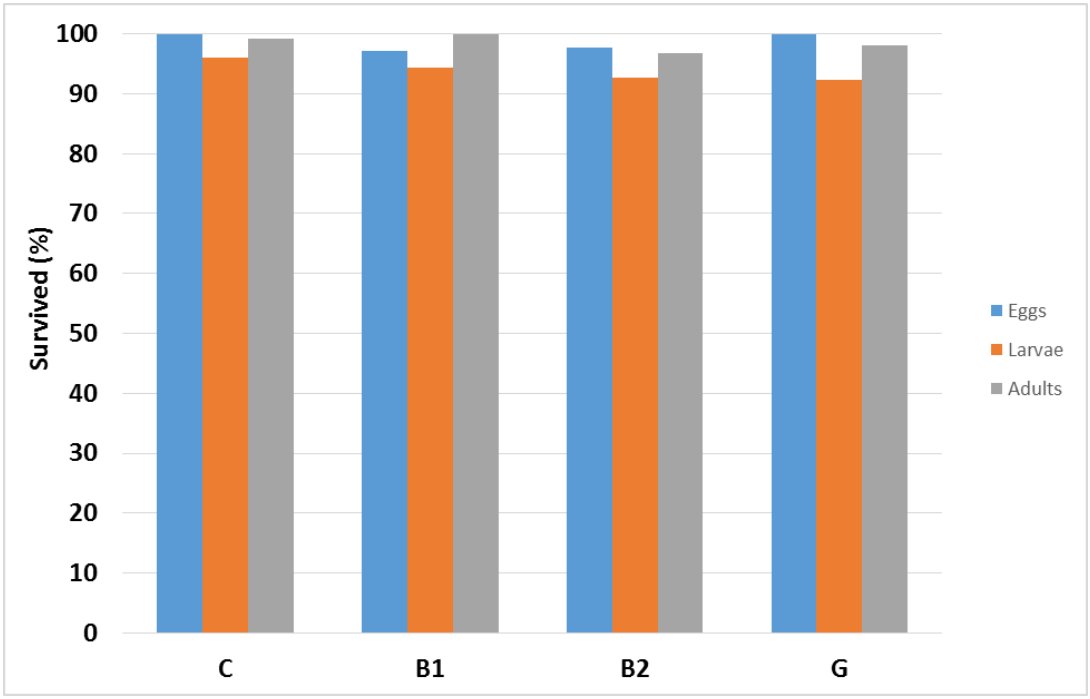


Figure 2.28: Second trial of the insecticidal effect of garlic smoke generators against eggs, larvae and adult *D. melanogaster*. C – Control, B1- Blank, B2 – Blank after 10 min, G- Garlic extract with 9% DAS3.

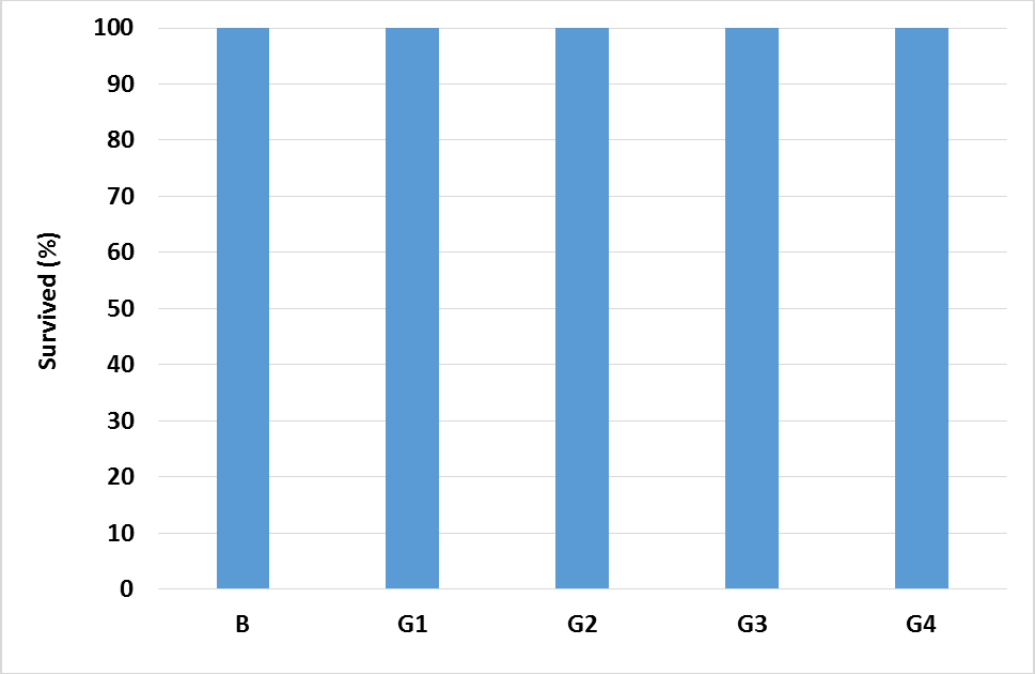


Figure 2.29: Third trial of the insecticidal effect of smoke generators against adult *D. melanogaster*. B- Blank, G1- Garlic extract (~3% TPS), G2- Garlic extract with 9% DAS3, G3- Garlic extract with 9% DAS3 with boxed organisms elevated, G4- Garlic extract with 9% DAS3 and 4.5% TWEEN20 with boxed organisms in very close proximity to smoke generator.

2.5 DISCUSSION

This chapter reports a comprehensive set of assays to determine the insecticidal properties of garlic products (garlic extract, natural garlic oils and synthetic garlic oil). *Drosophila melanogaster* was used as the model for these studies. The synthetic garlic oil, R22, had the greatest insecticidal effect compared to Mexican and China garlic oil and garlic extract. This is due to the garlic oils containing a higher proportion of longer chained diallyl polysulfides. R22 had a higher proportion of DAS3 of almost twice, shown in figure 2.1. DAS4 was the most effective diallyl polysulfide against *D. melanogaster* eggs and larvae (Figure 2.25). Garlic extract has good insecticidal properties that are comparable to the garlic oils, however, garlic extract consists of multiple different compounds that could facilitate its effects to that of the garlic oils which consists of just diallyl polysulfides.

The use of the microsprayer for the insecticidal studies gave a greater insecticidal effect with better consistency than the other methods used. This must be due to the microsprayer covering a greater area of the grape juice agar plates with the garlic solution as well as coating the eggs/larvae. Additionally, there was decreasing insecticidal effect from the egg to the third instar larval stage. This decreasing sensitivity could be due to the garlic having less of an effect on larger on more developed organisms. Ethanol was used to emulsify the garlic oil; however, ethanol can have an ovicidal effect but no such toxic effects were observed at the other life stages. This is because during the life cycle, the organisms become increasingly tolerant to the ethanol. The survival of *D. melanogaster* with ethanol is age-dependent (Bijlsma-Meeles 1979). Due to the ovicidal effect of ethanol, TWEEN20 was tested (Figure 2.4) and it was determined that it did not have ovicidal activity. Therefore, TWEEN20 was also used to emulsify the garlic oil and was tested separately with the garlic oils. It was used at a concentration of 3 % with water (which is comparable to the commercialized garlic products used by Ecospray Ltd).

The adult flies came from an outbred and mixed population but that the cohorts used for the insecticidal tests had been subjected to standardised culturing and collecting conditions to more easily detect the effects of the different treatments and to minimise any effects of random environmentally derived variation (e.g. body size variation, etc). The rationale was to use mixed sex cohorts of both sexes for the assays and this gives best field relevance,

where groups of individuals of both sexes are likely to be targeted by insecticides. Though each sex might be differentially susceptible to insecticides, this will not have any confounding effect on the investigations but could be interesting to investigate in the future. In addition, other traits such as fertility could be tested in the future, as an insecticide that is sterilising (but not lethal) might still be useful (though crops can still be damaged if females are trying to lay sterile eggs). Hence it is survival that is the most important indicator for these insecticidal studies, and therefore it was the trait upon which this thesis focused in the experiments.

For the insecticidal experiments, four different garlic products were studied including garlic extract, the natural garlic oils (Mexican and China), and the synthetic garlic oil R22. All life stages of *D. melanogaster* were studied which includes the eggs, 1st, 2nd, 3rd instar larvae, pupae, and the adults. For the initial insecticidal experiments, the organisms were studied in a closed environment and a range of garlic extract/oil concentrations were used, between 0-5% (v/v). Both garlic extract and garlic oils caused a concentration-dependent insecticidal effect on all *D. melanogaster* life stages tested (figures 2.4-2.10). A repellent effect was also observed for all garlic products where at least 50% of the larvae would try and get away from the garlic. It would be great to investigate this repellent effect further, such as using food assays.

The reduction of egg hatching shown in the insecticidal studies could be due to either direct contact with the garlic extract/oil solution or due to its volatile compounds. It has been demonstrated that the vapours from garlic have an insecticidal effect (Bhatnagar-Thomas and Pal 1974; Ekesi 2000). For example, Elkesi et al. (2000) used garlic extract at 5, 10, and 15 % (w/v) and found it to be effective against egg viability of *M. vitrata* and *C. tomentosicollis*. Ovicidal activity could also occur due to prevention of larval eclosure (emergence of the larva from an egg) where the larvae is fully formed (Jarial 2001).Jarial et al. (2001) found that *Aedes* eggs in deionized water undergo complete fracture producing free shell caps, but in garlic extract (6 %) only partial fracture was observed and the larvae remained enclosed in the shell. It has been suggested that garlic extract could strengthen the egg structure preventing it from hatching with fully developed embryos (Neveu, Grandgirard et al. 2002). Therefore, this suggests that garlic inhibits egg hatching

due to difficulties of breaking the eggs during larval eclosure. A mechanism for this has not been suggested, therefore, further investigation into the hardening of the shell with garlic treatment would be useful. Jarial et al. 2001 showed that the garlic extract treatment not only prevented shell fractures but only a small section of the exochorion was sloughed off and the large papillae displayed aeropyles.

Additional insecticidal experiments were performed on *D. melanogaster* eggs and adults. Due to the range of concentrations used of 0-5 % (v/v) garlic oil, there could be inaccuracy in the LC50 results that were obtained since the values were below 1 % garlic oil. Therefore, a concentration range between 0-1 % (figure 2.12) China garlic oil and R22 garlic oil were used. Mexican garlic oil was not used as it is no longer available. In the previous experiments, the results suggested similarity in the susceptibility to insecticidal activity between eggs and adults. However, from observing the results from the 0-1 % garlic oil studies, it can be suggested that the eggs are the most susceptible life stage of *D. melanogaster*. When comparing the garlic oils, there was little difference for the eggs and with the adults R22 synthetic garlic oil had the greatest effect.

The non-volatile experiments were more relevant to field application scenarios due to the open environment. This scenario simulates the target pest organism coming into contact with crop that has already been treated with garlic. The garlic treatments were still very effective and gave similar results to the spraying method (section 2.3.3). Therefore, contact only application with the release of volatile compounds provided effective control.

Table 2.3 shows the LC50 results from using the fumigation method. This was to study the fumigation toxicity effect of garlic oil. The synthetic garlic oil, R22, was used because this is the current garlic treatment being developed by Ecospray Ltd. The results showed that the volatiles alone were sufficient to cause an insecticidal effect. Garlic oil has been known for its success in fumigation studies, such as against the larval sciarid fly *Lycoriella ingénue*, with a LC50 of 0.87 µL/L air and various other insects that was storage grain pests (Huang, Chen et al. 2000; Mikhaiel 2011). DAS3 alone has been shown to have fumigation effect. It was found that DAS3 suppresses egg hatching at 0.32 mg/cm^2, and larval and adult emergence at 0.08 mg/cm^2 of *S. zeamais* and *T. castaneum* (Huang, Chen et al. 2000). With the eggs, larvae and adults, the results obtained from using different

methods were comparable (table 2.3). However, the garlic products and methods used did not affect pupae, most likely because of the thick pupal casing. Therefore, if garlic products are to be used in agriculture it is important to take in consideration the time of the year to target the most susceptible life stages i.e. the eggs and adults.

Timed fumigations studies were performed (figure 2.20) where *D. melanogaster* adults were treated with 0-5% (v/v) R22 garlic oil using the fumigation method. Adult survival was recorded over 30 minute intervals. An increase in R22 concentration resulted in an increase in rate of mortality. This further demonstrates that the rate of insecticidal activity of garlic products is concentration-dependent and rate-dependent.

Insecticidal studies of R22 garlic oil with copper sulfate ($CuSO_4$) were performed on *D. melanogaster* following successful nematicidal studies performed by a student in the Hamilton lab (UEA). R22 garlic oil with $CuSO_4$ caused a strong insecticidal effect. The addition of $CuSO_4$ the R22 garlic oil led to an increase in activity of approximately 50% against *D. melanogaster* (Table 2.5) compared to R22 garlic oil activity alone, suggesting a synergic effect. The synergic effect of copper has previously been studied (Bindesbol, Holmstrup et al. 2005; Cedergreen 2014; Vaidya, McBain et al. 2017) and is also known to cause toxicity to *D. melanogaster* (Hwang, de Bruyne et al. 2014). This increase in activity could be due to complexes forming that are very reactive against cellular thiols. Copper chloride and diallyl polysulfide complexes have been characterized by X-ray crystallography. However, the authors stated that Cu donor properties decrease in the series DAS1-DAS4, suggesting the Cu becomes less reactive the greater the amount of sulfurs in the complex(Salivon and Shkurenko 2007). Complexes of diallyl polysulfides with $CuSO_4$ could have different properties compared to $CuCl_2$ in which the decrease in Cu donor activity may not apply. This experiment could be taken further by using a larger range of $CuSO_4$ concentrations and possibly different copper forms such as $CuCl_2$ and test if increased activity (with garlic oil) is observed. The individual diallyl polysulfides could be tested as results may vary depending on the reaction of copper to the diallyl polysulfide. Kachur et al (1997) presented the observation of hydrogen peroxide production caused by copper-catalyzed thiol oxidation and induced apoptosis. DTT was used in which it is oxidized by formation a Cu2+-DTT complex via the formation of an oxygen-containing

intermediate. DTT oxidations involved the production of superoxide and hydrogen peroxide which ultimately leading to apoptosis. Similar copper complexes formed with polysulfides could induce further production of hydroxyl radicals compared to polysulfides alone and accelerate insecticidal effect.

A problem that can be associated with using garlic products for insecticidal procedures is the potent odour. Therefore, it was attempted to mask the odour by using cyclodextrins with the inclusion of diallyl polysulfides from garlic oil. Cyclodextrins (CD) are cyclic oligosaccharides characterized by a toroidal shape structure. There are three commonly used cyclodextrins consisting of six, seven, and eight glucopyranose units known as α-, β-, and γ-CD, respectively. β-CD can accommodate larger structures in its hydrophobic cavity (Bisson-Boutelliez, Fontanay et al. 2010; Di Donato, Lavorgna et al. 2016). Diallyl polysulfides from Mexican, China, and R22 garlic oil were encased in β-CDs in a 1 in 10 configuration. The inclusion was confirmed by NMR. This process was performed by colleague in the Hamilton lab, Dr Ryan Tinson (UEA). Insecticidal studies were performed using these constructs against *D. melanogaster* on the eggs, 1st, 2nd and 3rd instar larvae. Although odourless, the β-CD treatments did not cause an insecticidal effect. The reason is thought to be either the constructs are too large to enter cells or the diallyl polysulfides are not being released from the β-CD and cannot cause an insecticidal effect. Further investigation into the release of the diallyl polysulfides would be required for successful use as odourless garlic insecticides for commercial use.

The insecticidal effect of the individual diallyl polysulfides (DAS1-6) was studied. DAS1-6 was isolated from 10% (v/v) R22, synthetic garlic oil using the HPLC. DAS2-5 were tested for their insecticidal effect against *D. melanogaster*. DAS1 and DAS6 were not tested, as sufficient quantities were not obtained from the extraction. The eggs and the three larval stages were studied and for all DAS4 was the most effective at causing a lethal effect (figure 2.25). This was surprising as, in regard to the diallyl polysulfides, the longer the sulfur chain the more reactive they are. Therefore, it was expected that the longest sulfur chain tested (DAS5) would have the greatest insecticidal effect. However, along with becoming more reactive with cellular thiols, these diallyl polysulfides also increase in lipophilicity and could, therefore, get trapped in cell membranes, which would explain

these results (Block 2010). It is possible that there was some degradation of DAS5 after -20ºC degrees storage which would cause DAS5 to be less efficient than expected. This could be rectified by re-running a purified fraction after storage and before insecticidal experiments to ensure purification of the compound. Because these diallyl polysulfides have different molecular weights due to different numbers of sulfur atoms, the resulting concentration of the diallyl polysulfides will differ when equivalent proportions are used. In this study, a 2% (w/v) solution in 5 ml was used for each diallyl polysulfide. The final molar concentrations are shown in table 2.1. This could cause an impact on the results. Percentage was used for the concentrations to keep concentrations throughout the thesis unified. It would have been useful for this experiment additionally to have the same concentrations in terms of mg/L. This may have been a greater comparison of insecticidal effects between the diallyl polysulfides when it comes to concentrations, however, due to the differing number of sulfur atoms in the polysulfides a similar result to the one obtained would be expected.

The concentration of these diallyl polysulfides in the three different garlic oils (Mexican, China, and R22) were analysed. The concentrations of the diallyl polysulfides could explain the different insecticidal effects as seen in Table 2.2 which shows the LC50s of the different garlic solutions to compare with the diallyl polysulfide concentrations within the garlic oils. R22 garlic oil has higher concentrations of the longer chained polysulfides and has the greatest insecticidal effect. The R22 garlic oil had a DAS4 concentration of 30.21 %, Mexican and china garlic oils has concentrations of 22.46 and 18.05 %, respectively. Another example is DAS5 which in R22 had a concentration of 19.24 %, whereas in Mexican and China garlic oil were 6.02 and 4.37 %, respectively. Since the longer chained diallyl polysulfides are more reactive, as previously mentioned, this could explain the greater insecticidal effect of R22 garlic oil. Mexican and china garlic oil contained very similar concentrations of diallyl polysulfides and had more similar insecticidal effects. This shows evidence that the diallyl polysulfide content is important when it comes to the insecticidal effect of garlic products, and this should be put into consideration when manufacturing these products. Ecospray Ltd use R22 garlic oil in their products which, from the evidence presented, shows they are using the bester choice rather than using natural garlic oils that have a lesser insecticidal effect compared to synthetic. The synthetic

garlic oil can be spiked with sulfur which would aid in producing garlic oil that contains longer chained diallyl polysulfides, which would increase the insecticidal effect.

As well as giving evidence of the insecticidal effect of garlic products, it is important to establish a suitable application method. In collaboration with Octavius Hunt and Ecospray, garlic smoke generators were tested against adult *Drosophila*. The smoke generators from Octavius Hunt have not been previously tested scientifically. The results of the smoke generator experiments were very inconsistent (figure 2.27-2.29). The first trial showed that the garlic smoke was effective against *D. melanogaster*. However, in the subsequent trials none of the smoke generators were effective. There was inconsistent ignition among the different smoke generators. It is possible that batch problems, and potentially the expiration of active product ingredients, could the reason for this variation. Another factor that could have affected the results was the weather conditions of the trials. Weather temperatures varied between the trails which may have affected the trial outcomes, especially when using a polytunnel (as pictured) which leads to higher temperatures compared not outside. More experiments would need to be conducted, to include systematic tests of the shelf life and formulations of these products. However, the use of smoke generators remains a potentially useful method of garlic application for the future. Other application methods would be liquid spray or garlic covered earth pellets, both of which are used by Ecospray. The application of garlic products would depend on the crop and the pest in concerned.

In conclusion, garlic extract and garlic oils had strong insecticidal effects on the different stages of *D. melanogaster* and these effects were concentration- and rate-dependent. The synthetic garlic oil, R22, was the most effective. The effectiveness of the garlic solutions was also life-stage dependent where the eggs and adults were the most susceptible. This study shows the potential of using garlic-based products as effective insecticides.

3 BIOCHEMICAL MODE OF ACTION OF GARLIC-DERIVED DIALLYL POLYSULFIDES

3.1 INTRODUCTION

This chapter explores the effects of diallyl polysulfides at the biochemical level in *D. melanogaster*. It is important to know the fundamentals of GSH, reactive oxygen species and the pathways involved because they are major players in understanding diallyl polysulfides and their biochemical effects in insects. These pathways are described below.

Glutathione (GSH) is the most abundant non-protein thiol in mammalian systems and is converted to glutathione disulfide (GSSG) by peroxidase via the reduction of hydrogen peroxide (H_2O_2) and other peroxides (Meister and Anderson 1983; Khramtsov, Yelinova et al. 1989). GSH is acts as a scavenger of free radicals and other reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Johnson, Wilson-Delfosse et al. 2012). Diallyl polysulfides are able to react directly with GSH which leads to the generation of ROS such as H_2O_2 and superoxide (Munday, Munday et al. 2003; Chatterji, Keerthi et al. 2005). The diallyl polysulfides are able to regenerate and undergo further reactions to produce more ROS, which then lowers GSH levels. The production of ROS and depletion of GSH is very damaging to living cells and causes oxidative stress, leading to damage of membranes, proteins and peptides(Munchberg, Anwar et al. 2007).

The glutathione redox couple GSH/GSSG is important for maintaining and regulating cellular redox status. GSH/GSSG redox potential in vivo is estimated to be from -260 mV to -150 mV, depending on cellular conditions (Jones 2002). An increase in ROS levels would cause changes in cellular conditions and would require enhanced GSH activity to maintain redox status. If cellular systems are unable to counteract protracted oxidative stress and GSH levels decrease, it will lead to irreversible cell degeneration and cell death (Zhang and Forman 2012).

Glutaredoxin (GRX) is important for the oxidation of GSH to GSSG and function as GSH-dependent oxidoreductases (Rouhier 2010). GRX catalyses the deglutathionylation reaction when under oxidative stress which is important for the regulation of redox signal

transduction and sulfydryl homeostasis by regulating glutathionylation and deglutathionylation processes (Dalle-Donne, Milzani et al. 2008; Mieyal, Gallogly et al. 2008).

The conventional method of measuring GSH is performed using techniques such as fluorescence assays and high throughput chromatography (HPLC) with fluorescent probes, for instance monobromobimane (Vandeputte, Guizon et al. 1994). These techniques can measure total GSH and GSSG concentrations in cells, however, this can lead to inaccuracy and confusion of subcellular compartment-specific information in regard to the glutathione redox state in the cytosol (Morgan, Sobotta et al. 2011).

H_2O_2 is able to react with low-molecular weight thiols such as GSH and cysteine. This involves a nucleophilic attack from the thiolate on H_2O_2. Cysteine residues have increased reactivity with H_2O_2 as the electrostatic environment around the –SH group has increased acidity which results in a higher portion of the thiolate form. This increases the nucleophilicity towards H_2O_2 of the thiolate in cysteine residues (Ferrer-Sueta, Manta et al. 2011).

