Developmental Toxicity of Aluminium and Silver to *Drosophila melanogaster*

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Abstract

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Aluminium (Al) and silver (Ag), through human activities, are present in the environment at concentrations sufficient to cause toxicity. The aim of this study was to administer Al and Ag to the short lived model organism *Drosophila melanogaster*, so that developmental toxicity and potential ameliorative interventions could be examined over a compressed timescale relative to mammalian models.

Aluminium was administered to *Drosophila* in food as either the chloride salt or citrate complex at concentrations of 1, 10 and 100 mM and various developmental parameters were assessed. The lowest concentration to delay pupation relative to the control was 10 mM but this depended upon the food in which it was administered. Higher whole body tissue levels of Al were seen following Al citrate administration compared to $AlCl_3$, but Al citrate was less toxic as this did not did not impair larval viability at 100 mM; 100 mM $AlCl_3$ resulted in 100% mortality. Eclosion success was significantly impaired with either form of Al at 10 mM, but no difference was seen between the forms of Al. When *Drosophila* were fed $AlCl_3$ over their entire lifespan, a small but significant reduction in the lifespan of male flies was seen. No behavioural toxicity could be demonstrated. Existing studies have demonstrated significant tissue Al concentrations and toxicity whereas these have been minimal in this study. It is suggested that these differences may have a genetic component, with food composition exerting an influence also.

Silver, either as $AgNO_3$ or Ag nanoparticles (AgNPs) was administered in concentrations up to 500 μM and 10 mM, respectively. Either form of Ag, at 50 μM was sufficient to significantly retard pupation rate, although pupation or eclosion success was not impaired until 100 μM. The concentration-response relationship for $AgNO_3$ was steep with pupation success dropping to nearly zero by 300 μM; *Drosophila* in this study were far more sensitive to $AgNO_3$ than those in other reports. Animals exposed to AgNPs were still able to pupate at 500 μM, but these pupae were almost all non-viable when exposed to 400 μM AgNPs. At 1 mM and above, AgNPs, however, showed reduced toxicity compared to lower concentrations. The reasons for this are unclear. Both forms of Ag caused de-pigmentation in adults after larval exposure that may be explainable by inhibition of polyphenol oxidase enzymes by Ag (I) ions. The de-pigmentation was preventable by pre-loading larvae with Cu. Ascorbate prevented the de-pigmentation caused by AgNPs but not $AgNO_3$ suggesting that AgNP toxicity is due to Ag (I) ion release. Oxidation of AgNPs was found to be greatly accelerated by Fe (III) and Cu (II) ions in the presence of Cl^- ions. Although some of the results here conflict with the literature, developmental toxicity has been observed here, for both Al and Ag, and the variability across studies may provide an opportunity for dissecting the mechanisms behind Al and Ag toxicity through identification of the traits that confer sensitivity or resistance.

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Abbreviations

AEL	After egg laying
AgNP	Silver nanoparticle
ATP	Adenosine triphosphate
BAF	Bio-accumulation factor
BBB	Blood-brain barrier
CI	Confidence interval
CNS	Central nervous system
DAE	Dialysis associated encephalopathy
DNP	Dinitrophenol
GI	Gastrointestinal
HAS	Hydroxyaluminosilicate
HR	Hazard ratio
ICP-AES	Inductively coupled plasma atomic emission spectroscopy
JH	Juvenile hormone
LSPR	Localised surface plasmon resonance
MTD	Medium throughout development
NP	Nanoparticle
PVP	Polyvinylpyrrolidone
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TEM	Transmission electron microscopy
YP	Yeast pre-feeding

Chapter 1. Introduction

The Earth's crust contains a huge range of metals, many of which play a pivotal role in biochemistry (Cowan 1997). However, despite the biological essentiality of many metals, some are toxic. Toxic mechanisms vary but may involve mimicry of an essential metal (Astrin *et al*. 1987), binding to proteins and inducing conformational shifts in their structure (Macomber and Hausinger 2011), or induction of oxidative stress (Halliwell and Gutteridge 2007). The two metals under study in this thesis are aluminium (Al) and silver (Ag).

While a huge volume of literature surrounding the toxicity of Al and Ag exists, much of what is known centres on aquatic or microbial systems. Information relating to terrestrial animals contains many gaps and inconsistencies. Aluminium neurotoxicity to mammals, for example, is only obvious after parenteral administration (Parkinson *et al*. 1981), leaving its potential role in the neurodegenerative Alzheimer's disease, following long term exposure to low levels of the metal, controversial (Reusche 2002, Gupta *et al*. 2005). Environmental acidification is the main source of bioavailable Al for many organisms although deliberate human exposure occurs through its use in food additives and pharmaceuticals (Krewski *et al*. 2007). As a poison in humans, Ag has received little interest, with the condition of argyria considered disfiguring but otherwise inconsequential (Lansdown 2007), and subtle effects of Ag toxicity may pass unnoticed, despite the possibility that it may contribute to more serious pathologies. Historically, film photography was responsible for much of the Ag use (Purcell and Peters 1998), but recent technological advances in digital photography have considerably reduced demand, by more than 50% since 1999 (The Silver Institute 2009). However, Ag is increasingly used in the form of nanoparticles (NPs) (Project on Emerging Nanotechnologies 2013), and this necessitates not only the study of these novel particles but also the bulk material and its compounds, as the toxicity of silver nanoparticles (AgNPs) is in part rooted in their dissolution (Kittler *et al*. 2010).

As toxicants to terrestrial species, Al and Ag do not possess the notoriety of metals such as mercury (Hg), cadmium (Cd) and arsenic (As), and their effects may be less immediately obvious and therefore more difficult to identify and quantify. Furthermore, most work on Al and Ag involves acute or medium term studies, leaving questions regarding lifelong

exposure, especially through sensitive developmental periods, open. Such characterisation is essential as, for several reasons, developing organisms are often more vulnerable than mature organisms to toxic insults. Firstly, as the structure and function of the organism is most dynamic within this period, any interference with a process may have long-term consequences as it cannot be mitigated at later time, even after the poison has been removed (Kalia 2008). For instance, changes in brain microanatomy and neuronal connectivity after lead (Pb) exposure are permanent (Bellinger 2000, Lidsky and Schneider 2003), and epigenetic alterations would be carried for life and possibly passed to the next generation (Senut *et al.* 2012). Secondly, structures that protect sensitive organs, e.g. blood-brain barrier (BBB), when immature, may be more permeable to toxicants (Adinolfi 1985, Grandjean and Landrigan 2006). Finally, the high metabolic rate and cell turnover of many organ systems during this period creates an increased number of potential targets. This thesis aims to address some of the knowledge gaps regarding the developmental toxicity of Al and Ag to the model organism *Drosophila melanogaster*.

1.1. Aluminium

1.1.1. Biogeochemistry

Aluminium is the dominant metal in the earth's crust (8% by mass) and is the second member of Group 13 of the periodic table with an atomic number of 13. Under conditions compatible with life Al only exists in the +3 oxidation state; it is not redox active (Downs 1993). Aluminium is too reactive to be found naturally in its metallic state, but Al minerals abound (Cox 1989). Newly emerged crustal Al deposits are weathered by both chemical and physical processes, and as these minerals are modified, they pass through a succession of chemical forms, ultimately forming minerals of negligible solubility such as gibbsite, clays, or hydroxyaluminosilicates (HAS). Hydroxyaluminosilicates are especially notable for controlling Al dissolution through both thermodynamic and kinetic mechanisms (Exley 2003). Other mineral phases that bind Al include phosphates and hydroxides. However, the relative lithospheric abundance of silicon (Si) over phosphate ensures that the majority of Al is bound by Si and that phosphate remains unbound, soluble and available to biological processes (Exley *et al.* 1993).