Hydrogen peroxide (H_2O_2) is an important molecule for various cellular processes, such as β-oxidation of fatty acids and is generated as a by-product in respiratory chain activity. Though it is usually regarded as a damaging, unwanted molecule (D'Autreaux and Toledano 2007; Giorgio, Trinei et al. 2007). It is becoming clear that H_2O_2 can also have beneficial roles in cellular signalling and enzyme activity (Veal, Day et al. 2007; Groeger, Quiney et al. 2009). Conventional methods of measuring H_2O_2 usually have problems of specificity and lack of high temporal and spatial resolution. Redox-sensitive dyes, such as 2', 7'–dihydrodichlorofluoroscein, can also react with other cellular species, including metals, peroxidase, and cytochrome c. (Tarpey, Wink et al. 2004). Although there are new chemical probes that are more specific to H_2O_2, these probes still have low reaction rates, which would allow more time to perform the assay at a reasonable pace and adapt the experiment for easier comparison to other probes but higher reaction rates are more favourable due to obtain the result faster, and the measurements are based on absolute fluorescence intensity (Miller and Chang 2007; Dickinson, Huynh et al. 2010; Srikun, Albers et al. 2010).

The problems associated with conventional techniques to measure the redox potential of GSH/GSSG(E_{GSH}) and H_2O_2 can be overcome by using genetically encoded fluorescence-based probes. There are two of these fluorescent probes which consist of fusion proteins, constructed with a redox-sensitive green fluorescent protein (roGFP) which is fused with either the human glutaredoxin-1 (Grx1), to measure E_{GSH}, or peroxidase (Orp1) from *Saccharomyces cerevisiae*, to measure H_2O_2. These fluorescent probes permit real-time, subcellular compartment specific and non-disruptive measurement of the redox couple GSH:GSSG and H_2O_2(Gutscher, Pauleau et al. 2008; Gutscher, Sobotta et al. 2009).

It is increasingly becoming apparent that cellular redox changes are associated with various physiological and pathophysiological conditions. The increase in reactive oxygen species (ROS) is involved in many diseases (Schafer and Buettner 2001), including cancer (Yang, Ebbert et al. 2006). Cellular redox couples can be difficult to monitor as they vary significantly between subcellular compartments and can respond to endogenous and exogenous stimuli. Thus, it is becoming more important to establish research techniques to investigate cellular redox processes to obtain a more profound spatial and temporal understanding (Morgan, Sobotta et al. 2011).

ROS, such as H_2O_2 and superoxide, can cause cell damage and are usually rapidly inactivated via reduction with facilitation of superoxide dismutase (SOD), cytochrome c, catalase and glutathione S-transferases (GSTs) (Ahmad et al, 1991; Turrens, 2003). An imbalance of these enzymes and the increase in ROS will eventually lead to cell death. There are many synthetic enzymes that are known to suppress reduction enzymes (James and Xu, 2011; Buyukguzel, 2009; Adamski et al, 2003; Mamidala et al, 2011). For example, Buyukguzel (2009) investigated malathion, an organophosphorous insecticides, against Galleria mellonella (L.), larvae. It was found that the highest concentration of malathion caused a significant descrease in SOD and GSH content in the whole body of the insect.

There are many mechanisms that could be investigated to facilitate the understanding of the biochemistry that occurs with insecticidal treatment of diallyl polysulfides. Based literature, this thesis focuses on redox and oxidative stress as a mode of action.

3.2 METHODS

3.2.1 Insecticidal effect of hydrogen peroxide

The first experiment performed in this chapter was to explore the insecticidal effect of hydrogen peroxide. This was performed on the eggs and the three larval stages of *D. melanogaster*. The spraying method was used as previously described in section 2.2.5. Concentrations of 0-40 µM (with 5 µM increments) H_2O_2 in Milli-Q® water were used. The H_2O_2 treatments were made up from a 99% solution. Samples were in replicates of 4. Milli-Q® water was used for control.

3.2.2 Amplex red assay for detection of H_2O_2

of fluorescent detectors to measure ROS concentration in *D. melanogaster* cells. Amplex® Red (10-acetyl-3,7-dihydroxyphenoxazine) was used to measure H_2O_2. It is a colourless and non-fluorescent compound which reacts with H_2O_2 at 1:1 stoichiometric ratios to form resorufin, which is highly fluorescent (figure 3.1) (Zhou, Diwu et al. 1997). Due to the high fluorescence of resorufin, this assay has high sensitivity and can detect levels as low as 19 nM H_2O_2(Zhao, Summers et al. 2012).

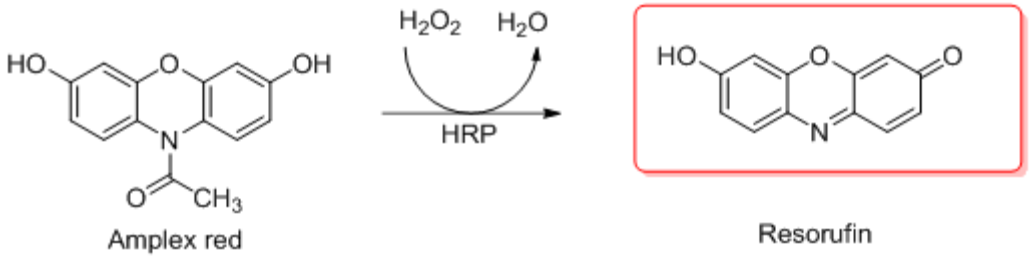


Figure 3.1: Amplex® Red reaction with H_2O_2 via Horseradish peroxidase (HRP) to produce resorufin.

Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit (Thermo Fisher Scientific) was used according to the protocol and the publication by Tran and Welte (2010). The Amplex red assay was first calibrated using known concentrations as shown in figure 3.2. The

buffer that came with the kit was used in both standard curve and following experiments, it was also used for standardisation for all experiments. A final concentration of 100 µM Amplex™ Red reagent was used. Samples tested were eggs, 1st, 2nd, and 3rd instar larvae of *D. melanogaster* and 100 of each life stage were treated with 2% (v/v) R22 garlic oil for 15 min. Samples were washed after treatment with PBS. Samples were then centrifuged for 10 min at 1300 rpm and the supernatant removed. NEM (20 µM) was added to each sample and incubated for 30 min. Samples were centrifuged for 10 min at 1300 rpm and supernatant was removed. An aliquot of 50 µl of glass beads (425-600 µm, Sigma-Aldrich) was added and the samples placed in a bead beater (Disruptor Gene, Scientific Industries) for 10 min. To 100 µl of the supernatant from the sample, 50 µl of the Amplex™ Red working solution was added and were incubated for 15 minutes at 25 °C (protected from light). Fluorescence intensity was read at 475ex/612em nm on a microplate reader (Tecan GENios) using the software Magellan™ as these were the closest filters the microplate reader had that fell within excitation/emission range. A gain of 60% was used. A buffer control was used for standardisation. Samples were replicated three times.

The investigations with the specific beat beater that was used showed that 1 min was not sufficient to achieve homogenisation and that in fact 10min gave a much improved and consistent homogenisation of this tissue. Variation in homogenisation time itself is not expected to confound the results and the inclusion of all the relevant controls helps to ensure the results are sound.

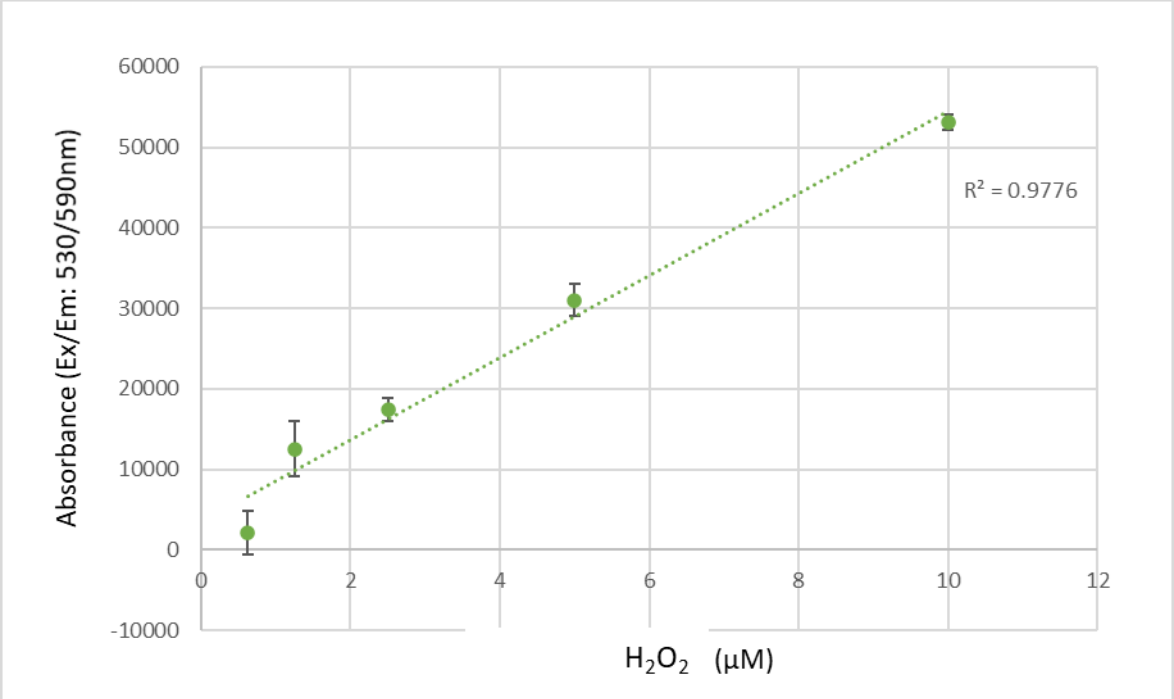


Figure 3.2: Amplex® Red standard curve. Concentrations used were 0.625, 1.25, 2.5, 5 and 10 µM H_2O_2. Shown are means ±SE, n=4.

3.2.3 MitoSOX to detect superoxide

Another ROS that has been reported to be produced following treatment with diallyl polysulfides is superoxide. MitoSOX™ Red is a mitochondrial targeted analogue of hydroethidine (HE) used to fluorescently detect superoxide (figure 3.3) (Zielonka and Kalyanaraman 2010). The MitoSOX Red assay performed according to protocol (Thermo Fisher Scientific). A final concentration of 5 µM, MitoSOX™ was used. Samples tested were eggs, 1st, 2nd, and 3rd instar larvae of *D. melanogaster* and 100 of each stage were treated with 2% (v/v) R22 garlic oil for 15 min. Samples were washed after treatment with PBS. Samples were centrifuged for 10 min at 1300 rpm and the supernatant then removed. NEM (20 µM) was added and incubated for 30 min. Samples were centrifuged for 10 min at 1300 rpm and the supernatant again removed. An aliquot of 50 µl of glass beads (425-600 µm, Sigma-Aldrich) was added and the samples were placed in a bead beater (Disruptor Gene, Scientific Industries) for 10 min. To 100 µl of the supernatant from the

sample, 50 µl of 5 µM MitoSOX™ reagent working solution was added, protected from light, and incubated for 15 minutes at 25 °C. Fluorescence intensity was read at 475ex/612em nm on a microplate reader (Tecan GENios) using the software Magellan™ as these were the closest filters the microplate reader had that fell within excitation/emission range. A gain of 60% was used. Each treatment was replicated three times.

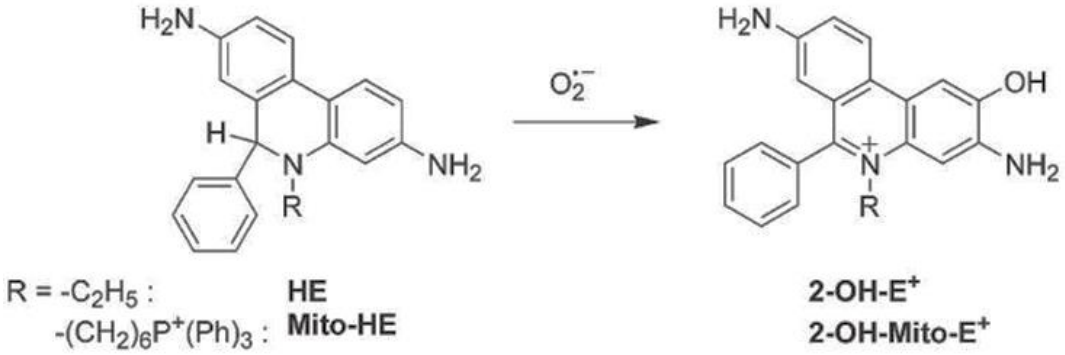


Figure 3.3: MitoSOX™ Red (Mito) reacts with superoxide to become fluorescent upon binding to nucleic acids.

3.2.4 DCFDA to detect ROS

2′-7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) was also used to detect ROS in *D. melanogaster* when treated with diallyl polysulfides. DCFH-DA is a cell permeable non-fluorescent compound used as an intracellular probe for ROS detection (figure 3.4). Intracellular esterases cleave DCFH-DA at two ester bonds, which produce 2′-7′-dichlorodihydrofluorescein (H_2DCF). H_2DCF is a non-fluorescent molecule that accumulates intracellularly and subsequent oxidation by ROS results in the highly fluorescent product 2′-7′-dichlorodihydrofluorescein (DCF). DCF is excited at 485 nm and emits at 530 nm (Bass, Parce et al. 1983; Royall and Ischiropoulos 1993).

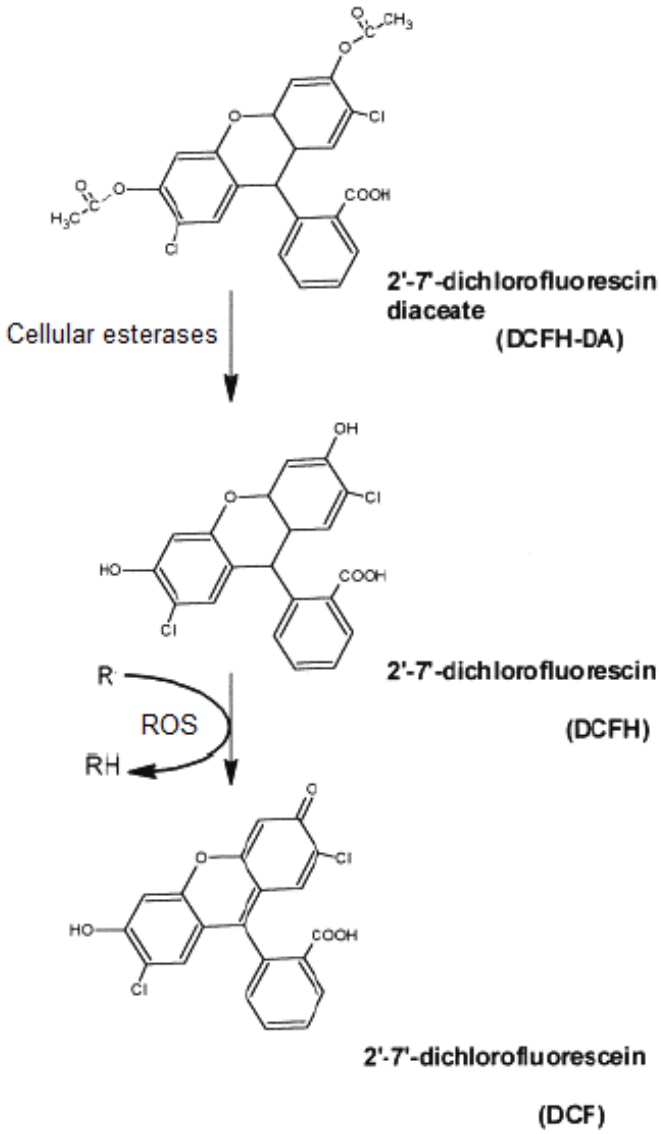


Figure 3.4: Mechanism of DCFH-DA. Intracellular esterases cleave DCFH-DA to produce DCFH. DCFH is oxidized by ROS to yield the highly fluorescent DCF.

The DCFDA (Thermo Fisher Scientific) assay was tested on third instar *D. melanogaster* larvae. Samples of 100 organisms were treated with 2% (v/v) R22 garlic oil for 15 min. Samples were centrifuged for 10 min at 13,000 rpm and the supernatant then removed. NEM (20 µM) was added to each and incubated for 30 min. Samples were centrifuged for 10 min at 13,000 rpm and the supernatant again removed. An aliquot of 50 µl of glass beads (425-600 µm, Sigma-Aldrich) was added and the samples placed in a bead beater (Disruptor Gene, Scientific Industries) for 10 min. To 100 µl of the supernatant from the

sample, 20μM DCFDA was added, and protected from light. Samples were incubated for 15 minutes at 25 °C. Fluorescence intensity was read at 485ex/535em nm on a microplate reader (Tecan GENios) using the software Magellan™. A gain of 20% was used. Samples were replicated three times.

3.2.5 HPLC with mBBr to measure GSH and cysteine

mBBr is a non-fluorescent compound that becomes intensively fluorescent when it reacts with thiols via a nucleophilic substitution, figure 3.9 (Kosower and Kosower 1987). GSH and cysteine concentrations of samples were calculated via HPLC analysis.

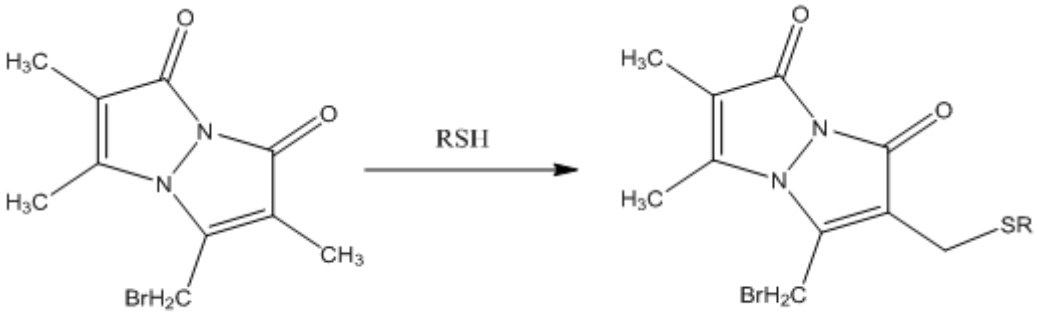


Figure 3.5: Monobromobimane reaction with thiols to become fluorescent.

Samples tested were eggs, 1st, 2nd, and 3rd instar larvae of *D. melanogaster* and 100 of each stage were treated with 2% (v/v) R22 garlic oil for 15 min. Samples were centrifuged for 10 min at 13,000 rpm and the supernatant then removed and dried in oven overnight. NEM (20 μM) was added and incubated for 30 min. Samples were centrifuged for 10 min at 13,000 rpm and the supernatant again removed. An aliquot of 50 μl of glass beads (425-600 μm, Sigma-Aldrich) was added and the samples were placed in a bead beater (Disruptor Gene, Scientific Industries) for 10 min. Three replicates of each sample. To 100 μl of sample50 mM Hepes, 5 mM ethylenediaminetetraacetic acid (EDTA), 3 mM mBBr (dissolved in CH_3CN) were mixed. The reaction was incubated for 15 min at 25°C in the dark. After 15 min Me_3SO_3H was added to a final concentration of 25 mM. Each sample

was taken up in separate 1 ml disposable sterile syringes, and any insoluble matter was removed by filtration through separate 0.2 μm solvent filters prior to HPLC analysis. The dried weight of the samples was used for normalisation in the calculations of cysteine and GSH concentrations

HPLC was used to separate cysteine and GSH mBBr adducts on a HiChrom ACE-AR C18 4.6 x 250 mm, 5 μm, column, and equilibrated at 37°C. The injection volume was 20 μl and was ran at 1 ml/min. Solvent A consisted of 0.25% v/v acetic acid and 10% MeOH, adjusted to pH 4 with NaOH. Solvent B consisted of 90% MeOH, 10% H_2O. Acetonitrile was used for as a negative control. Samples were eluted with the following gradient: 0-25 min, 1-45% solvent B; 25-27 min, 45-100% solvent B, followed by re-equilibration and re-injection. The retention times were 8.9 min for cysteine mBBr and 11.7 min for GSH mBBr. An excitation at 385 nm and emission at 460 nm was used for fluorescence detection, with 10X gain. Quantification was obtained by integration relative to the internal standard produced by previous C. Hamilton (UEA) group member. GSH and Cys were the main focus of this investigation as based on reviewing literature.

3.2.6 roGFP transgenic flies to detect changes in redox status

Before performing biochemical analyses, it was necessary to conduct insecticidal experiments on the roGFP *D. melanogaster* to confirm they showed comparable responses to garlic as did the wildtype to diallyl polysulfide treatment. Therefore, the same methods as described in section 2.4 were applied to all life stages, except pupae, of the roGFP *D. melanogaster* treated with synthetic garlic oil, R22. Concentrations of R22 used were 0-5% (v/v), all treatments were replicated by four.

To explore the *in vivo* redox processes affected by diallyl polysulfide treatment, roGFP transgenic *D. melanogaster* were used (table 3.1), which expressed either a mitochondrial E_{GSH} (Tubulin-Gal4, UAS-mito-roGFP2-Grx; P003) or a mitochondrial H_2O_2 probe (Tubulin-mito-roGFP2-Orp1; P018, and cyto-roGFP2-Orp1; P015).The line UAS-cyto-roGFP2-Grxwhich was previously mentioned was not used in this study as they did not unfortunately survive the shipping. The roGFP strains are UAS/GAL4 lines that were recombined already when received.

Table 3.1: roGFP strains used in redox study

roGFP line	Reference label
mito-Grx1-roGFP2	P003
cyto-roGFP2-Orp1	P015
mito-roGFP2-Orp1	P018

Using confocal microscopy (Leica SP2) the redox status of these probes was then determined. 1st instar larvae were used, as they were the most transparent of all the life stages, aiding visualisation of the fluorophores. As control treatments I used the exogenous reductant dithiothreitol (DTT) and the oxidant diamide. The synthetic garlic oil R22 at a sublethal concentration of 2% (v/v) was used as the insecticidal treatment. Exposure time for each of the treatments was 10 min. All samples were treated with 20 mM of the alkylating agent N-ethylmaleimide (NEM) prior to imaging. The organisms were adhered to a microscope slide using micro glue dots (Blu Tack).Excitation of biosensor fluorescence by the 405 nm and 488 nm laser lines was performed sequentially and line by line. The emission wavelength used was 509 nm. Images were collected and analysed by using Leica LAS AF software and Image J software to calculate redox potentials using the Nernst equation (equation 3).

3.2.7 Testing Diallyl polysulfide effects on Grx1-roGFP

Approximately 100 third instar *D. melanogaster* was added to 1.5 ml eppendorfs per sample. An aliquot of 100 µl of water and 50 µl of glass beads (425-600 µm, Sigma-Aldrich) was added and the samples placed in a bead beater (Disruptor Gene, Scientific Industries) for 10 min. Sample were centrifuged for 10 min at 13,000 rpm and the supernatant then added to 30K microcentrifuge filters. The samples were washed three times with 1X PBS, 10 min at 13,000 rpm each time. To a 96 well plate, 100 µl of filtrate was added with 50 µl of treatment. Treatments were water (control), 3% (v/v) R22 garlic oil, 8 mM GSH, 4 mM GSSG, 8 mM GSH with 4 mM GSSG, 7 mM GSH with 1 mM GSSG, and 6 mM GSH with 2 mM GSSG. The treatments used were to test a range of

different conditions to represent different scenarios such as high GSH levels. Fluorescence intensity was read at 450/535 nm and 485ex/535em nm on a microplate reader (Tecan GENios) using the software Magellan™. A gain of 20% was used. Samples were replicated three times

3.2.8 Statistical analysis

Data were analysed using one-way ANOVA (followed by Tukey's post hoc analyses) and t-tests, $P<0.05$ was considered significantly different. Analysis performed using ibm SPSS Statistics software and Microsoft Excel 2010(Excel 2010; SPSS 2016). The LC50s were calculated using GraFit software(GraFit 2016).

3.3 RESULTS

3.4 INSECTICIDAL EFFECT OF HYDROGEN PEROXIDE

An increase in H_2O_2 concentration leads to an increase in oxidative stress which results in a cascade of molecular events including apoptosis. Therefore, an increase of H_2O_2 concentration in cells ultimately leads to death of the organism. This is thought to be the case with *D. melanogaster* and is borne out by the results below. Since the garlic treatment was externally administered it would be plausible to assume the most effected cells, with an increase in ROS such as H_2O_2, would predominately occur in the external cells. Therefore, seeing the impact of treating the external cells directly with H_2O_2 would tell us what effects would occur. The LC50 values of H_2O_2 against *D. melanogaster* of life stages eggs, 1st, 2nd and 3rd instar larvae are shown in figure 3.6. The LC50 values were 3.44, 6.19, 14.89, and 30.77 µM, respectively.

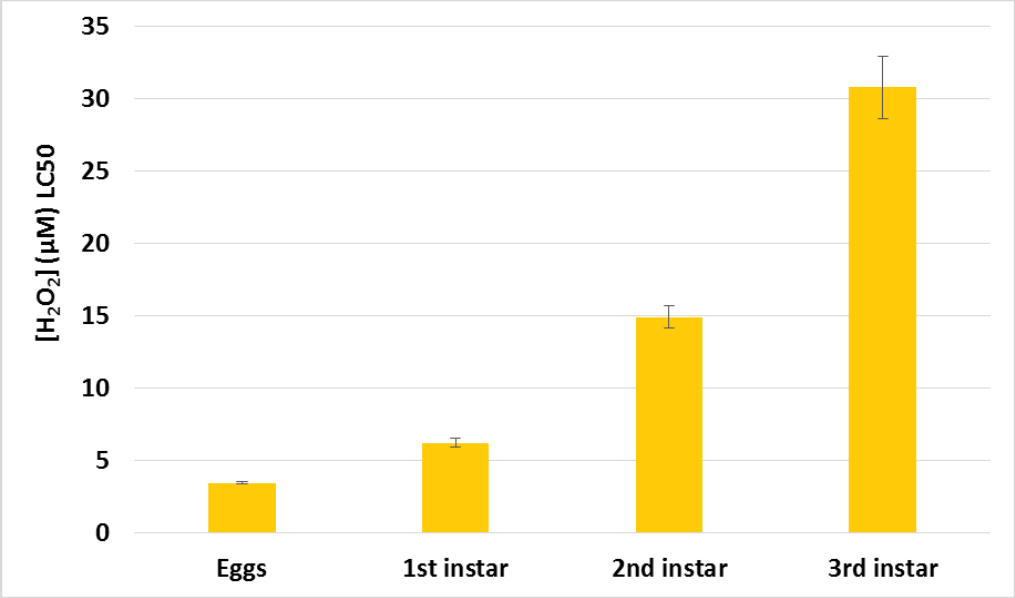


Figure 3.6: LC50 values of H_2O_2 against *D. melanogaster* eggs, 1st, 2nd, and 3rd instar larvae. The LC50 of H_2O_2were 3.44, 6.19, 14.89, and 30.77 µM, respectively. Shown are means ±SE, n=4.

3.4.1 Diallyl polysulfides cause an increase in ROS

One of the biochemical modes of action that causes lethality in organisms is the production of ROS, which causes oxidative stress. In order to detect the presence of this phenomenon in flies, fluorescent assays for the detection of ROS were used, as described above. The main ROS studied here was H_2O_2, with Amplex® Red being used for fluorescent detection. A standard curve (figure 3.2) of Amplex® Red was produced in order to use to determine H_2O_2 concentrations.

Mexican garlic oil caused the highest production of H_2O_2 in comparison to the other garlic treatments (figure 3.7). Garlic extract caused the lowest H_2O_2 production, with R22 garlic oil being slightly higher. Eggs gave the highest H_2O_2 readings with Mexican garlic oil. The results from the MitoSOX™ Red assay shows that all garlic oils tested resulted in higher detection of superoxide compared to garlic extract (figure 3.8).

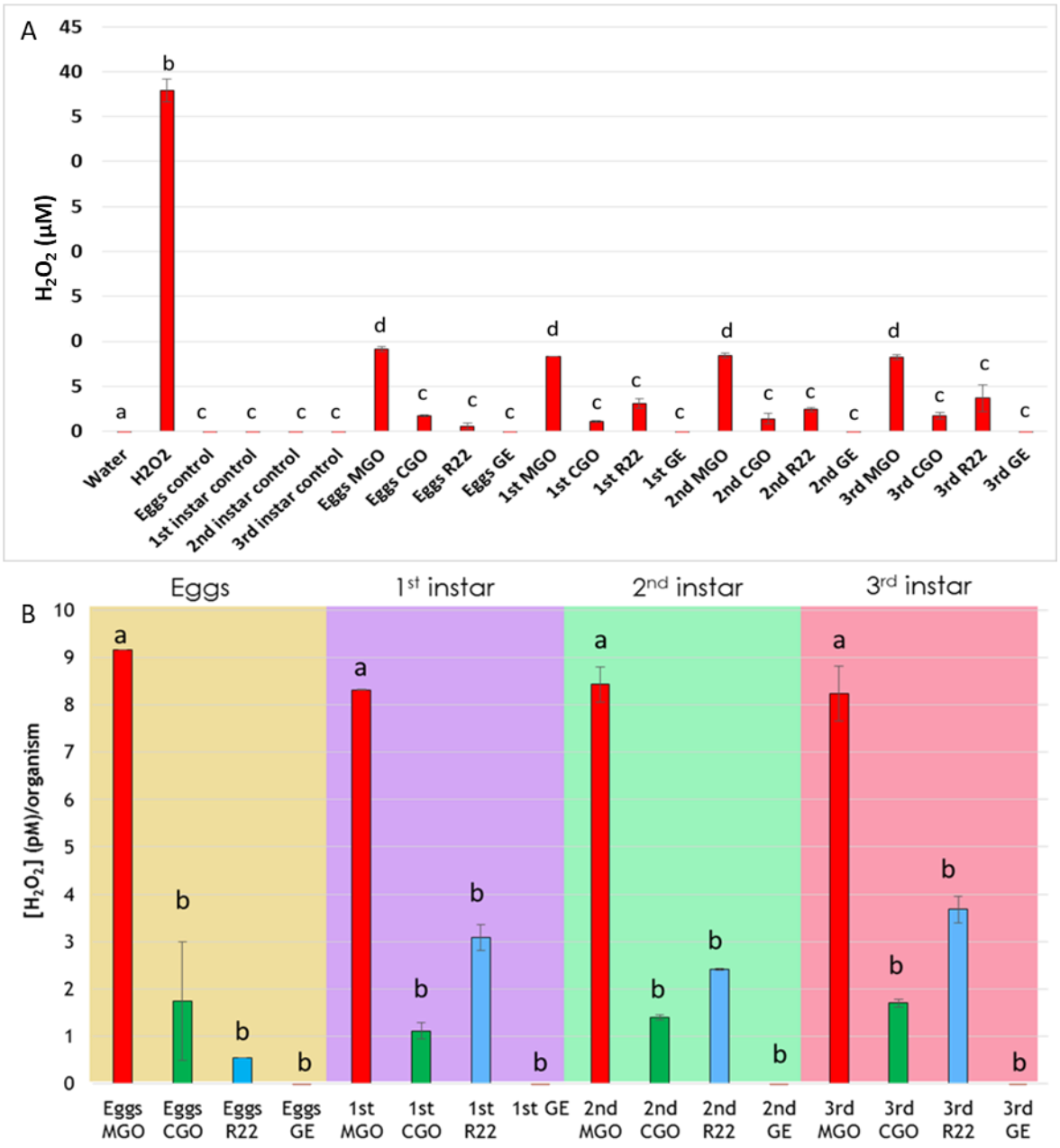


Figure 3.7: Amplex® Red assay to detect H_2O_2 levels in *D. melanogaster* eggs and larvae treated with garlic extract and oils, showing all controls. Water and no garlic treatments were negative controls and 38 µM H_2O_2 was used as the positive control. A) with the controls. B) without the controls. Bars show mean H_2O_2 levels µM /100µL ±SE. MGO; Mexican garlic oil, CGO; Chinese garlic oil, GE; garlic extract. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another ($P < 0.05$), n=4.

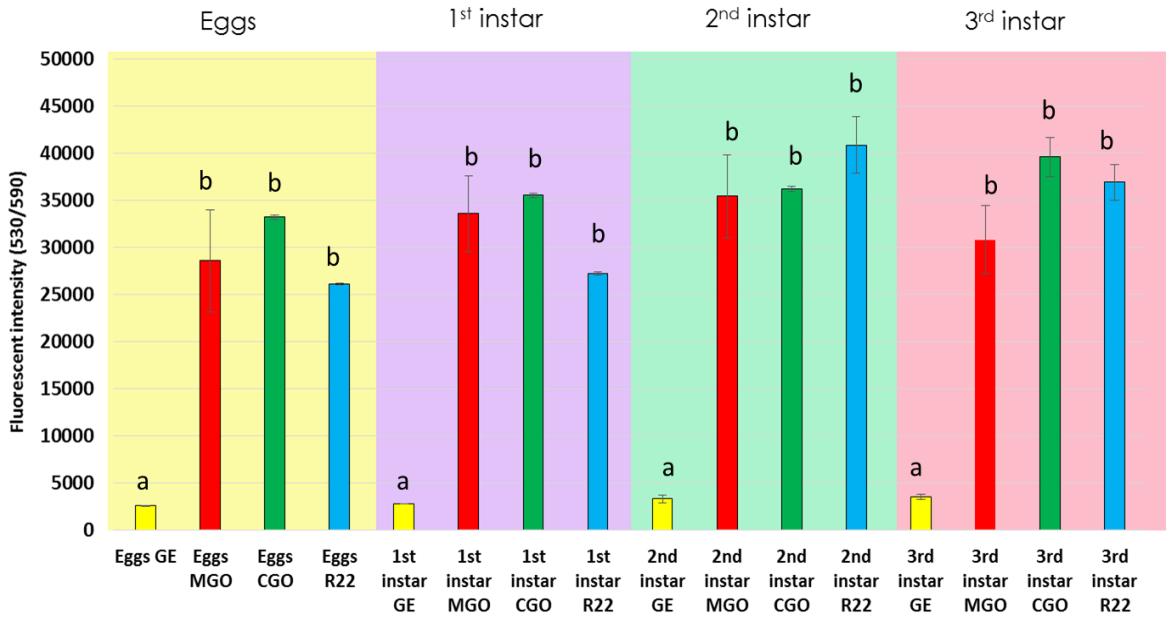


Figure 3.8: MitoSOX™ Red assay to detect superoxide levels in *D. melanogaster* eggs and larvae treated with garlic extract and oils. Bars show mean superoxide levels µM /100µL ±SE. MGO; Mexican garlic oil, CGO; Chinese garlic oil, GE; garlic extract. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another ($P < 0.05$), n=4.

DCFDA assay was used with *D. melanogaster* larvae treated with R22 garlic oil. Larvae were treated with 0-5% (v/v) R22 garlic oil. Figure 3.9 shows that as the garlic oil concentration increased the higher the fluorescence of DCF detected which suggests an increase in ROS. The results indicate that treatments containing higher concentrations of garlic oil resulted in higher levels of ROS detection.

To test for reactions of garlic oil with the assays Amplex® Red, MitoSOX™ Red and DCFH-DA were tested with R22 garlic oil. Figure 3.10 shows the synthetic garlic oil R22 and the ROS fluorescent assays. The results show that the Amplex® Red was significantly different from the control, which suggests that diallyl polysulfides react with Amplex® Red. However, this also suggests that diallyl polysulfides do not react with MitoSOX™ Red or DCFH-DA. There was a significant difference between the negative control (water) and Amplex® Red but not with MitoSOX™ Red.

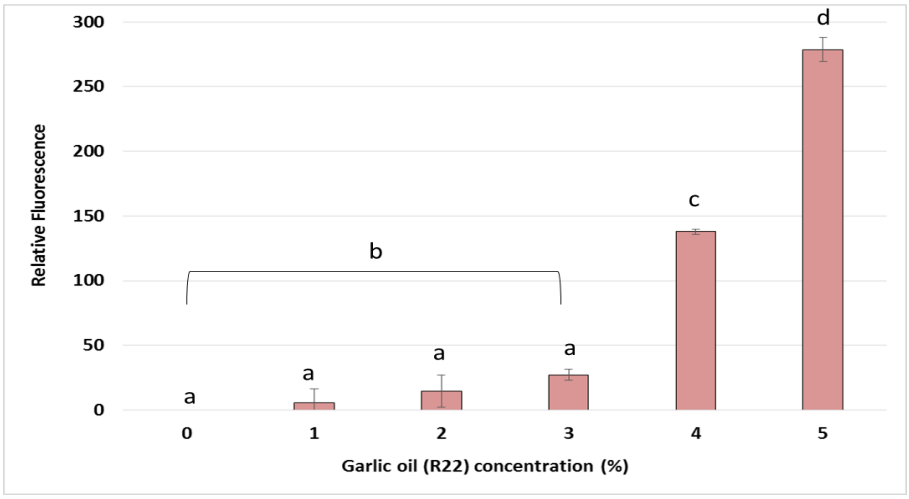
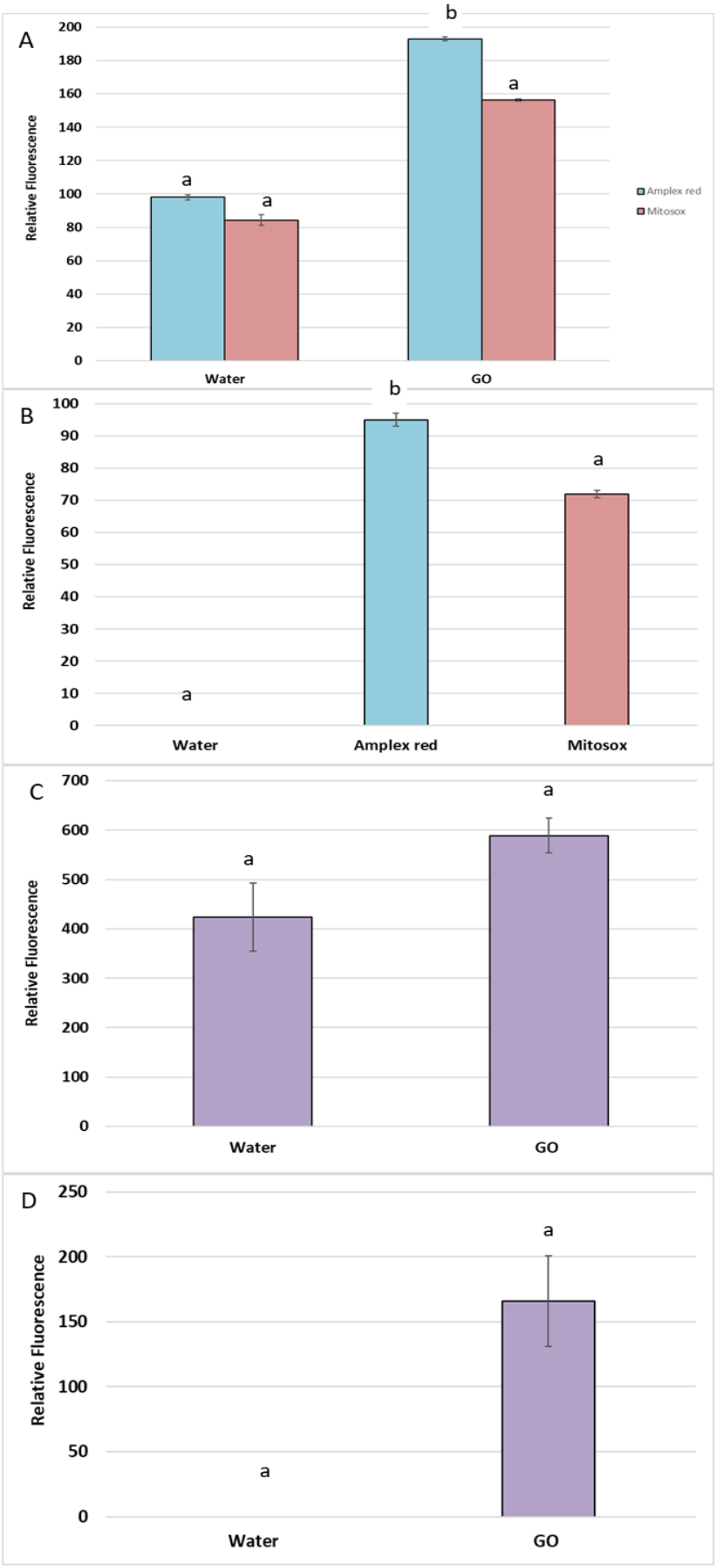


Figure 3.9: DCFDA assay of *D. melanogaster* larvae treated with R22 garlic oil. Larvae were treated with 0-5% (v/v) R22 garlic oil. Bars show mean ROS levels µM /100µL ±SE. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another ($P < 0.05$), n=3.


A
Relative Fluorescence
200
180
160
140
120
100
80
60
40
20
0
a
a
b
a
Water
GO
Amplex red
Mitosox
B
Relative Fluorescence
100
90
80
70
60
50
40
30
20
10
0
a
b
a
Water
Amplex red
Mitosox
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Relative Fluorescence
700
600
500
400
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200
100
0
a
a
Water
GO
D
Relative Fluorescence
250
200
150
100
50
0
a
a
Water
GO

Figure 3.10: R22 garlic oil study with Amplex® Red, MitoSOX™ Red and DCFH-DA, to detect reaction with R22. A) R22 with Amplex® Red (P= 0.0004) and MitoSOX™ Red (P= 0.076).B) R22 with Amplex® Red and MitoSOX™ Red with the subtraction of the water background. C) R22 with DCFH-DA (P= 0.218). D) R22 with DCFH-DA with subtraction of water background. Mean value of negative control (water) was subtracted from the treatment results to facilitate comparison. Error bars show ±SE. Bars show mean µM /100µL ±SE. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another (P < 0.05), n=3.

3.4.2 Decrease in GSH and cysteine levels

In addition to the production of ROS, diallyl polysulfides can also react with GSH and cysteine residues. To test this, monobrombimane (mBBr) was used to fluorescently detect GSH and cysteine in order to calculate concentrations and explore the effect when treated with garlic oil. Acetonitrile was used as a negative control with mBBr, however, a control of Mexican garlic oil with mBBr should have been performed. This would have given validation of the results.

Figures 3.11 and 3.12 show that GSH and cysteine concentrations were lower in *D. melanogaster* when treated with Mexican garlic oil. T-tests showed that there were significant differences between the control and garlic oil treatments for each time interval tested. Also shown is that the eggs and 1st instar larvae had higher concentrations of GSH than the 2nd and 3rd instar larvae. Table 3.2 shows in more detail the GS, n=4H concentrations in *D. melanogaster* eggs with and without garlic oil for comparison. Samples were replicated three times. Cysteine concentrations in the control treatment appeared to increase with age. The 2nd and 3rd instar larvae had significantly lower cysteine levels (protein and free cysteines) when treated with garlic oil.

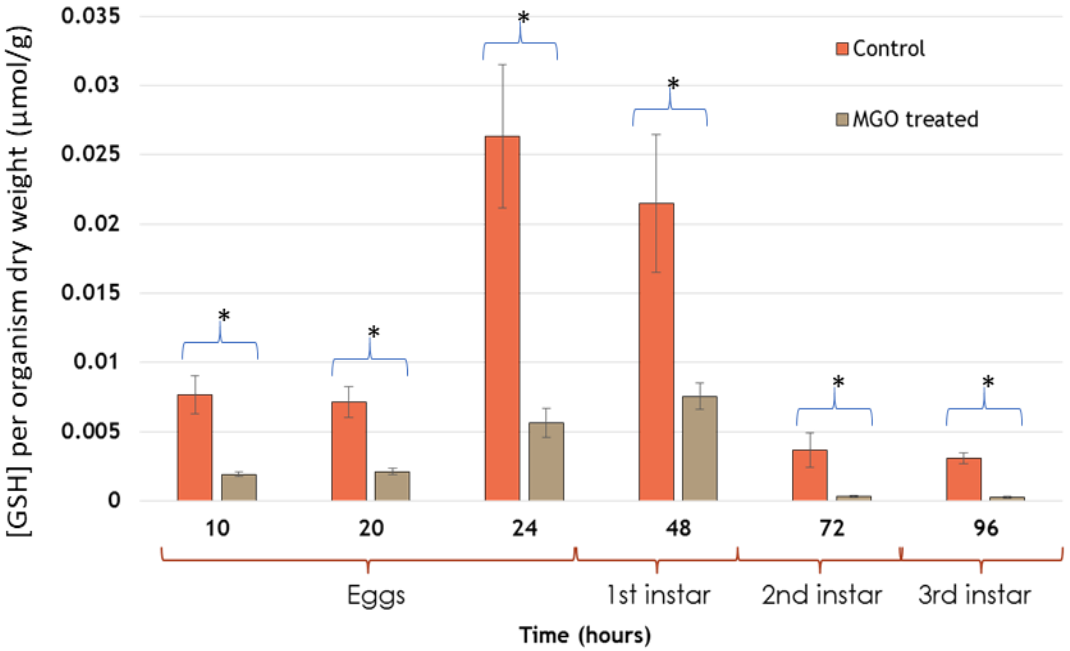

Figure 3.11: GSH concentrations of *D. melanogaster* with and without Mexican garlic oil treatment. The controls were treated with ethanol. Figure shows that GSH concentration in *D. melanogaster* is decreased when treated with garlic oil. Error bars show ±SE. * = P-value < 0.05 between control and garlic oil treated., n=4.