The chemistry of Al dictates its solubility in various physiological and environmental media and gives a chemical basis for its binding to biological targets. Aluminium has a

high affinity for oxygen-bearing ligands, and in aqueous solution it co-ordinates six water molecules. It is strongly hydrolyzing owing to its high charge density, and increasing pH results in successive loss of protons from aqua-ligands resulting in a predominance of $Al(OH)_4^-$ ions above pH 7.5. At pH ~6, the majority of Al is found as the triple de-protonation product $Al(OH)_3$, which has minimal solubility, and total dissolved Al is unable to exceed 1 µM and with negligible amounts of Al^{3+} (Martin 1988). Whether the undissociated complex, $Al(OH)_3^0$ (aq) exists at all is doubtful (Dezelic *et al*. 1971), but should it occur then its relevance is unknown although its electro-neutrality may allow it to pass through hydrophobic milieu.

The universality of Al minerals within soils and rocks creates a vast reservoir of the metal, but this is largely confined to the solid phase by virtue of its insolubility at circum-neutral pH. Direct industrial extraction of Al from tri-hydroxide ores causes a small increase in the environmental levels of soluble Al species (Martin 1988). More important is the inadvertent solubilisation of Al minerals through anthropogenic environmental acidification. Acidification may have several causes, but two of the most significant are acid mine drainage and acid deposition. Mining of mineral deposits, including coal, exposes iron pyrite containing overburden. Pyrite minerals are oxidised by atmospheric oxygen (O_2), releasing not only iron (III) (Fe (III)) ions but sulphuric acid that can leach away (Akcil and Koldas 2006). The combustion of coal liberates large quantities of sulphur dioxide (SO_2) and nitrogen oxides (NO_x) that, upon dissolution in rain water, form strong mineral acids (Hordijk and Kroeze 1997).

In addition to anthropogenic acidification, some environments are naturally acidic. Inorganic acids from natural sources include those released from iron pyrite oxidation in naturally exposed rock (Eppinger *et al*. 2007) and emissions from volcanic activity (Lohr *et al*. 2006). Environmentally important organic acids include humic acids, fulvic acids and low molecular mass acids such as citric, oxalic and lactic acids. The effects of these organic acids upon Al release from minerals are more complex than those of the mineral acids because of the large number of acidic species and their differing abilities to chelate metal ions and therefore shift solubility equilibria (Vance *et al*. 1995).

1.1.2. Toxicokinetics

1.1.2.1. Exposure, absorption and bioavailability

The bioavailability (proportion of the administered dose that is absorbed by the tissues) of Al is substantially affected by its route of administration and its speciation. Several routes of exposure to Al are available. Al is a potent surface acting poison of the gills in aquatic organisms (Poleo 1995, Exley *et al*. 1991) and need not be absorbed to produce deleterious effects (Dietrich and Schlatter 1989). Most systemic exposure in animals occurs through ingestion of contaminated water or food; as such, systemic uptake across the gill will not be considered here. Humans expose themselves to Al, sometimes in large concentrations, through its use in water purification, in cosmetics, as a food additive, and in pharmaceutical preparations (Krewski *et al*. 2007).

Although not the commonest route of exposure, intravenous or intramuscular administration results in the highest levels of bioavailability. Such exposure is known to occur in two situations. The first of these is contamination of dialysate used in haemodialysis procedures although the risks from this are much reduced today (Kerr *et al*. 1992). Perhaps of more concern is the use of Al to accentuate the immune response to an antigen. This property means Al has found application in many commercially available vaccines. Of course, although bioavailability would near 100%, actual absorption would be protracted as the intramuscular injection amounts to a depot preparation of poorly soluble Al. A particular issue with the use of Al in vaccines is that the target population is often infants or children, whose bodies are yet to fully develop and any toxic effects may have far reaching and permanent consequences (Tomljenovic and Shaw 2011, Tomljenovic and Shaw 2012). Another parenteral route of possible significance to the human population is transdermal absorption from antiperspirants (Exley 1998, Exley 2004, Guillard *et al*. 2004).

Despite the inherently high bioavailability of invasive parenteral administration, most chronic exposure to Al occurs through the enteral route. Reported oral bioavailability for Al varies widely from 0.001 – 24% in humans and varies inversely with the ingested dose (Greger 1993). The highest doses are received by renal patients receiving Al hydroxide phosphate-binders although these are being replaced where possible (Arenas *et al*. 2008). Most human exposure occurs through drinking water and food additives although

determining an accurate exposure history is notoriously difficult (Flaten 2001, Krewski *et al*. 2007). The mechanisms of Al absorption are poorly understood. Both transcellular and paracellular mechanisms have been proposed for the gastrointestinal (GI) absorption of Al into the plasma and which of these predominates is likely to be influenced by location within the GI tract, pH and available ligands (Devoto and Yokel 1994, Greger and Sutherland 1997). Although the acidic environment of the mammalian stomach and proximal duodenum would solubilise a large fraction of ingested Al, this could not easily be absorbed across cell membranes due to the high charge density and lipophobicity of the Al (III) ion, meaning that additional factors are required to facilitate absorption.

Active transcellular absorption processes have been proposed based upon *ex vivo* observations that adenosine tri-phosphate (ATP) depleting interventions, such as administration of dinitrophenol (DNP) (Feinroth *et al*. 1982), cyanide ions (Cochran *et al*. 1990), and glucose restriction (Feinroth *et al*. 1982), limit Al passage through the intestinal wall. However, the inhibitory effects of DNP could not be replicated in an *in vivo* model (Provan and Yokel 1988). Similarly, investigations of agents that affect calcium (Ca) channels have produced mixed results, leading to controversy over mechanisms that involve the sharing of Ca transporters (Provan and Yokel 1988). A passive, non-saturable, paracellular mechanism that depends upon a favourable electrochemical gradient for Al^{3+} has been proposed whereby Al is absorbed into the plasma by passing between enterocytes and directly through the interstitial fluid (Provan and Yokel 1988). Several studies (Powell and Thompson 1993, Cunat *et al*. 2000, Zhou and Yokel 2005, Aspenstrom-Fagerlund *et al*. 2009) confirm that this is the major contributor to Al absorption, accounting for up to 90% of the total uptake (Cunat *et al*. 2000). This is consistent with the notion that Al^{3+} ions, in terms of charge density, size, and coordination geometry, most closely resemble magnesium ions (Mg^{2+} ions), rather than Ca^{2+} or Fe^{3+} (Katz *et al*. 1996, Macdonald and Martin 1988); magnesium ions are absorbed into the plasma via a paracellular mechanism (Berthon 2002, Quamme 2008).

Complexation of Al by citrate significantly increases its bioavailability (Glynn *et al*. 2001). It has been proposed that this occurs in acidic compartments of the alimentary canal through the formation of an electro-neutral, lipophilic species that can therefore enter enterocytes (Martin 1986); nonetheless absorption from the stomach is poor because of a low surface area and thick walls (Whitehead *et al*. 1997). As such, there has been some

debate as to the mechanism by which citrate facilitates absorption of Al. Many workers are of the view that the lipophilic complex described above is responsible (Devoto and Yokel 1994), but it is now known that, by opening the tight junctions through sequestration of Ca^{2+} ions, citrate is able to enhance intestinal paracellular absorption (Devoto and Yokel 1994, Greger and Sutherland 1997, Taylor *et al*. 1998, Zhou and Yokel 2005). This is consistent with the finding that most Al absorption in mammals occurs intestinally, not gastrically (Yokel and McNamara 2001). Overall, the precise mechanisms of Al absorption from the gut are poorly understood with many, sometimes competing, hypotheses proposed. However, the majority of Al is likely to be absorbed into the plasma via a paracellular pathway in a manner analogous to Mg. The most notable knowledge gaps in mammalian enteral absorption pertain to transcellular absorption and include both how Al may actually enter and exit the enterocytes.

Literature regarding the bioavailability of dietary Al to invertebrates is more limited than for vertebrates. Enteral Al absorption has been demonstrated in invertebrates, including *Drosophila* (Massie *et al*. 1985, Wu *et al*. 2012b, Kijak *et al*. 2013), the land snail *Helix aspera* (Brooks *et al*. 1992, Brooks and White 1995), the crayfish *Pacifastacus leniusculus* (Woodburn *et al*. 2011), the nematode *Caenorhabditis elegans* (Page *et al*. 2012), and the earthworm *Eisenia andrei* (Zhao and Qiu 2010), but mechanistic details regarding its absorption are largely unknown. The model organism that has been characterised the best is the aquatic snail, *Lymnaea stagnalis*. Non-complexed, neutralised, and hence precipitated Al, is accumulated by *L. stagnalis* (Ballance *et al*. 2002). Extracellular polysaccharides secreted by the snail interact with the hydroxyl-Al species causing a further drop in aqueous solubility, but these complexes still remain bioavailable following ingestion of the polysaccharides (Jugdaohsingh *et al*. 1998). Fulvic acid surrogates decrease total bioavailability of Al to the snail whilst humic acids appear to have little effect (Walton *et al*. 2009). Phosphate, despite forming highly insoluble complexes with Al results in higher tissue uptake in *L. stagnalis* (Walton *et al*. 2009). The use of invertebrates to gain insight into the effects upon absorption of Al speciation, with a view to possible modelling of human disease, has not been reported.

1.1.2.2. Distribution and accumulation

Having entered the plasma, Al is bound mainly by the iron transport protein, transferrin, with citrate performing an important, if lesser, role (Martin 1988). Plasma ligands are expected to solubilise all blood Al, providing concentrations do not exceed 100 µg/l (Devoto and Yokel 1994). With higher plasma concentrations, it is proposed that colloidal precipitates appear, even in those with normal renal function (Parajon *et al*. 1989). It is difficult to compare the plasma concentrations of Al from even well designed animal studies as such measurements are not only time dependent but also vary with dose and route of administration. Other tissues may be less variable but still subject to qualitatively similar problems. These difficulties aside, it is still useful to gain an appreciation of the relative tissue concentrations of Al seen in different animals. A selection of blood values from the literature is shown in Table 1. It is apparent that even values that intuitively should be broadly similar, such as background levels in humans, vary widely. The reasons for this are not known but may represent the different exposure histories.

The distribution of Al to tissues other than the blood in mammals is affected by the route of administration. Parenteral Al exposure results in a broad distribution of the metal, which accumulates mostly in the bone, liver, kidneys, and spleen, and to a lesser extent the brain, muscles, heart, and lungs. Oral dosing of Al leads to a more restricted distribution, mainly in bones, blood, kidneys and liver. Smaller quantities are localised to the brain (Greger and Sutherland 1997). One mechanism proposed for central nervous system (CNS) penetration is transferrin-mediated uptake (Roskams and Connor 1990). However, the rapidity with which Al entered the brain's extracellular fluid in rats following intravenous Al citrate infusion suggests that a second carrier mediated (facilitated or active transport) process may be occurring, either alone or in tandem with transferrin mediated endocytosis (Yokel 2002).

Brain and bone concentrations of Al are elevated in mammals exposed to Al, and when orally administered, co-administration of citrate increases the absolute concentrations in these tissues. Brain concentrations of Al in renal patients that had died with dialysis associated encephalopathy (DAE) were ~4 fold higher than dialyzed patients without DAE and 6 fold higher than uraemic but non-dialyzed patients (McDermott *et al*. 1978). Ten

Table 1. Blood aluminium concentrations of different species, as reported in the literature

Species	Sample	Dosing Regimen	Reported Concentration (µM)	Reference
Human	Plasma	Background levels	0.037 – 0.185	Ganrot (1986)
Human	Serum	Background levels	0.06 (+/- 0.05 S.D)	Wang *et al.* (1991)
Human	Whole Blood	Background levels	~ 0.22	Taylor *et al*. (1998)
		Oral dose of 280 mg Al (elemental) + 3.2 g citrate. Mean peak concentration (~ 100 min after ingestion)	0.74 (+/- 0.11 S.D.)	
Rat	Plasma	Intestine perfused with 0 mM Al (pH 4) for 60 mins	~ 1.4	Cunat *et al.* (2000)
		Intestine perfused with 16 mM Al citrate (pH 4) for 60 mins	~ 5.2	
		Intestine perfused with 16 mM $AlCl_3$ (pH 4) for 60 mins	~ 1.3	
Rabbit	Plasma	Background levels	0.19 (+/- 0.14 S.D.)	Hewitt *et al*. (1989)
Rabbit	Serum	Background levels	3.85 (+/- 1.89 S.D.)	Yokel and McNamara (1988)
Trout (*Salmo mutto*)	Plasma	Exposure to water containing electrolytes but no Al at pH 5.2 for 96 hours	1.95 (+/- 0.44 S.D.)	Dietrich and Schlatter (1989)
		Exposure to water containing electrolytes and Al (400 µg/l) at pH 5.2 for 96 hours	No mean but ranged from 1.6 - 3.1 (n = 9). Not significantly different from line above	
Signal crayfish	Whole haemolymph	Fed food containing 420 μg_{Al}/g/day for 28 days	Below detection < 4.9	Woodburn *et al*. (2011)

week exposure of rats to Al citrate orally resulted in an ~18 fold increase in cortical Al concentration over control; bone, the major sink for Al, accumulated ~120 fold over control. Without citric acid co-administration, only bone levels were raised (slightly) (Slanina *et al*. 1985). Similarly, no differences in brain or bone Al concentrations were found in rats following 50 days of $AlCl_3$ administration. Al citrate, however, significantly

increased brain Al ~1.6 fold over control (Wu *et al*. 2012a). The effects of citrate in these studies are most likely explained by a total lower bioavailability of $AlCl_3$, but a more complex effect of citrate on the post-absorption distribution and/or elimination of the metal is possible.

As this thesis examines the developmental toxicity of Al, it is useful to consider how Al is distributed across the placenta and into the developing foetus. Although some work in mammals has found developmental toxicity of Al, implying placental transfer of the metal, studies concerning this are few. Twenty days of transdermal exposure to Al in pregnant mice showed transfer across the placenta, with amniotic fluid, foetal liver and foetal brain Al concentrations being raised by 20, 15, and 5% (Anane *et al*. 1997). However, a nine day oral study, in rats, found no Al in foetuses despite an elevation in placentas (Gomez *et al*. 1991). Human placentas contain ~0.5 $\mu g_{Al}/g_{DryTissue}$ whereas umbilical cords contain 0.3 $\mu g_{Al}/g_{DryTissue}$ although the reason for this difference is not known (Kruger *et al*. 2010).

The physiology and plasma chemistry of invertebrates differs from vertebrate, and the lack of a closed circulatory system and the consequent differences in blood flow to various organs will likely affect the distribution kinetics of Al (Brusca and Brusca 2003). To the knowledge of the author, direct measurements of invertebrate haemolymph Al concentrations have only been made once, in the signal crayfish *P. leniusculus*. *P. leniusculus* given Al in food showed no difference in haemolymph Al concentration from control as it was below detection (Woodburn *et al*. 2011). The two tissues that did show accumulation were the hepatopancreas and antennal gland. Al must therefore have been present in the haemolymph to allow translocation to the antennal gland.

Information regarding the accumulation of Al in terrestrial invertebrates is limited. Only three studies have examined Al accumulation in the versatile invertebrate model organism, *Drosophila*. In the first, flies that had been exposed to aluminium in food (10 mM) throughout life accumulated Al in a dose and time dependent fashion (Massie *et al*. 1985), attaining tissue concentrations of 226.4 $\mu g_{Al}/g_{DryTissue}$ by 12 days post eclosion. In the second, the concentration of Al in brain of flies exposed to $AlCl_3$ (10 mM) in the food (as adults) was 280 $\mu g_{Al}/g_{DryTissue}$, compared to 24.5 $\mu g_{Al}/g_{DryTissue}$ in controls (Wu *et al*. 2012b). In contrast to the values reported above, Kijak *et al*. (2013) found whole body tissue concentrations of only 47 $ng_{Al}/g_{DryTissue}$ after exposure to 4.5 mM Al in their food for

their entire lifespan (~50 days). For comparison, the earthworm *E. andrei* accumulates Al from soil contaminated with Al at soil concentrations of 20 – 100 mg_{Al}/kg_{Soil} to a peak tissue concentration of ~170 $\mu g_{Al}/g_{Tissue\ (wet\ or\ dry\ not\ specified)}$, but no soil concentration dependency was observed (Zhao and Qiu 2010). The terrestrial snail *H. aspresa* accumulates Al in both the digestive gland and kidney following a 24 hour feed of $Al(NO_3)_3$ (500 $\mu g_{Al}/g_{Feed}$) with tissue concentrations rising 5-6 fold in these organs 48 hours after Al feeding ceased (Brooks *et al*. 1992). Following exposure to $Al(NO_3)_3$ (4.8 mM) *C. elegans* have a whole body Al concentration 170 fold higher than control (Page *et al*. 2012).