Table 3.2: GSH concentrations of *D. melanogaster* eggs. HPLC analysis to study GSH concentrations when treated with Mexican garlic oil. Shown is the dry weight of the sample, peak area for GSH and the calculated GSH concentration.

	Eggs	GSH STD=3919.4mV/sec		
Sample	Dry weight (mg)	Area mV/sec	[GSH] (µmol/g)	Average (µmol/g)
Control 1	26.4	615.3	0.01575	0.05447
Control 2	34	2896.8	0.05771	
Control 3	12.6	1673.4	0.08995	
Mexican GO 1	10.8	276.6	0.01734	0.014227
Mexican GO 2	8.5	214	0.01705	
MexicanGO 3	18	220.4	0.00829	

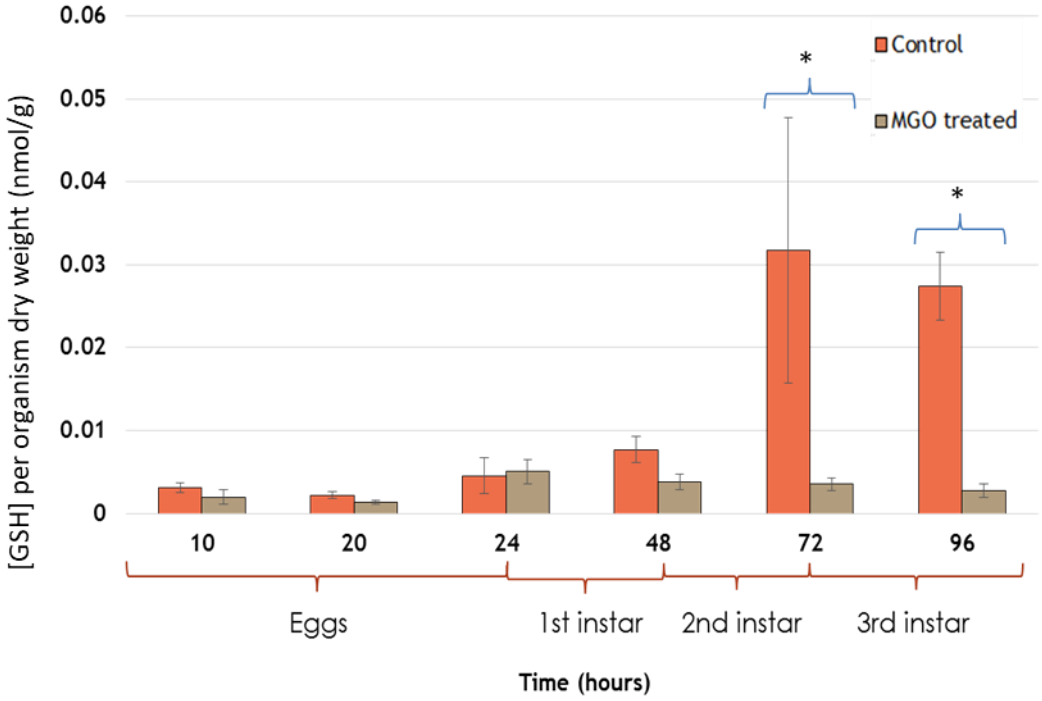

Figure 3.12: Free cysteine concentrations of *D. melanogaster* eggs and larvae treated with Mexican garlic oil. The 2nd and 3rd instar larvae had significantly lower cysteine levels (protein and free cysteine) when treated with garlic oil. Error bars show ±SE. * = P-value < 0.05 between control and garlic oil treated, n=3.

3.4.3 Insecticidal effects on roGFP transgenic *D. melanogaster*

Transgenic roGFP *D. melanogaster* were used to study GSH and H_2O_2 redox changes, but first, insecticidal studies were performed to assure similarity of the transgenic strains to the wildtype. The LC50s obtained for all three different roGFP strains were very similar to the wildtype from Chapter 2, as shown in table 3.3 and Figures 3.13- 3.15. Transgenic roGFP strains tested were: P015 (tub-GAL4>UAS-cyto-roGFP2-Orp1), P018 (tub-GAL4>UAS-mito-roGFP2-Orp1), P003 (tub-GAL4>UAS-cyto-Grx1-roGFP2). There were significant differences between the control and garlic treatments in each study.

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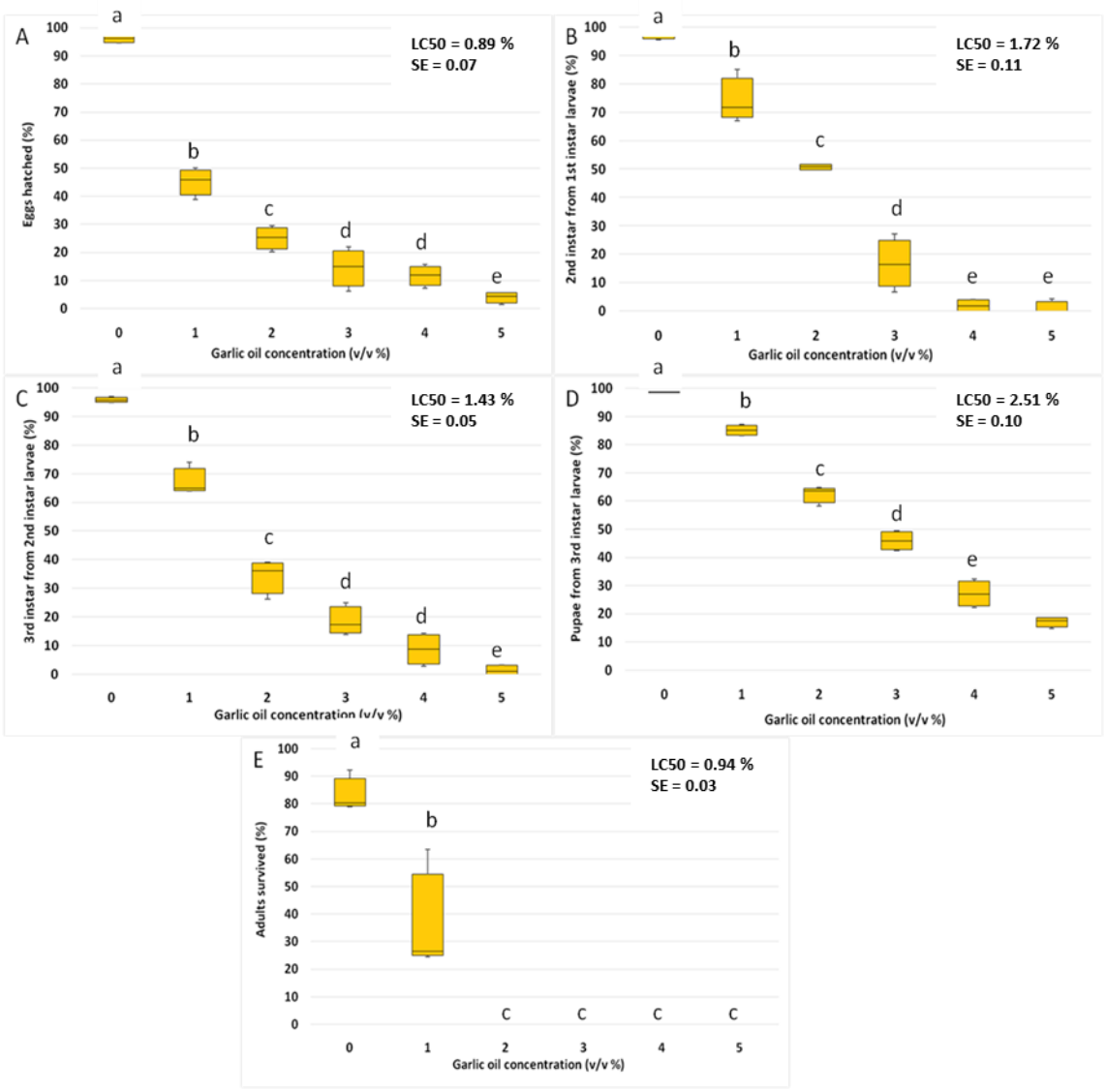

Figure 3.13: Insecticidal effect of R22 0-5% (v/v) against P015 *D. melanogaster* eggs, larvae and adults. P015 represents the tub-GAL4>UAS-cyto-roGFP2-Orp1strain. A) Eggs, F (5, 18)=193.2, P=0.0000069. B) 1st instar larvae, F (5, 18)=143.74, P=0.000014. C) 2nd instar larvae, F (5, 18)=171.65, P=0.0000092. D) 3rd instar larvae, F (5, 18)=592.66, P=0.00000042. E) Adults, F (5, 18)=123.56, P=0.000021. Ethanol was used as control. Boxes, lower/upper quartile; whiskers, minimum and maximum. SE= Standard error. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another (P < 0.05), n=4.

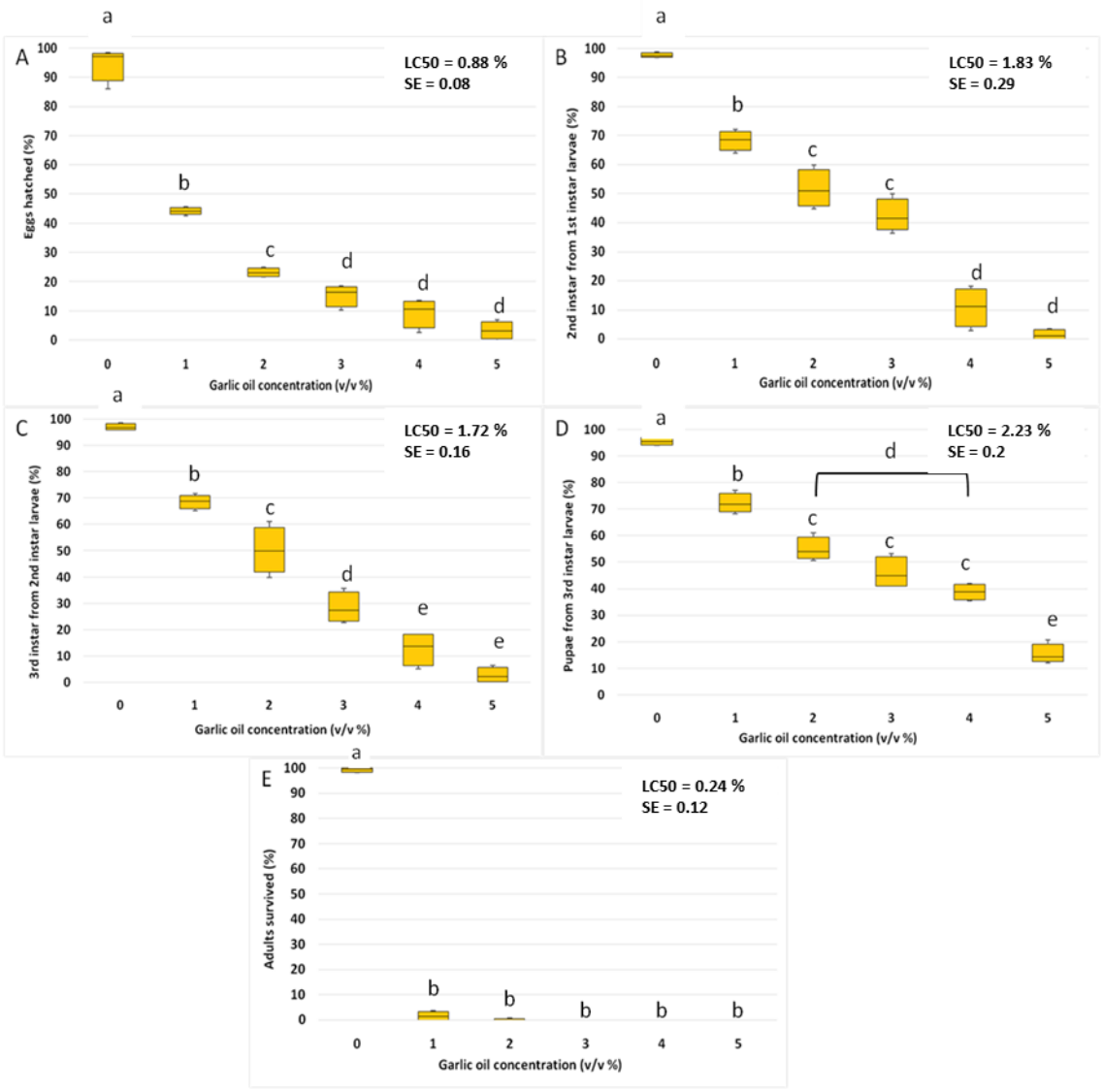

Figure 3.14: Insecticidal effect of R22 0-5% (v/v) against P018 *D. melanogaster* eggs, larvae and adults. P018 represents the tub-GAL4>UAS-mito-roGFP2-Orp1 strain. A) Eggs, F (5, 18)=111.62, P=0.000019. B) 1st instar larvae, F (5, 18)=164.61, P=0.00001. C) 2nd instar larvae, F (5, 18)=132.47, P=0.000018. D) 3rd instar larvae, F (5, 18)=206.17, P=0.0000059. E) Adults, F (5, 18)=528.11, P=0.00000056. Ethanol was used as control. Boxes, lower/upper quartile; whiskers, minimum and maximum. SE= Standard error. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another (P < 0.05).

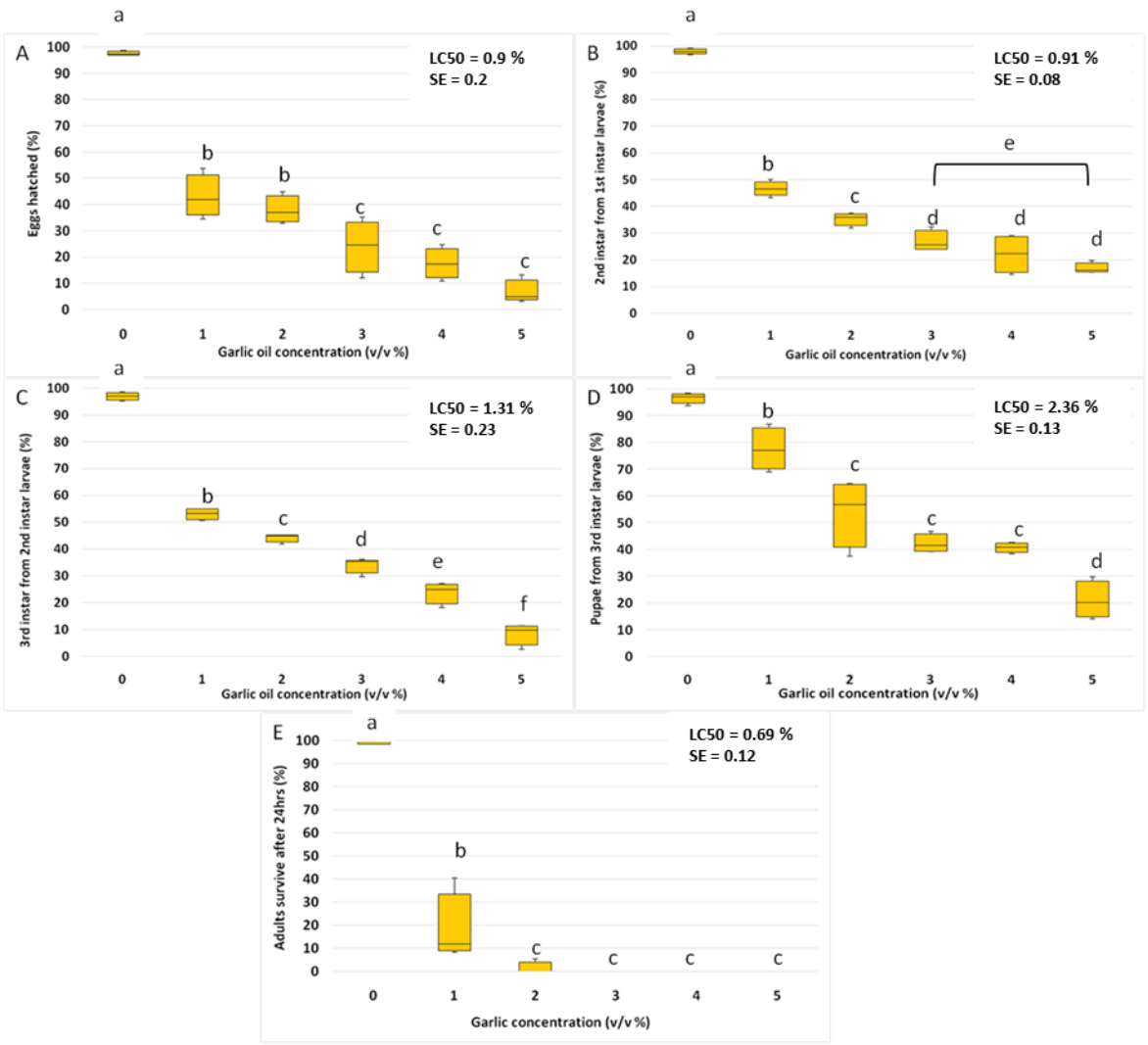

Figure 3.15: Insecticidal effect of R22 0-5% (v/v) against P003*D. melanogaster* eggs, larvae and adults. P003 represents the tub-GAL4>UAS-cyto-Grx1-roGFP2strain. A) Eggs, F (5, 18)=105.1, P=0.000031. B) 1st instar larvae, F (5, 18)=248.86, P=0.0000037. C) 2nd instar larvae, F (5, 18)=269.22, P=0.000003. D) 3rd instar larvae, F (5, 18)=68.3, P=0.00009. E) Adults, F (5, 18)=251.22, P=0.0000034. Ethanol was used as control. Boxes, lower/upper quartile; whiskers, minimum and maximum. SE= Standard error. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another (P < 0.05).

Table 3.3: Insecticidal study of R22 (v/v %) on the three strains of transgenic roGFP *D. melanogaster* eggs, larvae and adults. Wildtype values from Chapter 2 are also shown for comparison. LC50s are shown ± SE.

R22 (v/v %) LC50s ± SE	**P015[a]**	**P018[b]**	**P003[c]**	**Wildtype**
Eggs	0.89 ± 0.07	0.88 ± 0.08	0.9 ± 0.2	0.67 ± 0.34
1st instar	1.72 ± 0.11	1.83 ± 0.29	0.91 ± 0.08	0.98 ± 0.73
2nd instar	1.43 ± 0.05	1.72 ± 0.16	1.31 ± 0.23	1.31 ± 0.16
3rd instar	2.51 ± 0.1	2.23 ± 0.2	2.36 ± 0.13	1.79 ± 0.18
Adults	0.93 ± 0.03	0.24 ± 0.12	0.69 ± 0.014	0.75 ± 0.02

[a] P015 - cyto-roGFP2-Orp1, [b] P018 - mito-roGFP2-Orp1, [c] P003 - cyto-Grx1-roGFP2

3.4.4 Redox changes with garlic treatment

Figure 3.16 shows confocal images of mito-roGFP2-Orp1 1st instar *D. melanogaster* larvae with their calculated $E_{roGFP2,}$ which represents H_2O_2 levels. Figure 3.22 shows confocal images taken of mito-roGFP2-Grx1 1st instar *D. melanogaster* larvae with their calculated E_{GSH} which represents redox status of GSH/GSSG. For each treatment there were three replicates (section 6.3). Shown in figures 3.16 and 3.18 are the control (A), garlic oil treated (B), 2 mM diamide treated (C) and 20 mM treated (D). The E_{roGFP2}/E_{GSH} average was calculated using the equations shown in section 1.5.5. Figures 3.16 and 3.18 show the differences in fluorescence intensity in relation to the redox potentials. With a more negative E_{roGFP2}/E_{GSH}, the higher the fluorescence emitted when excited at 488 nm.

The ratios of the fluorescence intensities that represent the redox state of the prober oGFP2-Orp1 or roGFP2-Grx1 are shown in figures 3.17 and 3.19, respectively. For the mito-roGFP2-Orp1 larvae the garlic oil treatment appeared to show an effect on redox status. However, the t-test revealed that this was not significant. The diamide treatment was significantly different from the control and the DTT was not. For the mito-roGFP2-

Grx1, the garlic oil treatment showed a difference in redox status and was significantly different from the control, as was the diamide treatment. The DTT treatment was not significantly different from the control. Figure 3.19 shows that the DTT treatment result was more oxidized than the control, from looking at the box plot it shows that this is likely from one sample measurement and is possibly due to a technical error. Figure 3.18 and 3.19 shows confocal imaging of cyto-roGFP2-Orp1 at a higher quality with fluorescence intensities (figure 3.22). The distribution of the fluorescence is interesting, a high amount of fluorescence is seen on the external areas (including the gut) and the change in fluorescence with treatment appears to occur internally.

A crude mixture containing the roGFP2-Grx1 was extracted from the larvae to test reaction with diallyl polysulfides. Third instar larvae were lysed and the soluble protein was isolated from the lysate. Ultrafiltration was used to remove GSH and other thiols to prevent reaction with GRX. Garlic oil was added to the lysate and fluorescent intensities were measured to see if diallyl polysulfides react directly to the roGFP2-Grx1 probe. Figure 3.23 shows the fluorescent intensities when excited at 450nm and 485 nm. GSH and GSSG were also used to test with the roGFP-Grx1. The ratios of the results in figure 3.27 were calculated and are shown in figure 3.24 for an easier comparison between the two excitations.