Of the air (non-gill) breathing invertebrates, the pond snail *L. stagnalis* has probably been the most intensively investigated regarding Al toxicity and accumulation. The digestive gland (the detoxificatory organ equivalent to the crayfish hepatopancreas) and kidney (equivalent to the antennal glands of the crayfish) of *L. stagnalis* usually accumulated the highest concentrations of Al (Dobranskyte *et al*. 2006) and were the organs responsible for the majority of the body burden (Dobranskyte *et al*. 2004, Desouky 2006). However, different complexing agents, notably phosphate and fulvic acid, caused a redistribution of Al to other body compartments (Walton *et al*. 2009). Levels of Al in the snail digestive gland following exposure in water to neutralised Al at 500 µg/l were around 2000 µg/g at day 15 (Dobranskyte *et al*. 2004).

Invertebrates, lacking an internal skeleton, are unable to sequester metal ions within the bone matrix. Instead, one mechanism that they possess is the ability to form inert granules that can be stored, for various periods of time, either intra- or extra-cellularly (Brown 1982, Taylor and Simkiss 1984). These granules are found in many tissues but often reside in organs such as the hepatopancreas or kidney. Granule composition can be categorised on the basis of whether they contain primarily metals that are either hard (type A granules) or soft (type B granules) Lewis acids (Brown 1982, Taylor and Simkiss 1984). Both *H. aspersa* (Brooks and White 1995) and *L. stagnalis* (Elangovan *et al*. 2000) accumulate a considerable amount of Al in type B granules (despite Al being a hard Lewis acid) following oral ingestion. In *H. aspersa* Al is found in association with phosphate in granules within the digestive gland. However, work in *L. stagnalis* has shown that intracellular Si can be mobilised, as an alternative to phosphate, in response to Al ingestion, leading to the deposition of inorganic HAS containing granules in the digestive

gland (Desouky *et al*. 2002, White *et al*. 2008). Inorganic granules are found in *Drosophila* in the malpighian tubules. Two types of granules, both equivalent to type A granules, have been described; type I contains mainly Ca, Mg and phosphate and type II contains largely potassium (K), with lesser amounts of Ca and Mg (Wessing *et al*. 1992). They also accumulate barium and strontium when administered to *Drosophila* but, consistent with observations in *H. aspersa* and *L. stagnalis*, do not appear to sequester Al (Wessing and Zierold 1992). There are no reports of granules that contain soft metal ions in *Drosophila* malpighian tubules; these are, however, found in the midgut epithelium of the digestive system (Lauverjat *et al*. 1989), but no studies have investigated the presence of Al in these.

1.1.2.3. Elimination

The elimination of Al from an organism can be protracted; therefore with continued Al exposure accumulation within the tissues can occur giving the potential for long term toxicity. Single compartment elimination models are generally inadequate for Al (Priest 2004). This becomes evident when estimates for the half-life of Al are compared. The first-order elimination half-lives of Al from human blood and the remainder of the body have been calculated as 7 and 300 days respectively (Devoto and Yokel 1994). Wilhelm *et al*. (1990) examined plasma half-life estimates from eight studies and found that figures vary from 1.5 hours to 85 days. An overall body elimination half-life of seven years was proposed by Priest *et al*. (1995). The difficulties in determining accurate kinetic parameters for the elimination of Al are compounded by a multitude of external factors such as iron status and renal function (Devoto and Yokel 1994, Greger and Sutherland 1997). The primary route of Al elimination appears to be the urine; increases in bone accumulation of the metal are found in both rats and humans that have reduced kidney function (Greger and Sutherland 1997). A small proportion (1.8 - 10%) of parentally administered Al in humans is excreted in the bile (Klein *et al*. 1982). Likewise, dogs (Kovalchik *et al*. 1978), rats (Yokel and McNamara 1989), and rabbits (Klein *et al*. 1988) all eliminate a minor fraction of parentally administered Al via this route.

Data concerning the elimination of Al from invertebrates is even more limited. The author is aware of no studies that attempt to calculate formal toxicokinetic parameters for any invertebrate. Nonetheless, several studies give empirical data regarding changes in tissue

concentrations over time. Crayfish excrete a sizable proportion of accumulated Al over the first ten days following administration, but a large amount still remains and the timescale of its removal is unknown; the route of elimination is suggested in part to be via the antennal glands. Much of the Al which was not excreted was eventually stored in insoluble, biologically inert, inorganic granules, possibly indefinitely (Woodburn *et al*. 2011). *L. stagnalis*, by contrast, was shown in some studies to purge the entire digestive gland load of Al within 10-20 days (Desouky *et al*. 2003, White *et al*. 2008). Following a 24 hours pulse of Al, *H. aspersa* slowly eliminates Al over 12 days in the faeces, but considerable amounts remain in the digestive gland after this. Whether this remains permanently or is simply excreted too slowly to be detectable is not known (Brooks *et al*. 1992).

The mechanisms by which invertebrates excrete Al are not well studied but it is known that granule formation can facilitate excretion in some species (Brooks *et al*. 1992, Brooks and White 1995, Elangovan *et al*. 2000, Desouky 2006). *L. stagnalis* excretes significantly more Al when subsequently given Si strongly suggesting that inorganic granules containing HAS are preferentially excreted (White *et al*. 2008). The fate of accumulated Al in *Drosophila* is unknown, but it is unlikely that the granules (concretions) found in the malpighian tubules that function in Ca excretion (Wessing *et al*. 1992) are involved as Al has not been found to enter these (Wessing and Zierold 1992). However, as Si facilitates Al excretion by *L. stagnalis*, perhaps a similar mechanism may exist in *Drosophila*.

1.1.3. Toxicodynamics

The toxicity of Al appears universal; there are examples of detrimental consequences following Al exposure across diverse taxonomic groups. The fundamental mechanisms associated with this toxicity are oxidative stress, disturbance of metal ion homeostasis and disruptive ligand binding. Acute toxicity is rare in air breathing organisms but is a major threat to aquatic life with fish suffering toxicity at Al concentrations ranging from 100 – 400 $\mu g_{Al}/l$ in the water (Dietrich and Schlatter 1989). Although the gill is primary target in these organisms, there are multiple mechanisms that interact in a complex fashion. The expression of the toxicity varies with temperature, pH (Dietrich and Schlatter 1989), and ionic strength (Lydersen *et al*. 2002), and is most severe following a sudden change in environmental conditions that create transient, non-steady state conditions (Poleo *et al*.

1994). Ultimately, the actions of Al upon the gill are mostly a surface phenomenon involving precipitation of poly-hydroxy-Al complexes (Poleo 1995) and/or inhibition of certain enzymes (Exley *et al*. 1991), resulting in respiratory and/or osmoregulatory failure.

Acute oral lethality of Al to mammals has been little studied but 24 hour $AlCl_3$, LD_{50} values for rats, ranging from 700 mg/kg (Yellamma *et al*. 2010) to 3.5 g/kg (Rawy *et al*. 2013) have been reported. Fourteen day, oral LD_{50} (Al mass) values for rats administered Al nitrate or bromide are 261 and 163 mg/kg respectively (Llobet *et al*. 1987). Of far more concern is the toxicity that may arise after chronic exposure to Al, owing to its ability to accumulate in specific tissues. Whilst bone is the major mammalian sink for Al and experiences deleterious consequences (Kerr *et al*. 1992), the organ that experiences the most profound pathology is the brain (Savory *et al*. 2006, Kumar and Gill 2009); blood disorders including microcytic anaemia are also common (Farina *et al*. 2005). As chronic studies can be costly and time consuming, many studies monitor acute effects in an attempt to understand the toxic mechanisms but they can only provide partial insight. As an indication of the organ systems involved in Al toxicity, Table 2 displays examples of the more commonly observed effects in rats from both short and long term studies.

Table 2. Toxic effects of aluminium, as reported in the literature

Dosing Regimen	Toxicity observed	Reference
Daily i.p injections of 270 μg_{Al}/kg for two months	Impaired spatial learning and memory Decreased long term potentiation in CA1 neurons	Liang *et al*. (2012)
Daily oral gavage of 320 $mg_{AlCitrate}$/kg for 4 days	Neuron and astrocyte loss in CA1 and CA3 regions of the hippocampus	Silva *et al*. (2013)
430 $mg_{AlCitrate}$/l in drinking water	Reduced trace element content of bone at 60 days Reduced bone mineral density at 120 days	Li *et al*. (2011)
810 mg/l Al (as citrate) in drinking water	Reduced erythrocyte count Reduced haemoglobin Reduced haematocrit	Farina *et al*. (2005)
500 mg_{AlCl3}/l in drinking water	As above + reduced erythrocyte volume and diameter Increased reticulocyte count	Gromysz-Kalkowska *et al*. (2004)

Human diseases arising from Al toxicity includes DAE that may cause death within a year (Alfrey *et al*. 1976), osteodystrophy, and microcytic anaemia (Parkinson *et al*. 1981). The bone and blood phenomena seen in Al exposed patients are easily demonstrated in rats, but the neurological consequences are variable across species. Rabbits produce the most similar neurohistological pathology to humans in response to Al; when injected intra-cerebrally, Al phosphate caused the development of neurofibrillary tangles, similar to those now known to be caused by Al in DAE (Klatzo *et al*. 1965, Terry and Pena 1965). The neuropathology seen in Al exposed rabbits and humans bears some resemblance to the lesions seen in Alzheimer's disease and has led to controversy, still far from resolved, of Al being an aetiological factor in Alzheimer's disease. This hypothesis is strengthened by the finding of higher levels of Al in the brains of Alzheimer's patients compared to control (Perl 1988). Some, but not all, epidemiological studies of Alzheimer's disease have found a correlation between disease incidence and Al intake (Flaten 2001, Krewski *et al*. 2007).

The effects of chronic exposure to low doses of Al remain controversial, and despite its known toxicity, little attention has been paid to the possible effects of Al upon reproduction and development. As a now ubiquitous toxicant, Al will likely initiate any toxic effects during development and perhaps before conception (Exley 2003). Toxicity of Al to the reproductive organs has been demonstrated in both sexes and may have consequences beyond reduced fecundity. Female mice exposed to Al in drinking water showed histological changes in the ovaries including vascular congestion and loss of embryos and foetuses (Mohammed *et al*. 2008). Following subcutaneous administration of $AlCl_3$, male mice showed histological changes to the testes and decreased testicular mass. These males, when mated to unexposed virgin mice, sired embryos and foetuses that were more likely to die *in utero* and were of lower mass than unexposed counterparts (Guo *et al*. 2005). Male rats show hormonal changes following oral treatment with $AlCl_3$ (128 mg/kg) for 120 days with suppression of both testosterone and luteinizing hormone levels coupled with reduced expression of the androgen receptor (Sun *et al*. 2011). Developmental toxicity may present following pre-natal, maternal parenteral exposure to Al. Golub *et al*. (1987) administered 1000 μg/g Al lactate in food to pregnant and lactating mice; the pups showed evidence of impaired development with reduced crown to rump lengths, reduced organ and body weights and behavioural deficits (Golub *et al*. 1987). Impaired

neurological and cognitive function of mouse pups coincides with altered brain neurotransmitter levels when pregnant mice are exposed to 300 – 600 mg/kg Al (Abu-Taweel *et al*. 2012). Poirier *et al*. (2011) assign a "no observed adverse effect level" of 30 mg_{Al}/kg in pregnant rats.

There is little information regarding the toxicity of Al to terrestrial invertebrates and mechanistic details are primarily absent. The 48-hour LC_{50} of Al to *C. elegans* has been reported as ~670 µM (pH unspecified) (Chu and Chow 2002), but another study found no reduction in lifespan at 1.9 mM (pH 3) administered throughout life (Page *et al*. 2012). The 14-day LC_{50} of Al to the earthworm *E. andrei* was shown to be 316 $mg_{AlCl3}/kg_{DrySoil}$ (van Gestel and Hoogerwerf 2001). Given the lack of toxicity data to terrestrial invertebrates examination of dietary administration of Al to aquatic invertebrates, especially those that respire without gills, may provide some insight into toxic processes occurring in land invertebrates. *L. stagnalis* has been studied extensively with respect to Al toxicity and demonstrates significant toxicity in response to precipitated Al at neutral pH. The outward manifestation of this toxicity is depression of locomotion and feeding behaviour (McCrohan *et al*. 2000). Isolated neurons from this species have shown aberrant electrophysiological activity upon exposure to Al salts suggesting a possible, direct neurotoxic effect (Campbell *et al*. 2000). Independently of bioavailability, various ligands have been shown to affect the behavioural toxicity of Al to *L. stagnalis*: phosphate (Walton *et al*. 2009) and silicic acid (Dobranskyte *et al*. 2004) reduce behavioural toxicity whereas a fulvic acid surrogate increases toxicity (Walton *et al*. 2009).

Lifelong or developmental studies of Al toxicity in invertebrates are few. The nematode *C. elegans* exhibits reduced lifespan when exposed to a low concentration of Al in food from the larval stage, but paradoxically shows increased lifespan when exposed to higher concentrations. Other parameters that were negatively impacted by Al included development time, body length, fertility (Page *et al*. 2012). Of particular concern was the finding that toxicity could be passed from parent to offspring even in the absence of direct Al exposure in offspring. Whether this is a chemical legacy effect or an epigenetic phenomenon is not known, but trans-generational toxicity could have serious re-percussions as even removal of the toxicant from the environment may be insufficient to prevent further damage. When both generations received Al, toxicity was further enhanced (Page *et al*. 2012). Three reports of chronic developmental toxicity of Al exist for

Drosophila, all of which found a reduction in lifespan following $AlCl_3$ with threshold concentrations ranging from 0.5 mM to 5 mM (Massie *et al*. 1985, Wu *et al*. 2012b, Kijak *et al*. 2013). One of these studies noted severe neurodegeneration, characterised by large vacuoles throughout the brain (Wu *et al*. 2012b). Whilst all three studies concur that Al administration from hatching onwards has a detrimental effect upon lifespan, the period of highest sensitivity is less clear. Two studies suggests that larval (i.e. during development) through to adulthood exposure causes the greatest overall lifespan reduction by Al (Wu *et al*. 2012b, Kijak *et al*. 2013) whereas another found exposing flies during the adult period was no less toxic that exposure from hatching (Massie *et al*. 1985).

Human exposure to Al is likely to be as high as it has ever been and is likely to increase in both amount and the number of people exposed. Future increases in Al exposure may come from more processed food, a greater emphasis on disease prevention through vaccination, and expansion of water purification to regions that currently are not supplied with mains water. Although established as neurotoxic, the potential for Al to inflict developmental toxicity that may persist throughout life has received little attention. Growing bodies and nervous systems may be especially vulnerable to Al toxicity in same manner as they are to other metals (Domingo 1994).