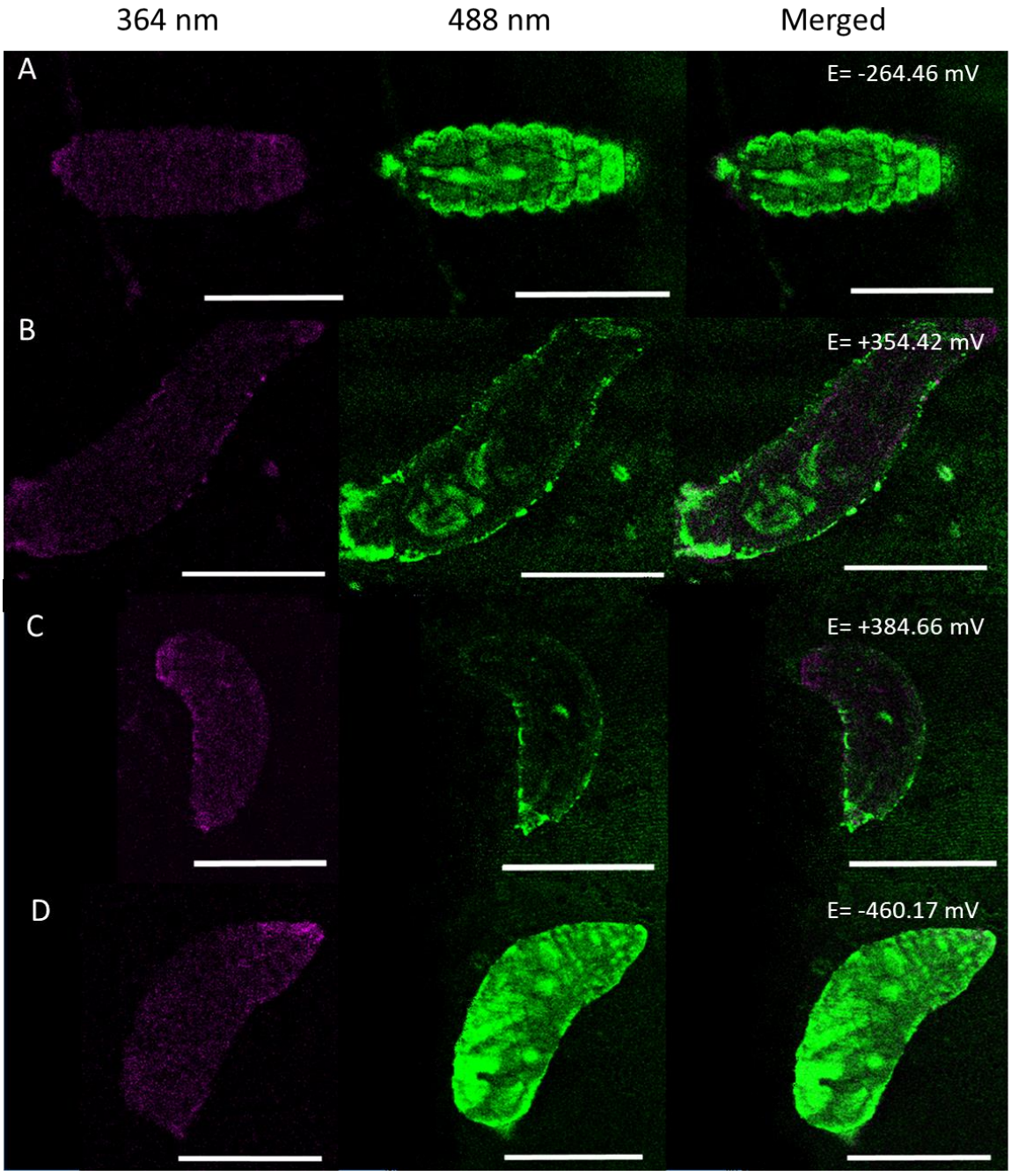

Figure 3.16: Confocal imaging and comparison of the four treatments on the mito-roGFP2-Orp1 (P015) 1stinstar *D. melanogaster*. A) untreated, B) 2% (v/v) R22 garlic oil, C) 2 mM diamide, D) 20 mM DTT. This figure shows the differences in fluorescence intensity in association to the redox potentials. With a more negative E_{roGFP2} the higher the fluorescence emitted when excited at 488 nm. Scale bar = 500 µm.

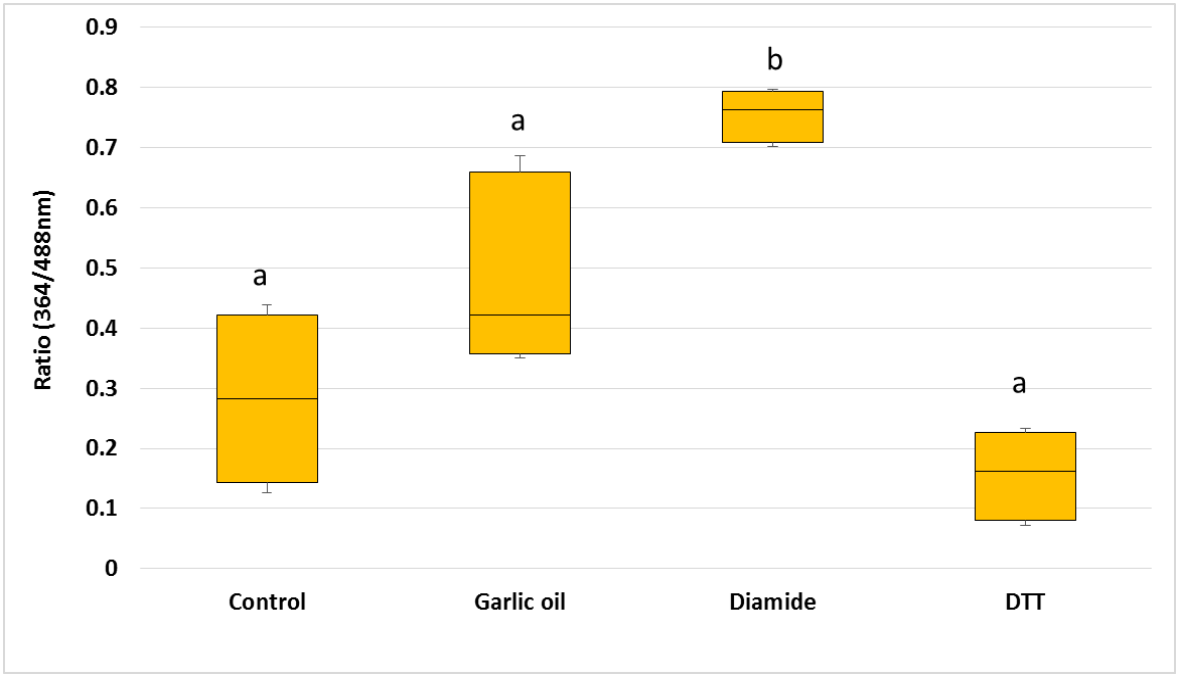

Figure 3.17: Box plots of 364/488 nm oxidation ratios of mito-roGFP2-Orp1 (P015). The diamide treatment was significantly different from the control. Boxes, lower/upper quartile; whiskers, minimum and maximum. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another ($P < 0.05$).

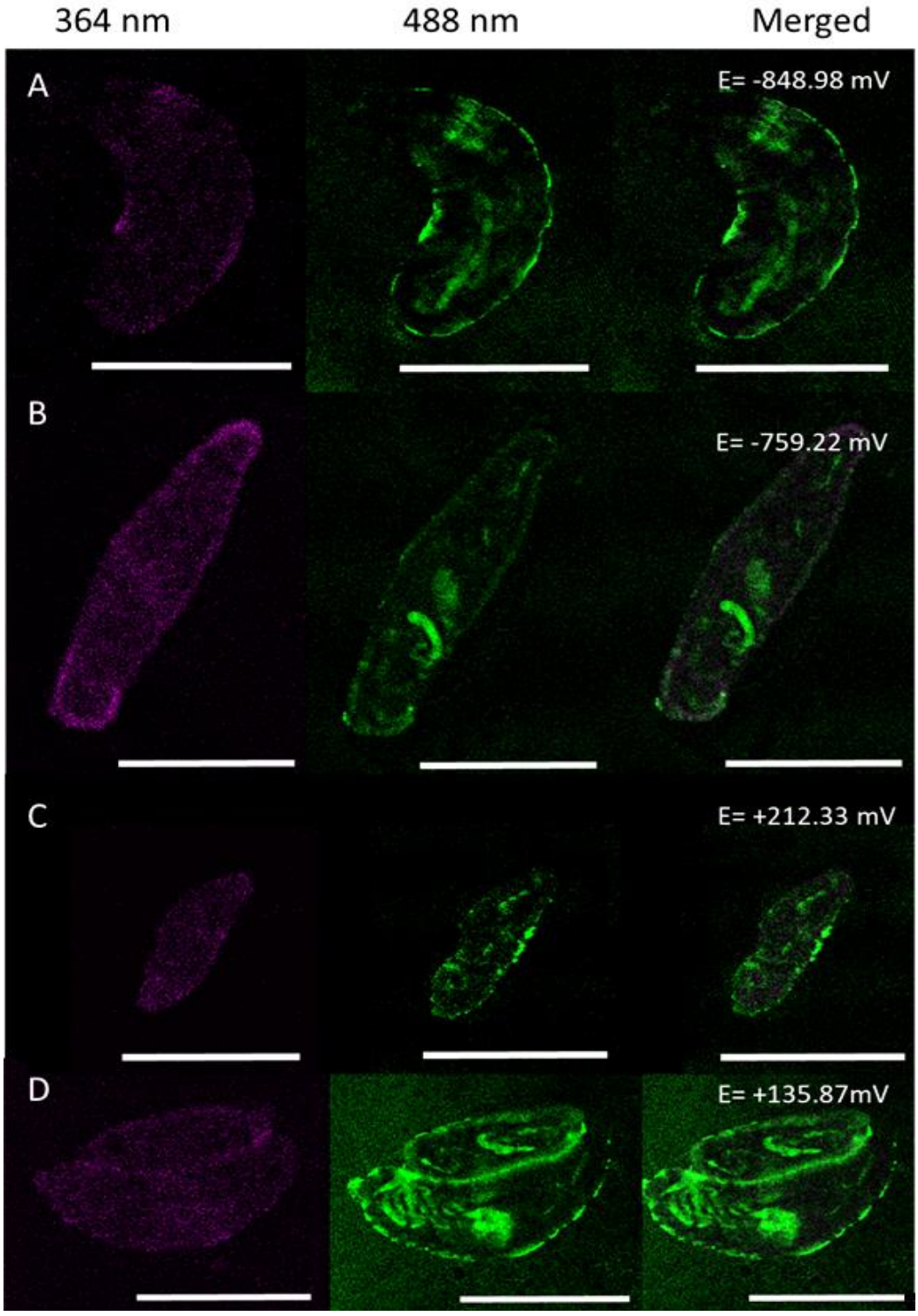

Figure 3.18: Confocal imaging and comparison of the four treatments on the mito-roGFP2-Grx1 (P003) 1stinstar *D. melanogaster*. A) untreated,B) 2% (v/v) R22 garlic oil, C) 2 mM diamide, D) 20 mM DTT. This figure shows the differences in fluorescence intensity in association to the redox potentials. With a more negative E_{roGFP2} the higher the fluorescence emitted when excited at 488 nm. Scale bar = 500 µm.

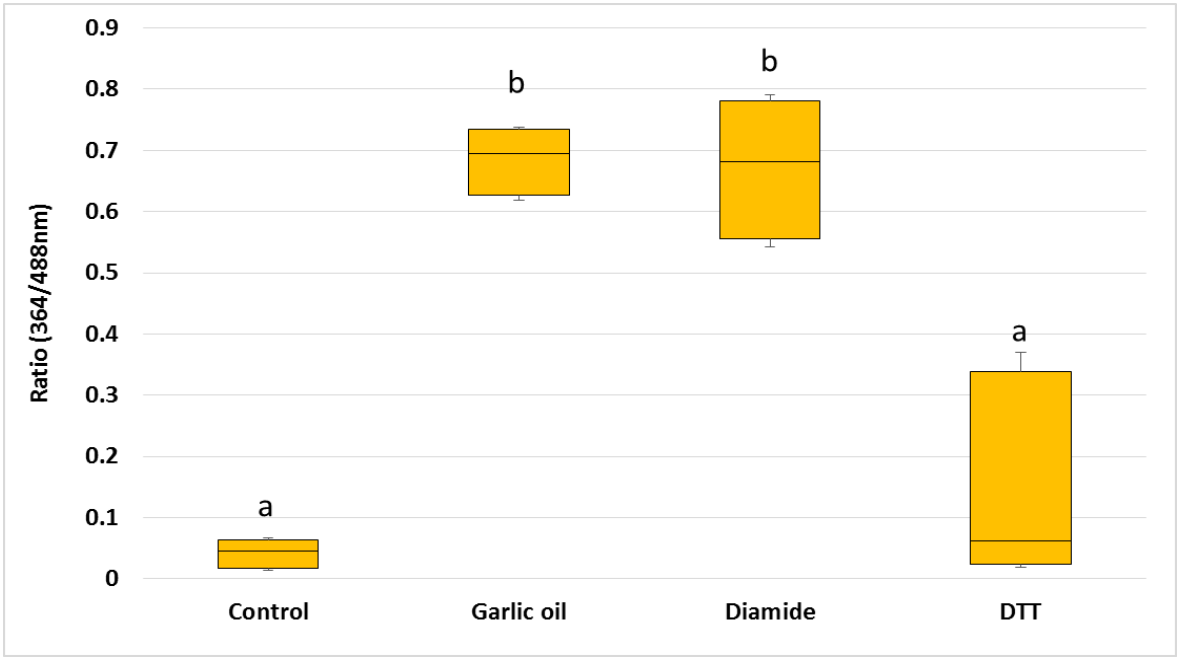

Figure 3.19: Box plots of 364/488 nm oxidation ratios of mito-roGFP2-Grx1 (P003). The garlic oil treatment showed a difference in redox status and was significantly different from the control, as was the diamide treatment. Boxes, lower/upper quartile; whiskers, minimum and maximum. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another ($P < 0.05$).

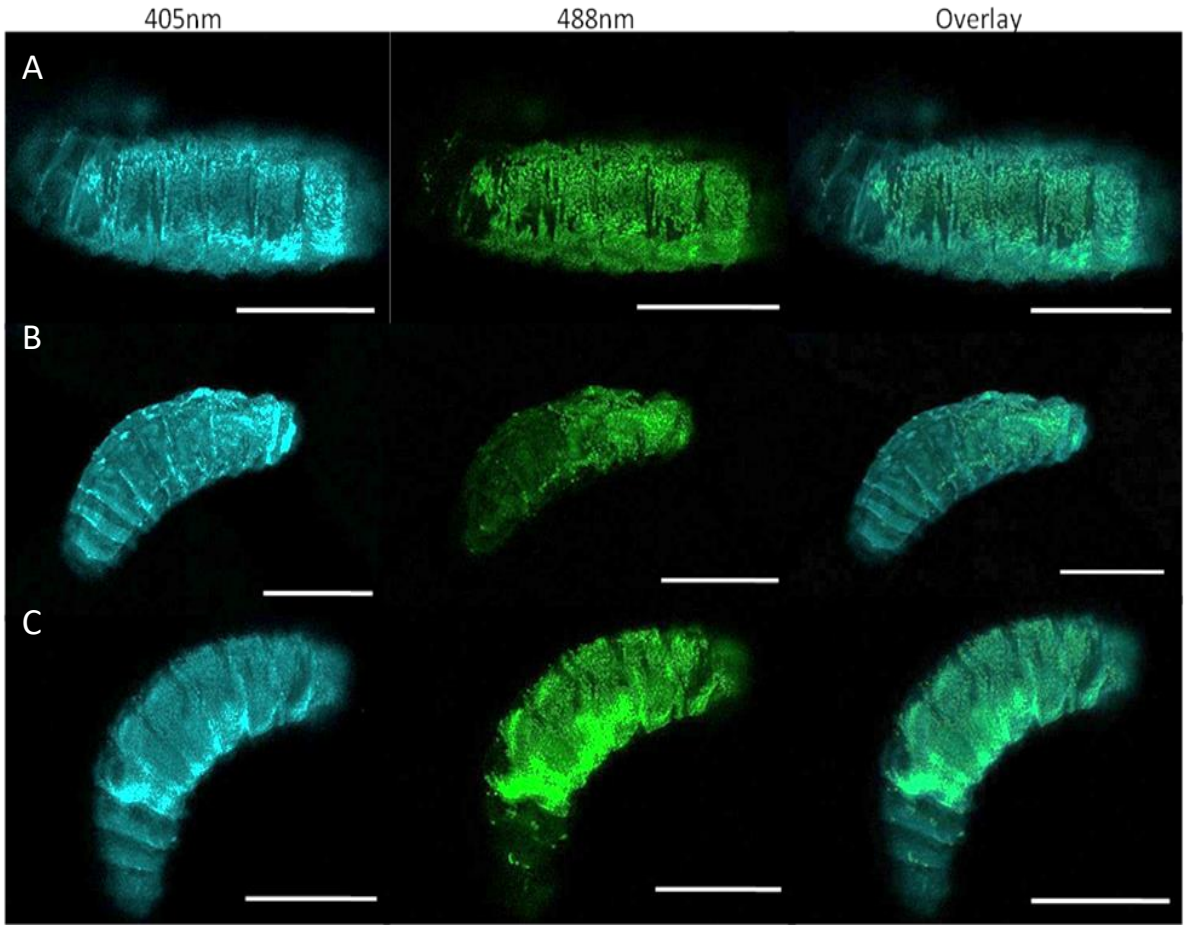

Figure 3.20: Confocal images using the Leica SP8 of cyto-roGFP2-Orp1 (P018) 1st instar *D. melanogaster* larvae which were untreated. A-C) Three replicates are shown with images taken at 405 and 488 nm with E_{roGFP2} of -231.01, -249.21, and -202.73 mV, respectively. The overlay of the images is at 50 % transparency. Scale bar = 500µm.

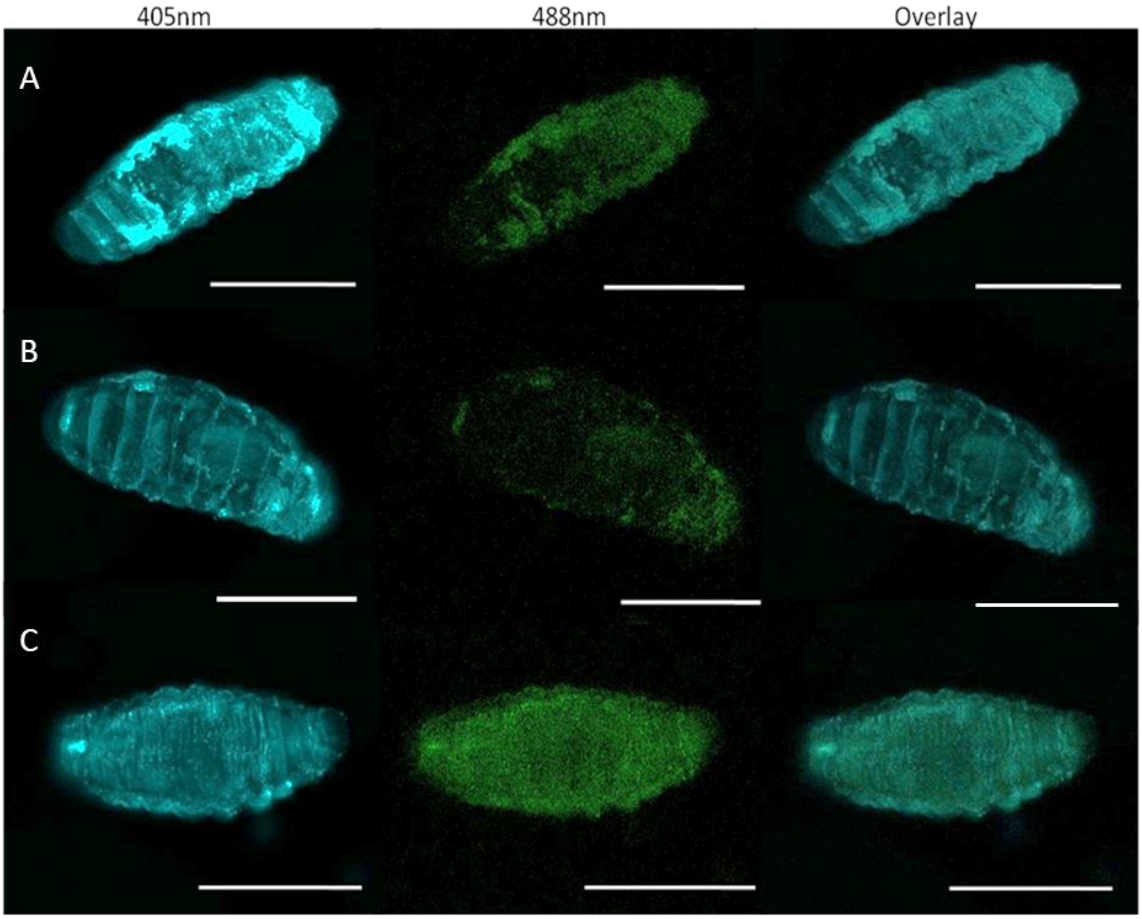

Figure 3.21: Confocal images using the Leica SP8 of cyto-roGFP2-Orp1 (P018) 1st instar *D. melanogaster* larvae which were treated with 2 % (v/v) R22 garlic oil. A-C) Three replicates are shown with images taken at 405 and 488 nm with E_{roGFP2} of +46.91, -6.41, and -0.22 mV, respectively. The overlay of the images is at 50 % transparency. Scale bar = 500µm.

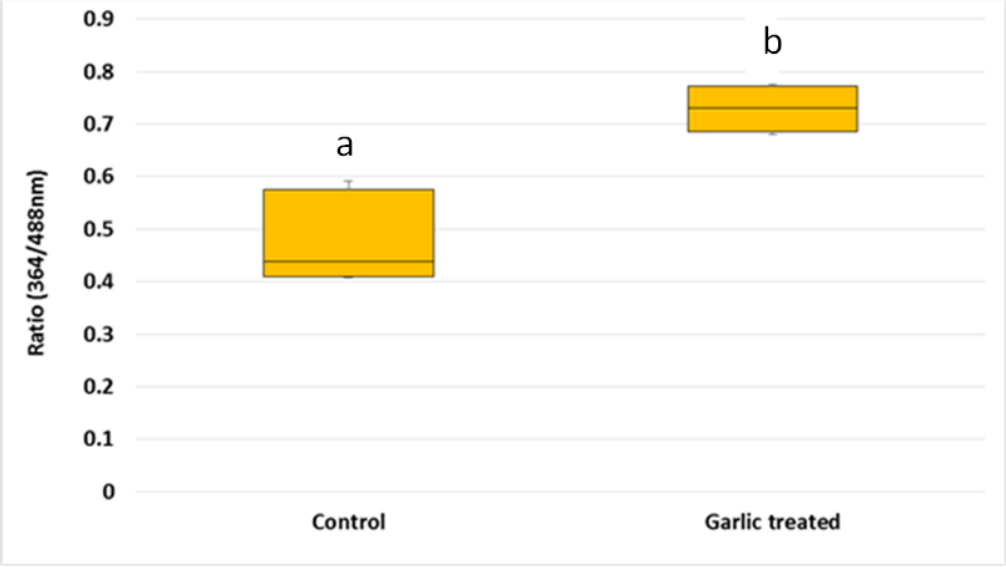

Figure 3.22: Box plots of 364/488 nm oxidation ratios of cyto-roGFP2-Orp1. The garlic oil treatment was significantly different from the control. Boxes, lower/upper quartile; whiskers, minimum and maximum. The data were analysed by one way ANOVA (P value <0.05 was considered significantly different) followed by Tukey post hoc comparisons. Differing letters (a, b, c, d, e, f) were significantly different from one another ($P < 0.05$).