1.2. Silver

Silver is a potent antimicrobial (Hwang *et al*. 2007, Pathak and Gopal 2012), good electrical conductor (Moosbrugger and Committee 2000) and the photosensitivity of specific compounds renders it ideal as a photographic medium (Vogel 2011). As an antimicrobial, Ag has received a resurgence of interest in the form of AgNPs (Morones *et al*. 2005, Lara *et al*. 2011). A large number of products incorporate AgNPs into fabrics and other materials as an antimicrobial for hygiene purposes and to prevent odour (Project on Emerging Nanotechnologies 2013). Considered fairly benign to mammalian systems (Petering 1976), Ag is highly toxic to aquatic biota (Ratte 1999) and microbes (Russell and Hugo 1994). Silver nanoparticles exhibit different properties and toxicology from both the bulk material and its compounds (Oberdorster *et al*. 2005, Schluesener and Schluesener 2013). The effects AgNPs in the long term are largely unknown.

1.2.1. The chemistry of silver ions as relevant to biological systems

Silver is a soft Lewis acid with an affinity for sulphur bearing ligands (Wiberg *et al*. 2001). In aqueous solution, in the absence of other ligands, the Ag (I) ion co-ordinates four water molecules (Lee *et al*. 2003). This Ag-aqua complex is only weakly hydrolysing and at pH 7 remains soluble to concentrations >200 mM, although at greater concentrations and/or higher pH Ag_2O precipitates from condensation of two molecules of AgOH (Wiberg *et al*. 2001). The solubility of Ag ions is heavily influenced by its co-ordinating ligands. One of the most biologically important ligands for Ag (I) ions is Cl^-, largely due to its abundance. The neutral AgCl complex is highly insoluble in water, but addition of a further Cl^- ligand to form the $AgCl_2^-$ complex enhances its solubility (Jonte and Martin 1952). The second most biologically relevant ligand is sulphur. Inorganic sulphide binds Ag, outcompeting Cl^- unless sulphide is limiting, producing a complex of minimal solubility (Adams and Kramer 1998). Organic thiols may alter Ag solubility in either direction depending on the specific ligand (Bell and Kramer 1999, Jacobson *et al*. 2005).

1.2.2. Toxicokinetics

1.2.2.1. Exposure, absorption and bioavailability

Silver has no utility in biology. Nonetheless, global deposition from natural sources in 1978 was estimated at 438 t (~17% of the total Ag discharged to the biosphere) (Purcell and Peters 1998). Ag concentrations in freshwater and seawater are extremely low at ~10 ng/l (Ratte 1999) and 100~300 pg/l (Luoma *et al*. 1995), respectively. Mining and processing increases the opportunity for release of bioactive forms of Ag. In 1997, an estimated 11500 t of anthropogenic Ag was deposited into the environment, representing 57% of that extracted during that year (Johnson *et al*. 2005). The photographic industry was once the largest consumer of Ag, accounting for 50% of the total US demand for the metal. However, with the decline of film photograph, by 2008, it accounted only 12.5% of demand (The Silver Institute 2009). Nonetheless, Ag based radiographic media are still used and with lax regulation emissions still occur (Grigoletto *et al*. 2011). It has been suggested that Ag from photographic effluents is not bioavailable as it is bound in stable thiosulphate complexes (Bard *et al*. 1976). Other sources of environmental pollution with Ag include the electronics industry (Ratte 1999), mining and smelting operations (Johnson *et al*. 2005), and some cloud seeding operations with Ag iodide (Cooper and Jolly 1970,

Curic and Janc 2013). Key sources for direct human exposure to Ag are its use in dental amalgams and as an antibacterial, particularly for burns treatment and water purification, representing the possibility of both transdermal and oral absorption. Other sources of human exposure include fumes from Ag containing solders and brazing alloys, and Ag acetate containing smoking cessation aids (Drake and Hazelwood 2005).

As vertical neighbours within the periodic table Ag shares many properties with copper (Cu) when in the +1 oxidation state (Wiberg *et al*. 2001), and much of the toxicokinetic behaviour of Ag (I) in mammals (and likely other organisms too) is due to chemical mimicry of Cu (I) (Bertinato *et al*. 2010). Copper (I) ions are taken up from the intestine by facilitated diffusion (along its concentration gradient), after reduction from Cu (II), by the high affinity Cu (I) transporter, Ctr1 into enterocytes (Prohaska 2008). From here it is passed to the Cu chaperone Atox1 before being dispatched across the basolateral membrane of the enterocyte by the ATP dependent copper pump ATP7A (Prohaska 2008). The apical Cu (I) ion transporter, Ctr1, is responsible for transporting Ag (I) ions into the enterocytes (Lee *et al*. 2002, Bertinato *et al*. 2010) and ATP7A is responsible for their exit into the portal circulation (Verheijen *et al*. 1998). At present there are no reports of the Cu (I) chaperone Atox1 binding or carrying Ag (I) ions, but this seems highly likely as this is how Cu (I) is passed from Ctr1 to ATP7A (Prohaska 2008). Binding by cellular thiols and proteins precludes passive diffusion across the gut wall (Lansdown 2010).

Oral bioavailability of Ag is poorly studied, and much information that exists relates to people that have been exposed, for indefinite periods of time, to unknown concentrations of the metal. This is surprising given the moderate cost of an Ag radioisotope (^{110m}Ag). However, using ^{110m}Ag, Furcher *et al*. (1968) found a wide species variability with rats, mice, and monkeys absorbing less than 1% and dogs nearly 10% of Ag administered in drinking water over a week. From these figures the authors estimate that bioavailability in humans is in the order of 4%, but such extrapolation has been criticised (Lansdown 2010). Holler *et al*. (2007) estimate that human bioavailability may be as much as 20% in some cases.

The mechanisms of Ag (I) ion uptake in invertebrates have received little attention. However, the Cu transporter Ctr1 is conserved throughout phyla from yeast to humans (Nose *et al*. 2006), suggesting that Ag (I) uptake made be similarly conserved. *Drosophila*

express three isoforms of the Ctr1 transporter, with Ctr1A being the likely ortholog to the mammalian transporter as, in knockdown studies, the human Ctr1 is able to substitute for the *Drosophila* Ctr1A (Hua *et al*. 2010). Copper uptake by Ctr1A is inhibited by Ag (I) ions, strongly suggesting that the latter are taken up via this route (Zhou *et al*. 2003). Bioavailability estimates for *Drosophila* are unavailable, but Armstrong *et al.* (2013) showed that following administration of ~500 µM Ag in food, for an unspecified period, the whole body tissue Ag concentration was raised to ~140 $\mu g_{Ag}/g_{Tissue(wet\ or\ dry\ not\ specified)}$ from levels that were undetectable in control samples.

Other invertebrate species have been used to determine absorption, but bioavailability figures are scant. Although some investigated species are aquatic, all bioavailability figures below refer to dietary ingestion of Ag as this is most relevant to this study. The oligochaete worm *Lumbriculus variegates* approximately quadrupled its total body tissue concentration of Ag after exposure to sediment contaminated with Ag sulphide, with a bio-accumulation factor (BAF) of 0.18 (Hirsch 1998); between 12 and 36% of ingested Ag was absorbed by the polychaete, *Nerels succinea* (Wang *et al*. 1999), and oral bioavailability of Ag to the snow crab *Chionoecetes opilio* was near 90% (Rouleau *et al*. 2000). Clearly, a wide range of bioavailabilities is evident amongst invertebrates, but direct comparisons are difficult due to the nature of the food substrate concentrations administered.

1.2.2.2. Distribution and accumulation

The distribution of Ag throughout the tissues is influenced by its chemical similarity to Cu. However, there are some notable differences. For example, Cu (I) absorbed from the rat intestine is complexed to macroglobulin α(1)-inhibitor III, or transcuprein, homologous to human α_2-macroglobulin, but Ag is not complexed by these proteins and appears to be carried in rat plasma by α_1-macroglobulin (Hanson *et al*. 2001). The major mammalian Cu reserve is ceruloplasmin and rats fed a diet containing AgCl incorporated Ag into this enzyme as demonstrated by loss of oxidase activity (Ilyechova *et al*. 2011). The Cu in ceruloplasmin is not freely exchangeable and the metallation is performed at the point of synthesis, suggesting that Ag must be transported to the appropriate cellular compartment. Although the mechanism for this has not been studied, the copper transporter ATP7B is important for adding copper to ceruloplasmin (Prohaska 2008) and is capable of

transporting Ag (I) ions (Ibricevic *et al*. 2010) also, so it is logical to hypothesise that the route is the same.

Comparison of tissue levels across studies and species needs caution but helps gain an impression of distribution patterns. Blood concentrations for species where the data are available are shown in Table 3. The tissue distribution of Ag in female rats, following a parenteral bolus of ^{110m}Ag (I), was widespread, with the largest amounts appearing in the liver, uterus, spleen, and ovaries, in descending concentration order. Levels in the brain were the lowest of all tissues and slow to equilibrate. The amounts of silver in the mammary gland, uterus, ovaries, adrenal gland, and liver were dependent upon the lactation status of the animal (Hanson *et al*. 2001) consistent with similar observations with Cu (I) (Donley *et al*. 2002), suggesting that important processes for the regulation of Cu in developing pups could be compromised. Babich *et al*. (2009) found that Ag incorporated into brain tissue was restricted to the pituitary gland and suggested that this is related to the fact that this structure has a high Cu demand. It is also outside the BBB suggesting Ag may not be able to penetrate it. Several other reports suggest that Ag enters the brain, but much of the Ag is found associated with the tissues of the BBB itself. The main sink for Ag in mammals is the skin. In the case of Ag excretion being exceeded by intake, large quantities of Ag are deposited in the papillary layer of the dermis, the connective tissue of the vasculature, and the corneas (Drake and Hazelwood 2005, Lansdown 2010).

This thesis is concerned with the developmental toxicity of Ag as there is currently little literature regarding this. Research suggests that maternal-foetal and maternal-milk transfer of Ag does occur, raising justifiable cause for concern. Analysis of *post-mortem* human foetal liver tissue as part of an investigation into sudden infant death syndrome yielded levels of Ag up to 6.5 fold higher (Lyon *et al*. 2002) than the liver concentration of adults (Zeisler *et al*. 1988). This suggests that Ag is likely localised to foetal tissues at higher concentrations than the adult population. Liver Ag concentrations fall with age and by 1 year (Lyon *et al*. 2002) approximate those found in adults (Zeisler *et al*. 1988), but for unknown reasons. Exposure of the newborn humans may continue as Ag (up to 42.0 µg/l) was found in human milk (Krachler *et al*. 2000). These findings are consistent with animal models. Pregnant rats distributed injected Ag to the developing foetus, suggesting placental transfer (Danscher 1981). Likewise, rats fed a diet containing AgCl before

conception and throughout pregnancy produced pups that showed signs of toxicity and reduced Cu levels (Shavlovski *et al*. 1995). This, of course, does not prove that Ag crossed the placenta as it may simply have inhibited the transport of Cu. Nonetheless, given that Cu ion transport mechanisms can transport Ag ions at the expense of Cu ions it is very likely (Bertinato *et al*. 2010, Ibricevic *et al*. 2010) that Ag ions can cross the placental barrier. Furthermore, Ag ions administered parentally to lactating rats enter the milk at concentrations similar to the plasma (Hanson *et al*. 2001)

There have been no studies of the distribution of Ag within the body of *Drosophila*. Most of the invertebrate species that have been studied with regard to organ distribution of Ag have been aquatic with Ag absorbed directly from the water column. Accumulation and distribution of Ag varies greatly with physiology but, as the examples below demonstrate, it appears that invertebrates have the capacity to accumulate far higher Ag concentrations than mammals. Table 3 shows that the American red crayfish, following Ag feeding for 80 days, has blood Ag levels around 10 fold higher those seen in rats or man. Indeed, the control levels in the crayfish are often equivalent to exposed rats. After 80 days exposure to Ag the hepatopancreas of American red crayfish contained 1000 $nmol_{Ag}/g_{Tissue}$ (equivalent to 108000$\mu g_{Ag}/g_{Tissue}$). Other tissues also accumulated Ag with the antennal gland and muscle having the next highest concentrations (Mann *et al*. 2004). *C. opilio*, described above in Section 1.2.2.1, distributed the majority of the ingested dose of ^{110m}Ag to the hepatopancreas by 14 days post-exposure (determined semi-quantitatively using autoradiography) (Rouleau *et al*. 2000). Hepatopancreas Ag levels of *C. opilio* caught wild from the St Lawrence Estuary were 20 $nmol_{Ag}/g_{WetTissue}$ (Rouleau *et al*. 2000). *C. opilio* does not accumulate significant Ag in the gonads but the marine invertebrate, *Diadema antillarum* deposited 0.5 $\mu g_{Ag}/g_{WetTissue}$ in this tissue and the majority of retained Ag was in the egg mass (~ 20 $\mu g_{Ag}/g_{WetTissue}$) following Ag exposure from the water column. The authors noted that this may have repercussions for reproduction and development (Bianchini *et al*. 2007). As a non-gill breathing organism this may have relevance to reproductive toxicity in terrestrial invertebrates.

Table 3. Blood silver concentrations of different species, as reported in the literature

Species	Sample	Dosing Regimen	Reported Concentration (µM)	Reference
Human	Plasma	Background (Burns patients)	Below Detection < 0.021	Wan *et al*. (1991)
		Concentration in Ag sulphadiazine treated burns patient (8 days)	~1.62	
Human	Whole Blood	Background in unexposed workers	Below detection < 0.0009	Armitage *et al*. (1996)
		Concentration from occupationally exposed workers	0.063	
Rat	Plasma	Background	Below detection < 0.016	Loeschner *et al*. (2011)
		Oral gavage with 9 mg/kg/day (split into two doses/day) Ag acetate for 28 days. Sampled day 29 (unknown hours after last dose)	~1.85	
Rat	Whole Blood	Background	Below detection < 0.046	Van der Zande *et al*. (2012)
		Oral gavage with 9 mg/kg/day $AgNO_3$ for 28 days. Sampled day 7 (unknown hours after previous dose)	1.05 +/- 0.01 (SEM)	
		Oral gavage with 9 mg/kg/day $AgNO_3$ for 28 days. Sampled day 21 (unknown hours after previous dose)	1.02 +/- 0.03 (SEM)	
		Oral gavage with 9 mg/kg/day $AgNO_3$ for 28 days. Sampled day 29 (unknown hours after last dose)	0.57 +/- 0.04 (SEM)	
American Red Crayfish	Haemolymph	80 days feeding on fish diet containing no Ag	~1	Mann *et al*. (2004)
		80 days feeding on fish diet containing Ag 21.3 nmol/g_{Food}	~10	

1.2.2.3. Elimination

Rates of elimination of Ag differ greatly across species with the largest divide between invertebrates and vertebrates. For some invertebrate species, Ag accumulates even when administered in low doses. Silver can accumulate in mammals to very high concentrations as Ag selenides and sulphides in the skin (argyria) (Drake and Hazelwood 2005). These

deposits are essentially immobile and remain for life. However, it has been stated that Ag should not be considered a cumulative poison in mammals as excretion is usually efficient and it is only following chronic ingestion of large doses that such accumulation occurs (Lansdown 2010). The chemical similarly of Ag and Cu suggests that excretory mechanisms may be similar. In healthy rats, Cu is excreted primarily in the bile with less via intestinal secretion and a small fraction through the urine (Linder and Roboz 1986). The precise manner in which Cu enters the bile is poorly understood, but it appears that ATP7B is a key mediator for either its direct paracellular transport (Hernandez *et al*. 2008) into the bile duct or incorporation into excretory vesicles (Harada *et al*. 2000, Cater *et al*. 2006, Hernandez *et al*. 2008). As Ag is transported by ATP7B in some instances (Ibricevic *et al*. 2010) it is reasonable to hypothesise that this occurs during excretion also. Such a notion is supported by a study by Sugawara and Sugawara (2000), who exposed both control and ATP7B deficient rats to subcutaneous $AgNO_3$ and analysed the secreted bile. Fractions of the bile shown to contain Ag in control rats were devoid of the metal in ATP7B deficient rats. However, a second Ag containing fraction was found in both rat strains, suggesting a second ATP7B independent mechanism operating as well.