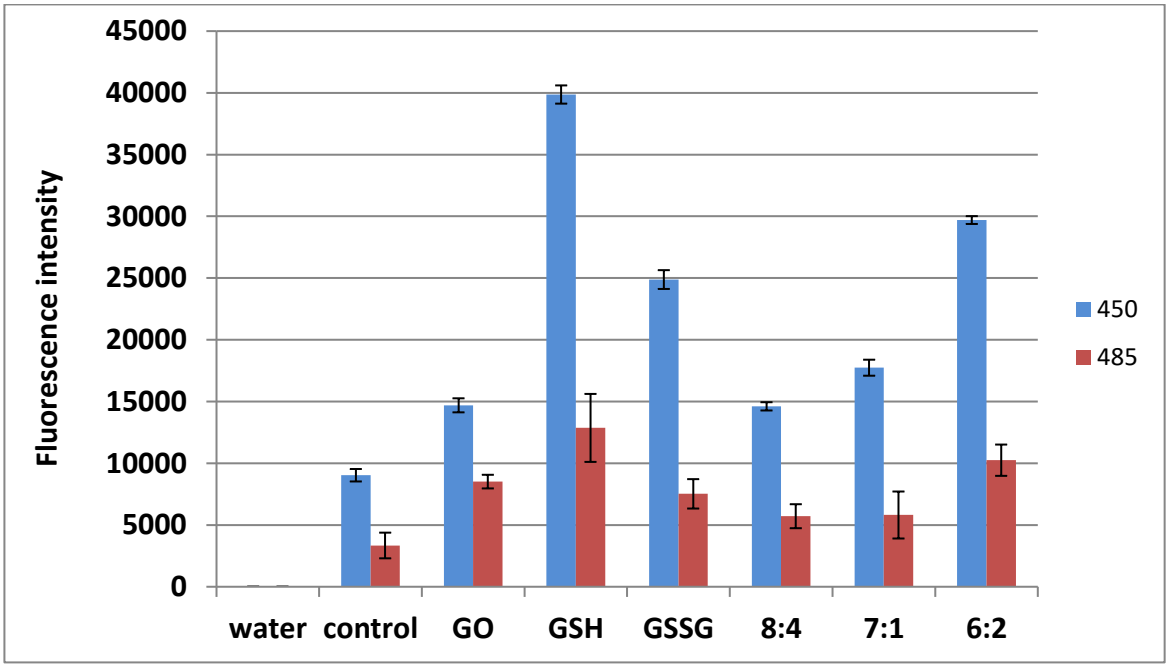

Figure 3.23: Fluorescence intensities of isolated roGFP-Grx1 with garlic oil, GSH, and GSSG. Excitation at 450 and 485nm, emission at 535nm. Control was untreated. GO, R22 garlic oil; GSH at 8 mM; GSSG at 4 mM; 8:4, 8 mM GSH and 4 mM GSSG; 7:1, 7 mM GSH and 1 mM GSSG; 6:2, 6 mM GSH and 2 mM GSSG. Error bars show ±SE.

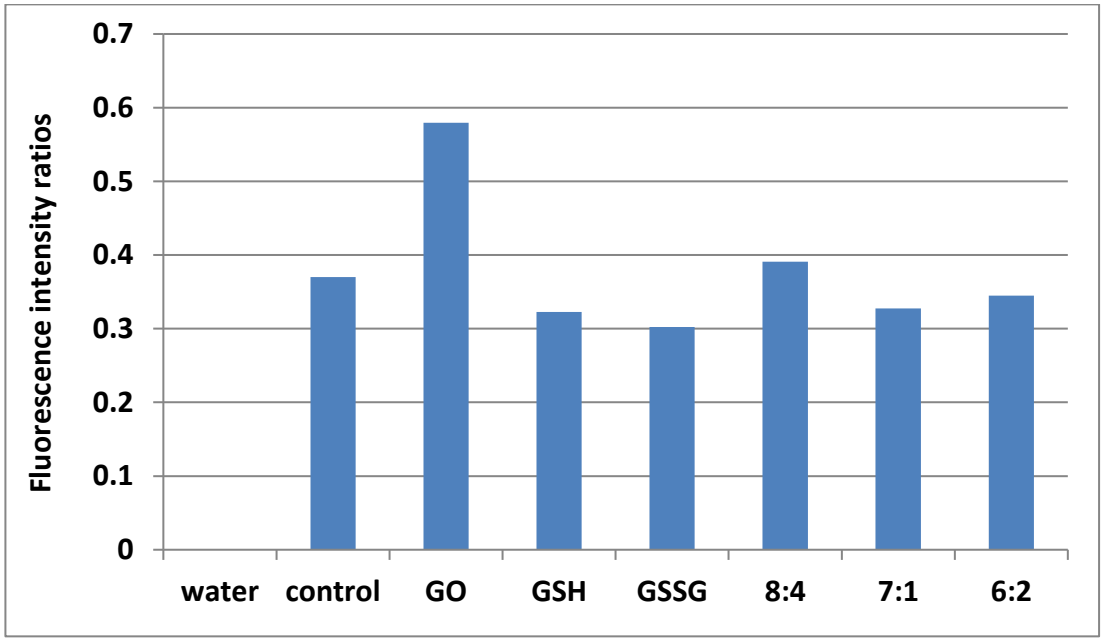

Figure 3.24: Fluorescence intensities ratios of isolated roGFP-Grx1 with garlic oil, GSH, and GSSG. Excitation at 450 and 485nm, emission at 535nm. Control was untreated. GO, R22 garlic oil; GSH at 8 mM; GSSG at 4 mM; 8:4, 8 mM GSH and 4 mM GSSG; 7:1, 7 mM GSH and 1 mM GSSG; 6:2, 6 mM GSH and 2 mM GSSG.

3.5 DISCUSSION

This chapter focused on the biochemical mode of action of diallyl polysulfides, using *D. melanogaster* as a model. One of the mode of actions of diallyl polysulfides is the increase in ROS, leading to oxidative stress which can ultimately lead to apoptosis (Munchberg, Anwar et al. 2007). To study if this occurs when treating *D. melanogaster* with diallyl polysulfides, fluorescent dyes were used to detect ROS. Amplex® Red was used to detect H_2O_2 and the results showed that H_2O_2 levels were higher when individuals were treated with Mexican garlic than china garlic oil, R22 and garlic extract. From the insecticidal studies, china and Mexican garlic oil had similar effects, which could suggest that ROS in addition to H_2O_2 are being produced in the presence of china garlic oil. Garlic extract gave no production of H_2O_2, but this was expected as garlic extract contains low levels of diallyl polysulfide. Eggs had high H_2O_2 concentrations, relative to the larval stages, which could be something to do with development and the need for more protection (Hyslop, Hinshaw et al. 1995).

Insecticidal studies of H_2O_2 were performed on *D. melanogaster* on successive life stages eggs, 1st, 2nd and 3rd instar larvae. An increase in H_2O_2 concentrations leads to specific oxidation of signalling proteins which results in a cascade of molecular events including apoptosis (Munchberg, Anwar et al. 2007). This oxidative stress would eventually lead to death of the organism which is thought to be the case when *D. melanogaster* are treated with diallyl polysulfides. Therefore, it was of interest to study the insecticidal effect of H_2O_2. Since the garlic treatment in Chapter 2 was administered externally it would be reasonable to believe the most effected cells, with an increase in ROS such as H_2O_2, would predominately occur in the external cells. Therefore, seeing the impact of treating the external cells directly with H_2O_2 would tell us what effects would occur. However, the problem of surface treatment is the physical barriers of the organisms, such as the shell of the eggs and the waxy cubicle of the larvae. In future, to complement this experiment, it would be useful to use labelled/derivatised compounds to locate areas most affected. H_2O_2 concentrations of 0-40 µM (with 5 µM increments) were used against *D. melanogaster* and this resulted in a concentration-dependent insecticidal effect. With an increase in H_2O_2 there should be higher reaction activity with low molecular weight thiols such as glutathione and cysteine (Ferrer-Sueta, Manta et al. 2011). Therefore, there should be an

increase in oxidative stress and a decrease in free thiols. There would also be an increase in reactivity with signalling proteins such as phosphatases, kinases and transcription factors, which would lead to cell death (Rhee and Woo 2011; Brigelius-Flohe and Maiorino 2013). However, another possibility is instead of an increase in H_2O_2 production, there could be a decrease in antioxidant capacity. It is possible that the diallyl polysulfides can interact with antioxidants, such as SOD, which would lead to higher levels of H_2O_2 (Han et al, 2003). Further studies of this would be very interesting to see what is actually causing the changes in ROS levels when treating with diallyl polysulfides.

Superoxide levels were studied using MitoSOX™ Red. All three garlic oils tested (Mexican, Chinese and R22) gave very similar results of increased superoxide in comparison to the control. The garlic extract gave similar results to the Amplex® Red assay, which showed a low level of increase in superoxide compared to the garlic oils.

Recently, DeLeon et al.(2016a,b) showed that polysulfide compounds from garlic can mimic ROS detection in Amplex® red, MitoSOX™ Red and roGFP assays, representing a potential confound for these assays. However, the authors reported no such effect of DCFDA (DeLeon, Gao et al. 2016; DeLeon, Gao et al. 2016).The results from my DCFDA assays to detect levels of ROS showed that Mexican garlic oil treatment in *D. melanogaster* also caused an increase in ROS detection, suggesting that the results of my assays using Amplex® red, MitoSOX™ Red and roGFP were legitimate. However, DCFDA is nonspecific and cannot with any accuracy indicate concentrations of specific ROS. A difference of the work by DeLeon and colleagues is that they used hydrogen polysulfides, whereas this study was focused on diallyl polysulfides. Conversely, diallyl polysulfides can react with low molecular weight thiols in cells and allyl persulfides and hydrogen polysulfide species can form (Jacob 2006). These sulfur species could react with the fluorescent dyes and cause a false positive result. Thus, these results are inconclusive and further studied would need to be performed to analyse the accuracy of the fluorescent dyes in the presence of diallyl polysulfides.

The fluorescent dyes were tested with garlic oil alone, it was found that the Amplex® Red did react with garlic oil. However, garlic oil with MitoSOX™ Red and DCFDA did not show any significant differences. These results further support that the results for the

MitoSOX™ Red and DCFDA assays were sound. However, it is likely that the Amplex® Red assay is not accurate due to increase in fluorescence in the presence of garlic oil.

In addition to the production of ROS, diallyl polysulfides can also react with low molecular weight thiols such as GSH and cysteine residues on proteins. GSH acts as an antioxidant by reacting with ROS. In the presence of diallyl polysulfides, GSH is reduced into GSSH and ROS generation increases(Munchberg, Anwar et al. 2007). Additionally, diallyl polysulfides can react with cysteine residues on proteins and form S-allyl modified proteins. Cysteinyl-S-allylation can result in protein dysfunction (Anwar, Burkholz et al. 2008). Diallyl polysulfides are also lipophillic and can bind to membranes and hydrophobic sites on proteins (Ariga and Seki 2006; Schneider, Ba et al. 2011).

D. melanogaster eggs, 1st, 2nd, and 3rd instar larvae were tested for their GSH and cysteine concentrations, following Mexican garlic oil treatment. Monobrombimane (mBBr) was used to fluorescently measure GSH and cysteine concentrations and to determine the effect of treatment with garlic oil. HPLC analysis was used for mBBr detection. It was found that GSH and cysteine concentrations were lower in *D. melanogaster* when treated with Mexican garlic oil than in the control. Eggs and 1st instar larvae had higher concentrations of GSH than the 2nd and 3rd instar larvae. The average GSH concentrations per egg for the control were 0.054 µmol/g and the garlic treated was 0.014 µmol/g. The reported normal GSH concentrations in *D. melanogaster* eggs are 0.015-0.03 µmol/g (Beaver, Klichko et al. 2012). Although, both concentrations fall within the normal GSH concentrations, the garlic treated eggs still exhibits much lower GSH than the control. This suggests that diallyl polysulfides in Mexican garlic oil are reacting with, and lowering, GSH, in accord with previous work (Munchberg, Anwar et al. 2007).

Mexican garlic oil treated *D. melanogaster* larvae also had lower cysteine levels than untreated controls. It was also noticed that cysteine concentrations in the untreated larvae increased with age, which is likely to be related to the different developmental stages. As with GSH, this shows that diallyl polysulfides in garlic oil could be reacting with cysteine residues, potentially leading to detrimental effects in cell leading to eventual death.

Because of their tracheal respiratory system, insects are particularly exposed to ROS and depend on high concentrations of intracellular GSH. The [2GSH]/[GSSG] ratio can serve

as an in vivo indicator for oxidative stress response in *Drosophila* as demonstrated by Sohal *et al.* (1990). To explore the *in vivo* redox processes effected by diallyl polysulfide treatment, enzyme-coupled roGFP transgenic *D. melanogaster* were used. These genetically encoded redox probes are targeted to specific subcellular compartment. The probes used were expressing a mitochondrial E_{GSH} probe (Tubulin-Gal4, UAS-mito-roGFP2-Grx1; P003), a mitochondrial H_2O_2 probe (Tubulin-mito-roGFP2-Orp1; P018) or a cytosolic H_2O_2 probe (Tubulin-cyto-roGFP2-Orp1; P015).The oxidoreductase (Grx1) probe specifically and reversibly reports roGFP2 oxidation by GSSG, and the peroxidase (Orp1) probe specifically and reversibly reports roGFP2 oxidation by H_2O_2. These probes are pH insensitive in the range of 5.5 to 8.5 (Schwarzlander, Fricker et al. 2008).

Insecticidal studies were first performed on these roGFP flies to confirm they are physiologically similar to the wildtype in terms of susceptibility to diallyl polysulfides. All life stages except pupae were studied. The pupae were excluded, as data from the wildtype insecticidal experiments showed that this stage was not susceptible to garlic treatment at any of the concentrations used. The results obtained showed that the roGFP flies had similar sensitivity to the wildtype and were hence suitable to use in the subsequent tests.

Using confocal microscopy, the redox status of GSH and H_2O_2 probes was determined. 1st instar larvae were used as they were transparent and amenable to imaging. The garlic treatment resulted in a more positive E_{roGFP2} compared to the control with the Orp1-roGFP2 probe. This suggested the oxidation of Orp1 due to an increase in an oxidizing environment i.e. an increase in H_2O_2. This indicates that diallyl polysulfides in garlic oil caused an increase in H_2O_2 levels, which was consistent with the results from the fluorescent assays. The garlic oil treatment affected redox status; however, this result was not significant.

Similar to the Orp1-roGFP2, the garlic oil treatment led to a more positive E_{GSH} with the Grx1-roGFP2 probe. This suggests a more potent oxidizing environment with an increase in GSSG and a decrease in GSH. Such changes in the GSH/GSSG redox couple states can be very damaging to the cell. The change in GSH/GSSG ratio suggests that the diallyl polysulfides in garlic oil react with GSH. This is consistent with published data (Munchberg, Anwar et al. 2007), see Chapter 1, but also the mBBr results. In both mBBr

and roGFP2 studies there were lower levels of GSH when organisms were treated with garlic oil. The distribution of the fluorescence is interesting, a high amount of fluorescence is seen on the external areas (including the gut) and the change in fluorescence with treatment appears to occur internally. This could suggest that there is higher proportion of the reduced form in environmentally exposed cells, which would facilitate protection from external harms. It would be interesting to know which parts of the organisms are affected the most. Perhaps using a more powerful microscope at higher magnification would give greater insight of what organs are largely affected by garlic treatment and give us a greater understanding of what occurs with the treatment. This could be taken even further by examining at a cellular level; comparing cells from different organs or even comparing external cells that were directly in contact with the garlic to the more internal cells.

Crude larval extracts containing Grx1-roGFP2 was tested with diallyl polysulfides, GSH and GSSG. There was not much difference between the treated samples and the control (untreated). There was likely to be other compounds still within the separated filtrate which may have caused interference. The use of GSH and GSSG did not validate the viability of the roGFP in these crude extract preparations as it would have been expected to show a more noticeable difference when compared to the control. Another possible limitation is the excitations used; 450 nm and 485 nm were used (due to filter selections on the miscroplate reader), whereas the optimal excitation wavelengths are 405 nm and 488 nm. This should not have caused much disturbance to the results as the wavelengths used still full within the excitation range shown in figure 1.20, which shows the spectra of the fully oxidised and fully reduced roGFP. This study should be repeated with more purified Grx1-roGFP2.

In conclusion, the results in this chapter gave new insight into the biochemical mode of action of diallyl polysulfides in *D. melanogaster*. We showed that treatment with garlic oil leads to an increase in ROS, and a decrease in GSH and cysteine levels. An increase in ROS levels would cause changes in cellular conditions and would require enhanced GSH activity to maintain redox status. If cellular systems cannot counteract prolonged oxidative stress and GSH levels decrease, it will result in irreversible cell degeneration and cell death (Zhang and Forman 2012). This will eventually lead to death of the organism. These

studies were the first to be done using garlic oil on insects. Knowing how they work gives confidence in the efficiency of diallyl polysulfides. This is important information as it confirms the potential of using garlic-based products as insecticides.

4 CONCLUSION

Garlic is well known for its bactericidal and fungicidal effects, which are due to the presence of organosulfur compounds that are produced when the garlic is crushed. Previous research from bacterial studies shows that diallyl polysulfides can react with glutathione, leading to the generation of reaction oxygen species (ROS) such as hydrogen peroxide. However, until the research described in this thesis it had not previously been tested whether these same biochemical activities would occur in insects. The known ROS-generating properties of garlic constituent compounds suggested that garlic products could have insecticidal effects, and hence could be used to control serious insect pests such as the cabbage root fly, which continues to be a significant problem for crops with the genus *brassica*. However, until this thesis research the details of insecticidal action in Diptera had not been investigated. The research is important because of the ever-pressing need for new and environmentally benign insecticides for the control of serious UK agricultural pests such as the cabbage root fly, spotted wing fruit fly and vine weevils, due to the toxicity problems that are associated with the use of many current insecticides.

In chapter 2 it was shown how effective garlic-based products are by performing various insecticidal experiments. Used were garlic extract, natural garlic oil and synthetic garlic oil. The polysulfide content of these garlic treatments was parallel to the insecticidal effect. Four main insecticidal methods were used; placing organisms onto treated surface, direct contact via spraying, treated surface with an open environment and fumigation. All methods were effective. Tests were performed with garlic oil with the addition of copper. The copper appeared to increase the insecticidal effect, possibly a synergic effect. Further studies into this would be of great interest as a way of maximizing the insecticidal activity of diallyl polysulfides. Studies were carried out in order to attempt at masking the potent odour of diallyl polysulfides which is unfavourable for fruit growers. Diallyl polysulfides were inserted into β-cyclodextrins; however, they had no insecticidal effect likely due to problems of entering the cells. Further research should be made into this as it would make garlic-based products more attractive to potential users.

Chapter 3 focused on the biochemical effect of diallyl polysulfides in *D. melanogaster*. First, fluorescent assays were performed and showed that the treatment of garlic oil caused

an increase in ROS such as H_2O_2 and SO_2. HPLC analysis was performed to study GSH and cysteine levels when treated with Mexican garlic oil. It was shown that garlic oil treatment caused a decrease in GSH and cysteine levels. Transgenic roGFP2 *D. melanogaster* were used to further look into these biochemical activities. It was shown that the treatment of garlic oil caused a more oxidizing environment which lead to a depletion in GSH levels and an increase H_2O_2. This further verifies the theory of the biochemical mode of action of diallyl polysulfides in insects.

My thesis research showed that diallyl polysulfides derived from garlic can be used as an effective insecticide. Using *D. melanogaster* as a model it was demonstrated that it is the diallyl polysulfides within garlic products that provide insecticidal activity and that these components work in a concentration-, rate-, and life-stage dependent manner. It was found that the higher the concentration of diallyl polysulfides, the more effective the insecticidal effect. From the results in this thesis, R22 (synthetic garlic oil) was the most effective which is likely due to the higher proportion of DAS3 compared to the other garlic oils. The eggs and adults were the most effective and the pupae were the least susceptible. This information is expected to be of great interest for crop growers as it clearly shows which life stages are optimal to target, which to avoid and thus what is potentially the most effective timing for insecticide application. It also informs as to the type of pest monitoring information needed to make decisions on the optimal insecticide application timings. Some insecticidal studies of diallyl polysulfides have been performed previously (Prowse, Galloway et al. 2006) and we significantly extended these studies here to show how these components work in various different formulations. This work was important as it demonstrated that the application of diallyl polysulfides can be adjusted within the various formulations tested, according to need. For example, direct application for eggs and larvae, spraying for adults. Importantly, the tests of different methods also showed that the perceptions by the insects of volatile garlic compounds alone are sufficient to cause lethality. This gives the potential for increased flexibility and variation in formulation, as it suggests that direct contact of the pest and garlic products is not needed for the products to be effective. It also showed that masking of garlic odours, though perhaps increasing customer palatability, would be unlikely to be useful for control. Finally, we also investigated the diallyl polysulfide content of various garlic products. It was confirmed that

it is the diallyl polysulfides that are key, this is important information as it can be used to highlight the insecticidal potential of various garlic products on the market and under development. Further research into formulations would be beneficial, such as developing garlic oils with various diallyl polysulfide content to find which is the most effective. Applying the insecticidal methods used in this thesis to a field setting would give information as to what is most suitable. Additionally, insecticidal studies performed on current pests such as *Drosophila suzukii* would greater confidence in the potential of using diallyl polysulfides to combat such pests.

Although many studies previously have been performed on the effectiveness of diallyl polysulfides as insecticides, biochemical studies of the mode of action in insects had not been conducted until the research described in this thesis. Knowing and understanding how diallyl polysulfides work and what cellular components they react provides greater insight and confidence for the commercial use of garlic products, as well as suggesting further routes for engineering increased efficiency. The results in this thesis showed that diallyl polysulfides are not only able to enter eukaryotic cells but can also react with cellular compounds. We showed for the first time that treatment with garlic oil leads to an increase in ROS, and a decrease in GSH and cysteine levels. In this thesis the average GSH concentrations per egg for the control were 0.054 µmol/g and the garlic treated was 0.014 µmol/g. Although, both concentrations fall within the normal GSH concentrations, the garlic treated eggs still exhibits much lower GSH than the control. This suggests that diallyl polysulfides in Mexican garlic oil are reacting with and lowering GSH. Diallyl polysulfides also cause changes in redox couples as demonstrated by using roGFP transgenic *D. melanogaster*. It is very likely that diallyl polysulfides interact with other thiols and cellular components and further research of the biochemical effects of diallyl polysulfides would be advantageous. These could include looking at biochemical effects with isolated diallyl polysulfides. In, chapter 2, it was shown that DAS4 had the most insecticidal effect out of the diallyl polysulfides isolated. It would be interesting to test the isolated diallyl polysulfides (DAS1-DAS6) and compare their effects on the biochemical activities studied in this thesis i.e., if they react with cellular thiols, cause increase in ROS, and cause changes in redox couple.

We were also able to conduct initial tests of further formulation of garlic products in realistic field trials in the smoke generator experiments. This highlighted some of the complexities involved in taking laboratory-based systems into the field. Though these first tests were not conclusive, they were important to show that the reliability of ignition in the products tested needed to be improved. In addition, better resolution of the relative garlic components within the smoke generators would also have been useful to determine diallyl polysulfide content, ratios and thus potential efficacy. The interaction of garlic product volatility, which we showed is important for insecticidal activity, with the smoke generators would also need to be investigated to check that the smoke dissemination was not masking activity overall. Along with this, tests of garlic component penetrance with distance from source would be useful as well as investigations of shelf life, and as a proxy, the diallyl polysulfide profile over time. Prior to wide-scale roll out, field trials on other insect models including pest species themselves would be necessary to see how effective diallyl polysulfides are in a real life setting. We concluded that the use of using smoke generators has potential value as an application method, but that further work is necessary for a full evaluation.

The results from this thesis could be used to develop new strategies for controlling pests such as the cabbage root fly and the spotted wing fruit fly. Principles that would need to be considered are (i) life stage of pest of interest, (ii) use of the right formulations, (iii) method of application and (iv) environmental conditions. In conclusion, diallyl polysulfides would make a great, natural insecticide that is low cost, effective and has lower adverse effects. Various methods can be used in their application which can benefit many crop growers. Since diallyl polysulfides are naturally occurring they would be much more affordable, and they are less likely to have adverse effects such as toxicity to human. Additionally, they would have lower shelf life compared to synthetic insecticides which would prevent associated environmental problems.

There are still many unanswered questions and this subject needs much more investigation. Future avenues such as the possibility developing combined mixtures of diallyl polysulfides with natural, already developed, insecticides; test for environmental stability, what is the half-life of diallyl polysulfide-based insecticides in field environments; the use

of additional *Drosophila*/alternative models to determine the molecular basis for the insecticidal properties of polysulfides; or more widely the use of waste materials from the agricultural production of alliums to generate oils. Future experiments should focus on developing more direct methods of analysing diallyl polysulfides in model organisms such as using fluorescent labelling of the allyl groups for *in vivo* detection of diallyl polysulfides. Additionally, more analytical data of diallyl polysulfides to investigate their chemical properties in a field environment and *in vivo*.

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6 APPENDIX

6.1 STATISTICAL ANALYSIS OF THE INSECTICIDAL EXPERIMENTS USING SPRAYING METHOD

Table 6.1: LC50s of garlic extract/oil insecticidal experiments. LC50±SE

GraFit LC50s (% ± SE)	Dipping/ pipetting	Spraying	Eggs	1st instar	2nd instar	3rd instar	Pupae	Adults
Garlic Extract	+		0.82 ± 0.09	2.17 ± 0.16	3.38 ± 0.13	2.55 ± 0.14		
		+	0.52 ± 0.17	2.74 ± 0.15	2.8 ± 0.15	3.81 ± 0.18	>5	>5
Mexican garlic oil with ethanol	+		0.15 ± 0.15	0.64 ± 0.1	1.42 ± 0.06	2.03 ± 0.2		
		+	0.23 ± 0.23	0.79 ± 0.01	1.99 ± 0.19	2.34 ± 0.1	>5	0.52 ± 0.03
Mexican garlic oil with 3% TWEEN20	+		0.57 ± 0.03	1.48 ± 0.15	1.75 ± 0.1	2.02 ± 0.07		
		+	0.45 ± 0.26	0.99 ± 0.12	1.13 ± 0.18	1.61 ± 0.17	>5	0.49 ± 0.05
China garlic oil with ethanol	+		0.54 ± 0.05	1.87 ± 0.26	1.42 ± 0.12	2.43 ± 0.21		
		+	0.62 ± 0.05	1.25 ± 0.26	1.72 ± 0.03	1.76 ± 0.25	>5	0.29 ± 0.11
China garlic oil with 3% TWEEN20	+		0.19 ± 0.15	1.12 ± 0.05	1.46 ± 0.08	2.59 ± 0.18		
		+	0.37 ± 0.05	0.56 ± 0.05	1.19 ± 0.17	2.84 ± 0.05	>5	0.26 ± 0.13
R22-TMR3 with 3% Tween		+	0.67 ± 0.3	0.98 ± 0.7	1.31 ± 0.6	1.79 ± 0.18	>5	0.75 ± 0.02

Table 6.2: P-values of garlic extract/oil insecticidal experiments

P-values	Dipping/pipetting	Spraying	Eggs	1st instar	2nd instar	3rd instar	Pupae	Adults
Garlic Extract	+		1.63E-05	4.29E-05	1.22E-03	2.36E-04		
		+	6.38E-05	2.75E-06	1.86E-05	2.02E-04	3.66E-01	2.22E-03
Mexican garlic oil with ethanol	+		1.21E-03	2.26E-05	7.78E-05	1.94E-05		
		+	7.53E-04	2.18E-05	9.50E-07	3.21E-05	3.43E-01	0.00E+00
Mexican garlic oil with 3% TWEEN20	+		2.44E-06	4.90E-07	1.39E-05	6.27E-05		
		+	1.45E-05	1.60E-06	1.41E-06	1.51E-05	9.85E-01	0.00E+00
China garlic oil with ethanol	+		0.00E+00	9.85E-05	9.50E-07	2.90E-04		
		+	2.50E-06	4.63E-06	3.44E-05	1.04E-06	5.03E-01	0.00E+00
China garlic oil with 3% TWEEN20	+		2.48E-04	6.13E-05	5.65E-06	6.50E-05		
		+	1.07E-05	2.02E-06	8.14E-06	1.24E-05	1.88E-01	2.00E-07
R22-TMR3 with 3% Tween		+	0.00E+00	6.55E-06	4.54E-05	5.50E-06	7.91E-01	5.10E-07

Table 6.3: F-values from ANOVA of garlic extract/oil insecticidal experiments

F-values	Dipping/pipetting	Spraying	Eggs	1st instar	2nd instar	3rd instar	Pupae	Adults
Garlic Extract	+		136.446	92.4	23.525	46.306		
		+	78.719	279.113	129.425	49.3	1.44	18.566
Mexican garlic oil with ethanol	+		23.595	119.665	72.651	127.228		
		+	28.75	121.56	428.31	103.871	1.51	2340.214
Mexican garlic oil with 3% TWEEN20	+		293.142	557.87	145.616	79.292		
		+	143.211	346.562	364.75	140.709	0.125	1403.945
China garlic oil with ethanol	+		399.806	66.033	428.281	42.57		
		+	290.912	226.462	101.023	411.8774	1.1	696.352
China garlic oil with 3% TWEEN20	+		45.38	80.027	209.131	78.154		
		+	161.765	316.192	180.538	152.324	1.688	793.16
R22-TMR3 with 3% Tween		+	491.518	197.058	90.305	211.406	0.623	547.533

6.2 STATISTICAL ANALYSIS OF THE INSECTICIDAL EXPERIMENTS USING NON-VOLATILE METHOD

Table 6.4: LC50s of non-volatile garlic extract/oil insecticidal experiments ± SE

Non-volatiles LC50s ± SE	Eggs	1st instar	2nd instar	3rd instar
Garlic Extract	>5	>5	>5	>5
Mexican garlic oil with ethanol	1.15 ± 0.12	1.23 ± 0.08	1.41 ± 0.11	1.95 ± 0.12
Mexican garlic oil with 3% TWEEN20	1.89 ± 0.18	1.45 ± 0.08	1.71 ± 0.14	2.10 ± 0.12
China garlic oil with ethanol	0.67 ± 0.02	0.74 ± 0.09	2.15 ± 0.24	2.47 ± 0.16
China garlic oil with 3% TWEEN20	0.59 ± 0.19	1.87 ± 0.13	1.67 ± 0.05	2.77 ± 0.13
R22-TMR3 with ethanol	0.67 ± 0.03	1.16 ± 0.12	1.34 ± 0.20	1.64 ± 0.17

Table 6.5: P-values of non-volatile garlic extract/oil insecticidal experiments

Non-volatiles P-values	Eggs	1st instar	2nd instar	3rd instar
Garlic Extract				
Mexican garlic oil with ethanol	6.88E-05	2.84E-06	3.87E-06	1.06E-05
Mexican garlic oil with 3% TWEEN20	5.29E-05	5.25E-06	5.52E-05	1.54E-05
China garlic oil with ethanol	3.00E-07	7.03E-06	9.90E-06	2.88E-05
China garlic oil with 3% TWEEN20	1.82E-05	7.82E-06	1.27E-05	3.28E-04
R22-TMR3 with ethanol	2.39E-05	5.52E-05	1.92E-05	2.32E-04

Table 6.6: F-values from ANOVA of non-volatile garlic extract/oil insecticidal experiments

Non-volatiles f-values	Eggs	1st instar	2nd instar	3rd instar
Garlic Extract				
Mexican garlic oil with ethanol	76.34	275.473	243.489	162.218
Mexican garlic oil with 3% TWEEN20	84.92	215.32	83.458	139.591
China garlic oil with ethanol	677.842	191.495	166.913	98.497
China garlic oil with 3% TWEEN20	130.748	183.473	151.175	60.169
R22-TMR3 with ethanol	117.132	83.453	127.959	298.9

6.3 CONFOCAL IMAGES

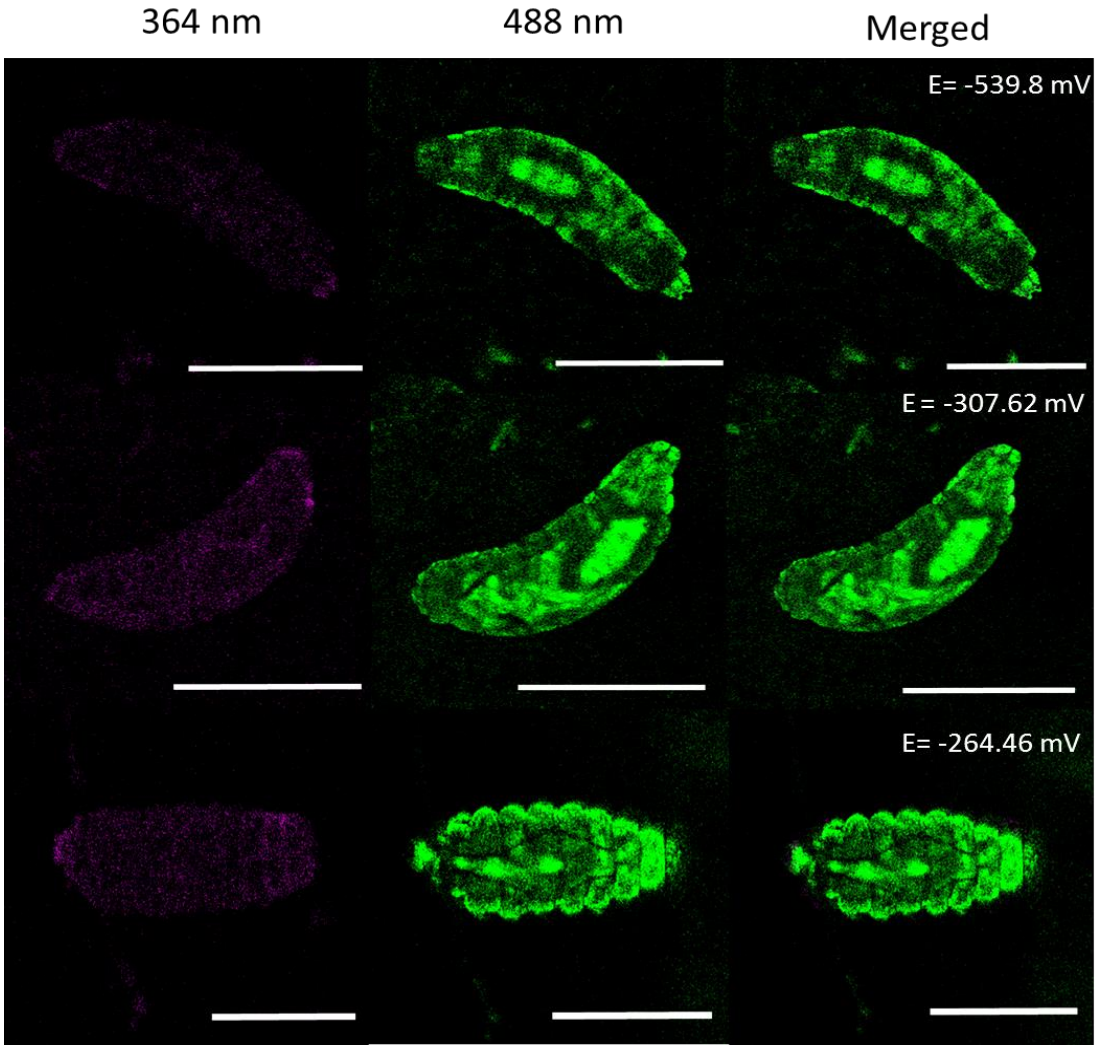

Figure 6.1: Control confocal imaging of mito-roGFP2-Orp1 1st instar *D. melanogaster*. Organisms were untreated. Shown in the figure are three replicates. E_{roGFP2} was calculated and shown with corresponding organism. The E_{roGFP2} of the organisms were -539.8 mV, -307.62 mV, and -264.46 mV. The average E_{roGFP2} was -370.62 mV. Scale bar = 500 µm.

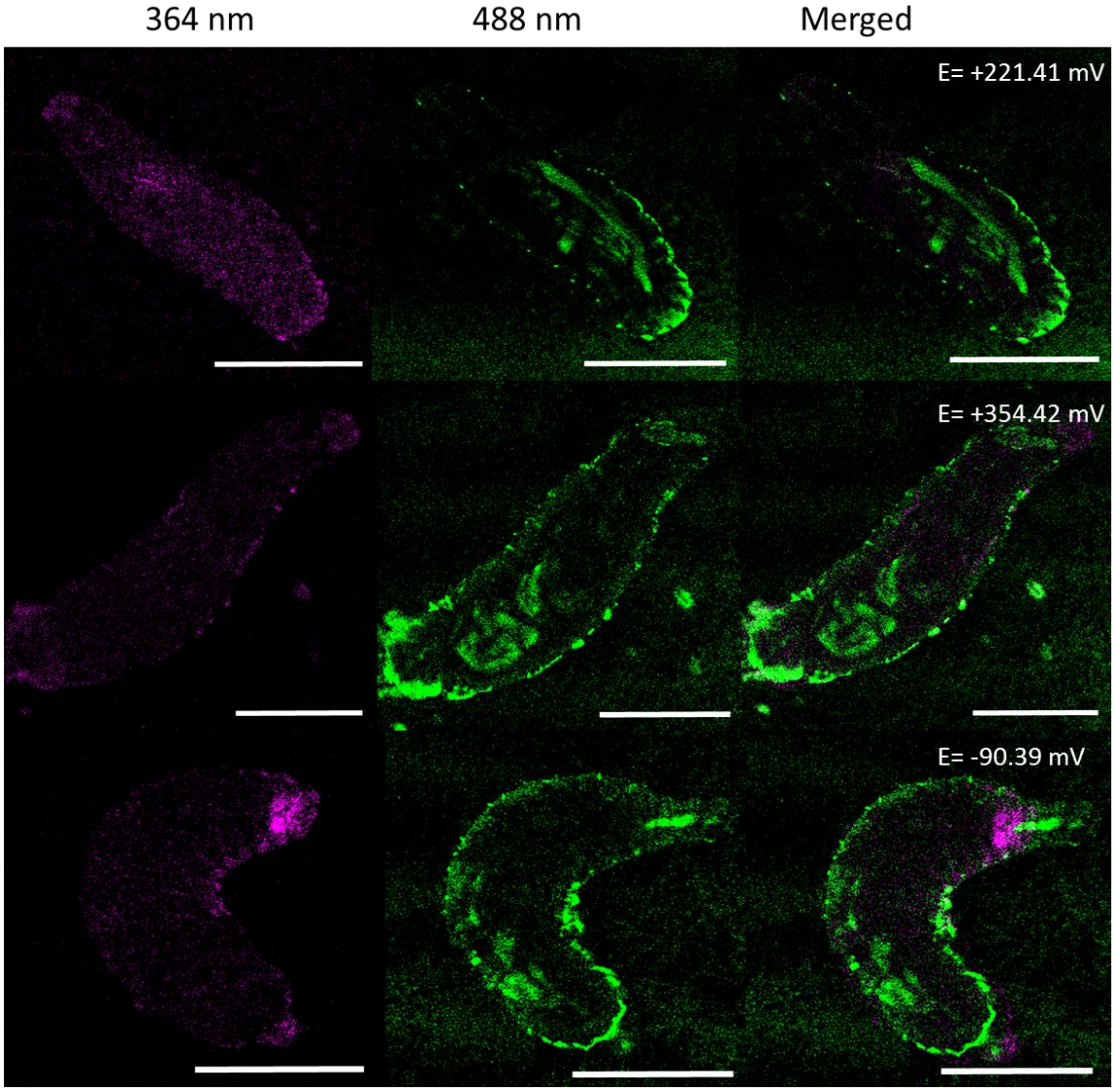

Figure 6.2: Garlic oil treated confocal imaging of mito-roGFP2-Orp1 1st instar *D. melanogaster*. Organisms were treated with 2% (v/v) R22 garlic oil. Shown in the figure are three replicates. E_{roGFP2} was calculated and shown with corresponding organism. The E_{roGFP2} of the organisms were +221.41 mV, +354.42 mV, and -90.39 mV. The average E_{roGFP2} was +151.81 mV. Scale bar = 500 µm.

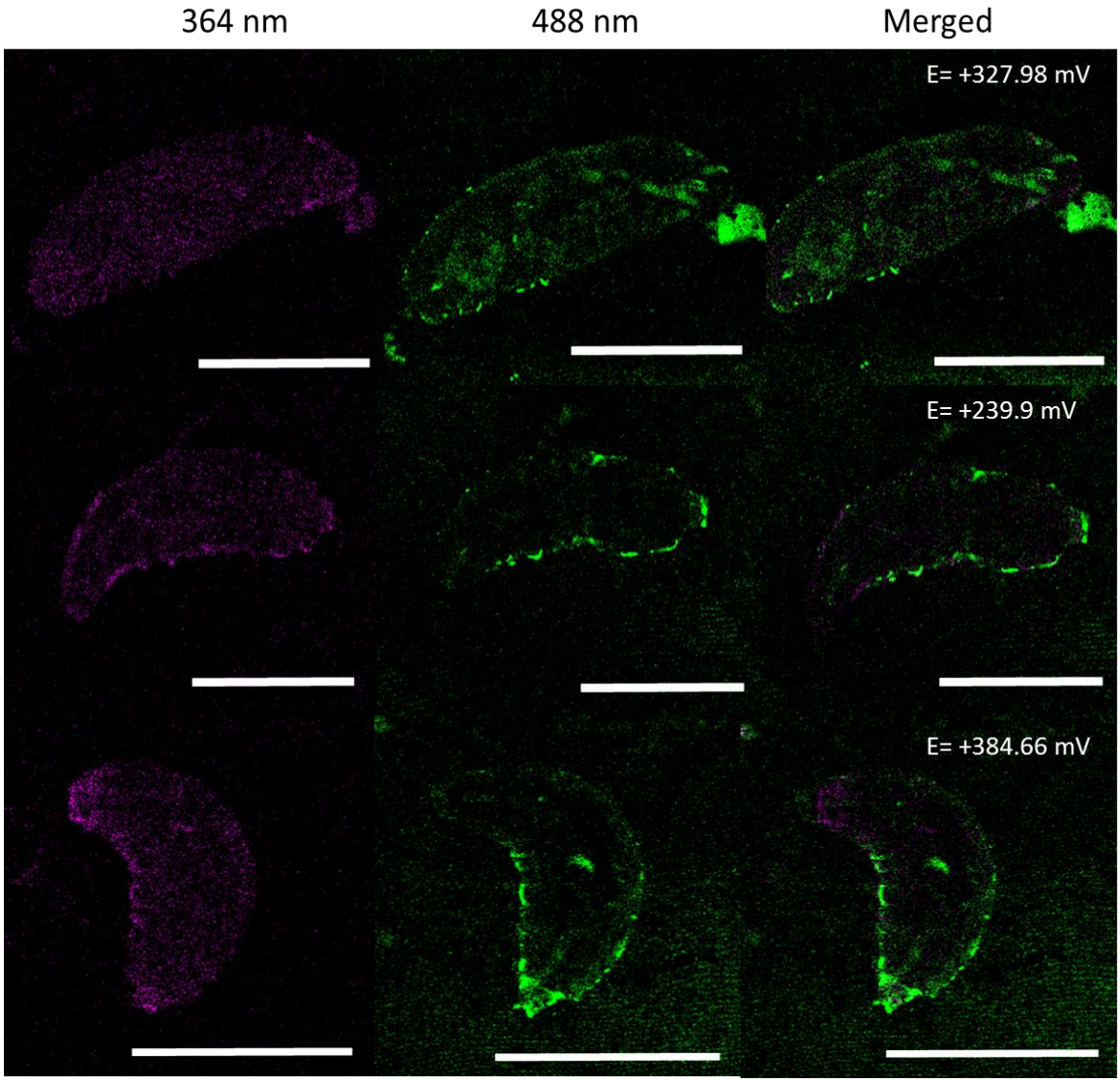

Figure 6.3: Diamide treated confocal imaging of mito-roGFP2-Orp1 1st instar *D. melanogaster*. Organisms were treated with 2 mM diamide. Shown in the figure are three replicates. E_{roGFP2} was calculated and shown with corresponding organism. The E_{roGFP2} of the organisms were +327.98 mV, +239.9 mV, and +384.66 mV. The average E_{roGFP2} was +317.51 mV. Scale bar = 500 µm.

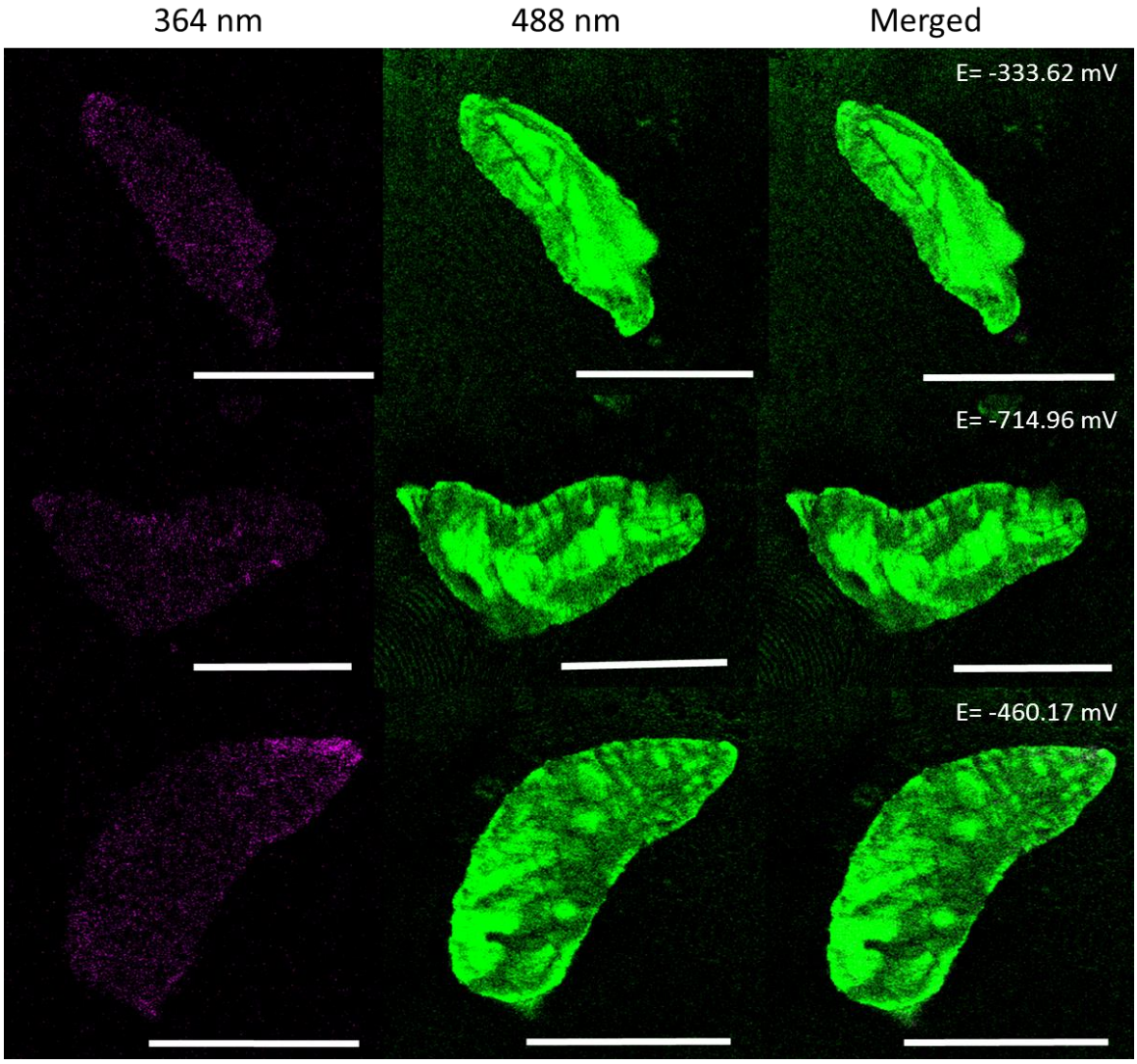

Figure 6.4: DTT treated confocal imaging of mito-roGFP2-Orp1 1st instar *D. melanogaster*. Organisms were treated with 20 mM DTT. Shown in the figure are three replicates. E_{roGFP2} was calculated and shown with corresponding organism. The E_{roGFP2} of the organisms were +333.62 mV, -714.96 mV, and -460.17 mV. The average E_{roGFP2} was -502.91 mV. Scale bar = 500 µm.

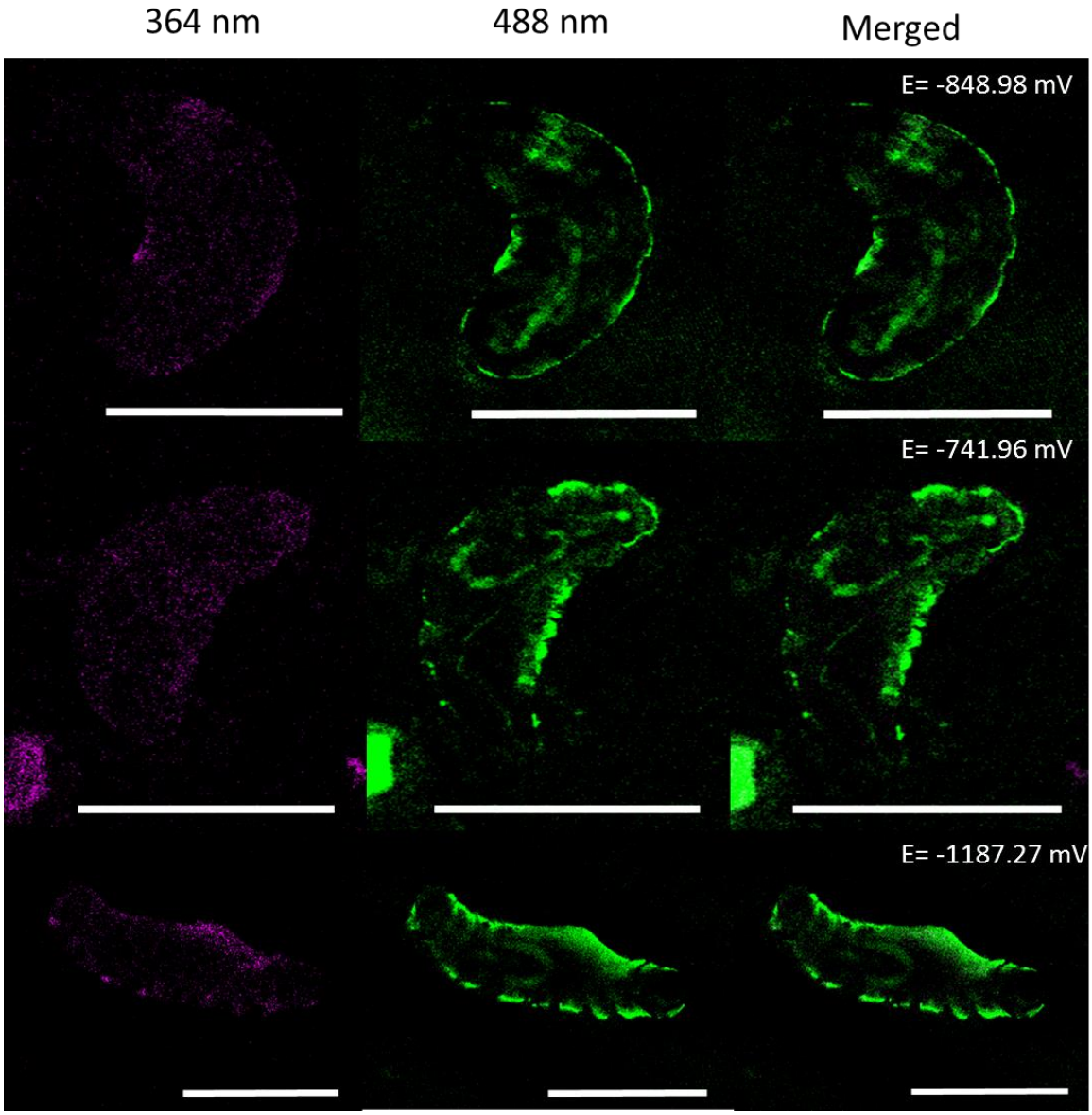

Figure 6.5: Control confocal imaging of mito-roGFP2-Grx1 1st instar *D. melanogaster*. Organisms were untreated. Shown in the figure are three replicates. E_{GSH} was calculated and shown with corresponding organism. The E_{GSH} of the organisms were -848.98 mV, -741.96 mV, and -1187.27 mV. The average E_{GSH} was -926.07 mV. Scale bar = 500 µm.

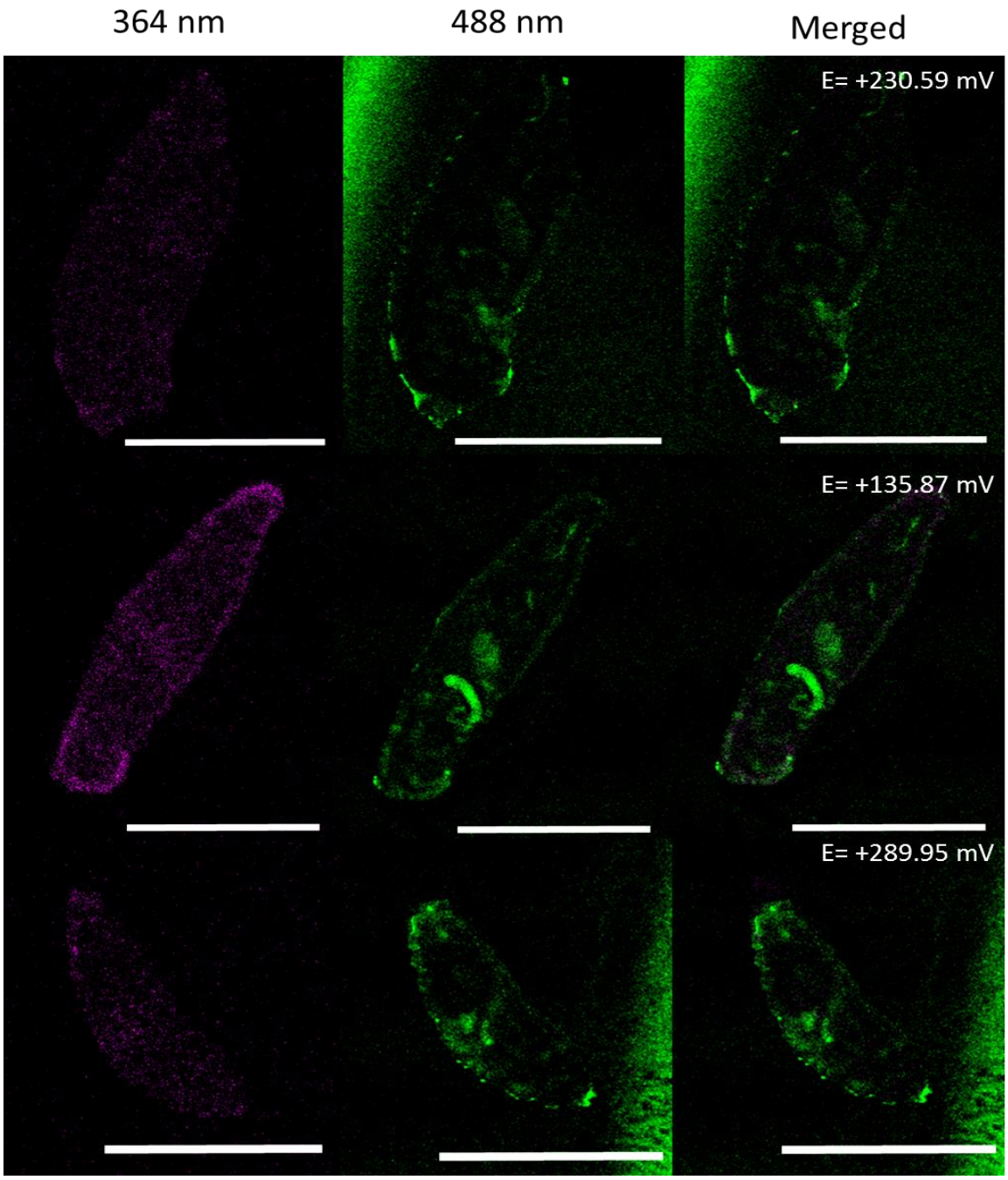

Figure 6.6: Garlic oil treated confocal imaging of mito-roGFP2-Grx1 1st instar *D. melanogaster*. Organisms were treated with 2% (v/v) R22 garlic oil. Shown in the figure are three replicates. E_{GSH} was calculated and shown with corresponding organism. The E_{GSH} of the organisms were +230.59 mV, +135.87 mV, and +289.95 mV. The average E_{GSH} was +218.8 mV. Scale bar = 500 µm.

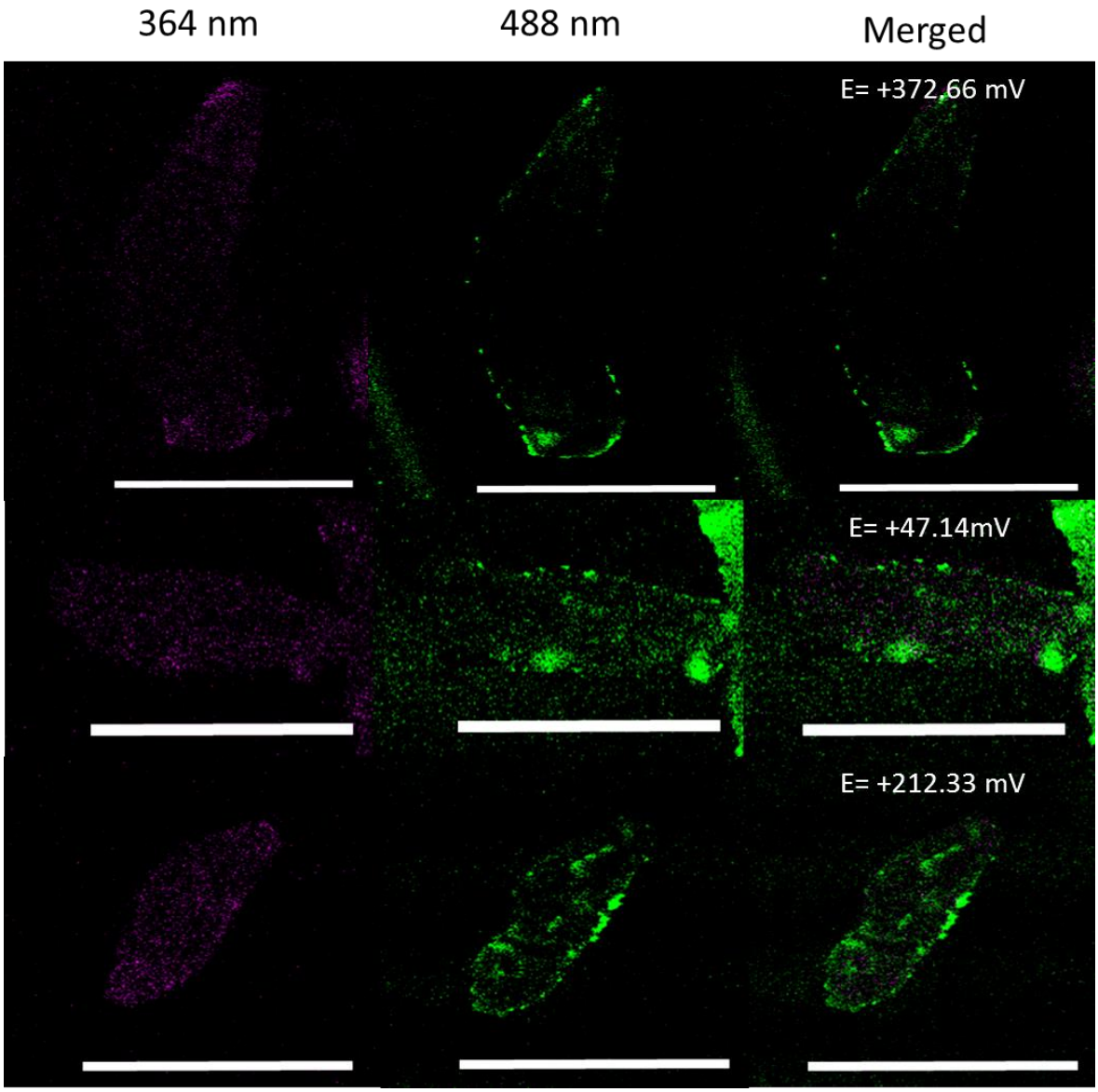

Figure 6.7: Diamide treated confocal imaging of mito-roGFP2-Grx1 1st instar *D. melanogaster*.Organisms were treated with 2 mM diamide. Shown in the figure are three replicates. E_{GSH} was calculated and shown with corresponding organism. The E_{GSH} of the organisms were +372.66 mV, +47.17 mV, and +212.33 mV. The average E_{GSH} was +210.71 mV. Scale bar = 500 µm.

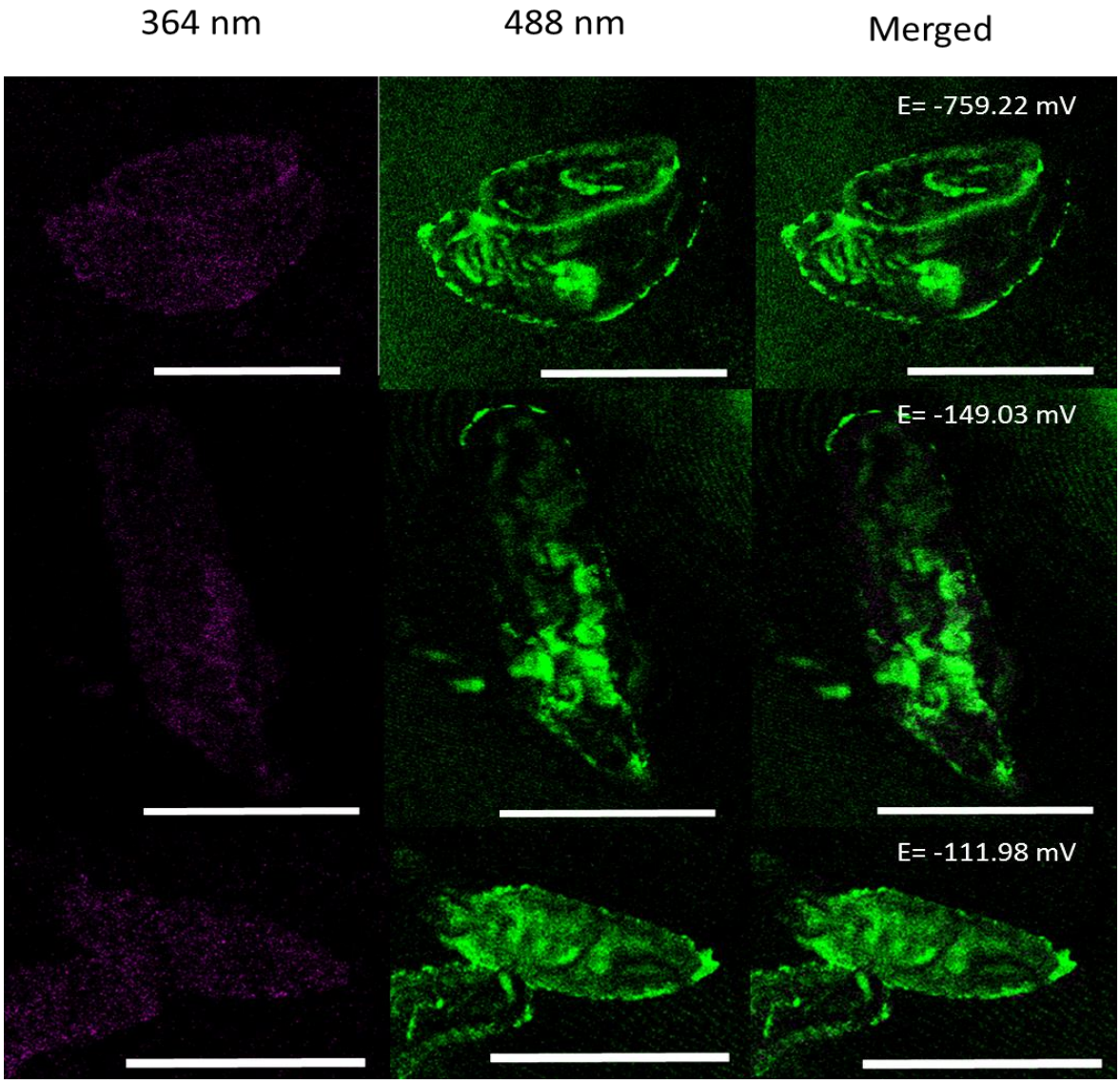

Figure 6.8: DTT treated confocal imaging of mito-roGFP2-Orp1 1st instar *D. melanogaster*. Organisms were treated with 20 mM DTT. Shown in the figure are three replicates. E_{GSH} was calculated and shown with corresponding organism. The E_{GSH} of the organisms were +759.22 mV, -149.03 mV, and -111.98 mV. The average E_{GSH} was -340.076 mV. Scale bar = 500 µm.

6.4 BOXES USED FOR INSECTICIDAL STUDIES

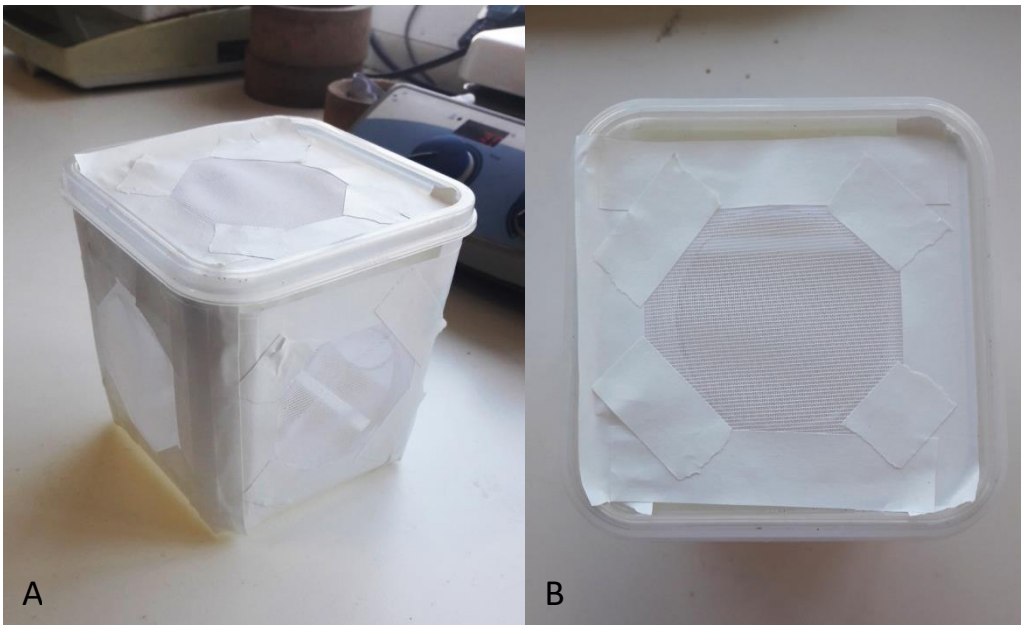

Figure 6.9: Images of the boxes used for the fumigation method. Holes were cut on the sides and covered in mesh.

Figure 6.10: Image of boxes used for pupae and adult studies.