Although Ag is not considered a cumulative poison in mammals (Lansdown 2010), its excretion may not be as rapid from all tissues as such a statement would suggest. For example, rats injected subcutaneously with 100 mg/kg Ag sulphadiazine eliminated only 6.6% of the total dose in five days and forty percent of the total amount remained in the tissues after seven weeks. Of all the tissues analysed, the brain showed no elimination and may even have increased its Ag burden over time through bodily re-distribution (Sano *et al*. 1982). Another Ag elimination study using rats, rabbits, and dogs found considerable interspecific differences with dogs and rabbits excreting Ag 10 and 100 fold slower than rats, respectively. Urinary excretion in rats was minimal at 0.5% over five days (Klaassen 1979). To the knowledge of the author, the only rate constant estimates that have been made for Ag in mammals were based upon a synthesis of literature data. Bachler *et al*. (2013), calculated first order rate constants for both humans and rats of 9×10^{-3} min^{-1} (equivalent to $t_{½}$ = 77 mins) and 3.54×10^{-3} min^{-1} (equivalent to $t_{½}$ = 195 min) for biliary and urinary excretion, respectively. Elimination kinetics of Ag are clearly complex and far from understood. Furthermore, slow elimination of small quantities from potentially sensitive compartments (e.g. the brain) may be masked in models that do not specifically consider such tissues as separate compartments.

Elimination of Ag from invertebrates has received some attention although not in *Drosophila*. What data exist suggest a wide range of elimination half-lives. When *L. stagnalis* was exposed to Ag ions in the food substrate, significant body burdens were measured. The elimination half-life was shown to be greater than 15 days probably by a considerable margin (Croteau *et al*. 2011). The slow elimination kinetics in *L. stagnalis* are mirrored by *C. opilio* in which the elimination half-life was estimated to be in the order of 1000 days (Rouleau *et al*. 2000). In contrast, *Daphnia magna* briskly eliminate ingested Ag with calculated biological elimination half-lives of 1.45 and 1.95 days for juveniles and adults respectively. Following waterborne exposure, these half-lives increased to 3.64 and 2.67 days for adults and juveniles respectively (Lam and Wang 2006).

1.2.3. Toxicodynamics

Waterborne ionic Ag is acutely toxic to freshwater aquatic organisms with LC_{50} values ranging from ~2.4 nM for neonate daphnids to 610 nM for adult crayfish (Bianchini *et al*. 2002); the mechanism of toxicity involves inhibition of the Na^+/K^+ ATPase and carbonic anhydrase enzymes that are responsible for maintaining osmotic homeostasis (Bianchini and Wood 2003, Morgan *et al*. 2004). Toxicity of Ag to terrestrial animals has received minimal attention probably due to its perceived low toxicity in mammals, including man (Lansdown 2007, Lansdown 2010). However, case-reports of serious toxicity in humans and the limited experimental data that does exist suggest that further study is warranted. Silver nitrate, a freely ionisable and thus more toxic form, has a 24 hour LD_{50} of 36 mg/kg and 120 mg/kg after intraperitoneal injection in the rat and rabbit respectively. Oral 24-hour LD_{50} values are approximately three times higher in both species (Tamimi *et al*. 1998). Following acute oral $AgNO_3$ challenge the main pathologies were gastrointestinal and pulmonary oedema and necrosis plus ascites; intraperitoneal injection caused focal fat necrosis, acute inflammation and widespread oedema (Tamimi *et al*. 1998).

The most common condition associated with Ag exposure in man is argyria along with the associated argyrosis. These disorders occur when excretory capacity for Ag is exceeded and are thought to be the result of deposition of Ag-S/Se complexes in the skin and cornea respectively, producing a blue-grey hue following photo-reduction of soluble Ag-protein species and re-oxidation to insoluble precipitates (Drake and Hazelwood 2005). The condition usually develops slowly over months or years and requires a total ingested

elemental Ag equivalent of between 2 and 6 g (Jonas *et al*. 2007). Although usually considered solely cosmetic (Lansdown 2010), there are case reports to suggest that other symptoms could be attributed to Ag toxicity, including neurological and psychiatric effects (Dietl *et al*. 1984, Rosenblatt and Cymet 1987, Ohbo *et al*. 1996, Mirsattari *et al*. 2004). However, case studies are only anecdotal and very difficult to attribute to Ag with any degree of certainty (Lansdown 2007). Argyria and argyrosis can be induced experimentally in rats (Berry and Galle 1982) with evidence of Ag accumulation within the brain (Rungby and Danscher 1983b). Furthermore, argyric mice exhibit hypoactivity (Rungby and Danscher 1984). Isolated case reports exist for renal toxicity (Rosenman *et al*. 1987, Chaby *et al*. 2005, Mayr *et al*. 2009).

Virtually no information exists regarding the potential for developmental toxicity of ionic Ag in vertebrates. Ag can interfere with Cu transfer across the mammalian placenta resulting in ceruloplasmin and superoxide dismutase (SOD) deficiency together with significantly higher post-implantation death than control and an increased incidence of foetal abnormalities including, cryptorchidism and hydronephrosis. In addition to Ag being teratogenic, 100% of Ag exposed foetuses that survived to term died within 24 hours of birth (Shavlovski *et al*. 1995). To the knowledge of the author, the only other vertebrate organism in which an *in vivo* study of the developmental toxicity of Ag ions has been made concerns the embryos of the zebrafish *Danio rerio*. Exposure to a concentration of 3 µM Ag post fertilization was sufficient to induce embryonic dysmorphology and reduce survival. Furthermore, even at 100 nM Ag, swimming behaviour of hatched larvae was impaired suggesting impaired neurodevelopment (Powers *et al*. 2010b), a hypothesis supported by an *in vitro* study that found altered differentiation in a PC12 cell line used as a model of neurodevelopment (Powers *et al*. 2010a).

Toxicological data regarding terrestrial invertebrate exposure to Ag is scant and acute studies are absent. Most available studies concern development. Larval *C. elegans* show 50% inhibition of growth at 0.6 µM when administered $AgNO_3$ in water (Yang *et al*. 2012). The earthworm *Eisenia fetida* produces fewer cocoons when exposed to $AgNO_3$ in soil for 28 days at concentrations above ~95 $mg_{Ag}/kg_{DrySoil}$ (Shoults-Wilson *et al*. 2011). When *E. andrei* were incubated for 28 days at a concentration of 15 $mg_{Ag}/kg_{DrySoil}$ and the resulting cocoons left to incubate for a further 28 days, a significant decline in the number of juveniles relative to control was seen (Schlich *et al*. 2013). However, cocoons were not

counted, so how much of this was due to reproductive toxicity of the parental worms compared to direct developmental toxicity in the progeny cannot be known.

Using *Drosophila*, Kroman and Parsons (1960) demonstrated a dose related decline in the number of emerging adults following exposure to $AgNO_3$ contaminated food, with a toxic threshold of ~ 9 mM. The main interest of these authors, however, was the genetics of pigmentation, for which they were exploiting the de-pigmenting effects of Ag in *Drosophila* that had recently been assigned a mechanistic basis (Yaffe 1955). Interference with the developmental event of pigmentation was discovered by Rapoport (1939), who administered $AgNO_3$ to larval *Drosophila* at ~4.7 mM and found adults emerged from their pupal case with cuticle coloration described as similar to the 'yellow' mutant strain. De-pigmentation of the bristles and malpighian tubules was also seen, together with softening of the cuticle (Di Stefano 1943). Melanin synthesis is dependent upon the activity of the enzyme tyrosinase that is responsible for oxidising a wide range of phenolics that ultimately polymerise to form melanin, in addition to cross linking proteins to harden and stiffen the cuticle (sclerotization) (Wright 1987, Andersen 2010). Immediately after emergence from the pupal case, the cuticle of adult flies is both soft and devoid of pigmentation. However, shortly after eclosion, the cuticle is sclerotized and melanised, irreversibly, under the control of the neurohormone, bursicon (Fogal and Fraenkel 1969, Mendive *et al*. 2005, An *et al*. 2012). Yaffe (1955), using haemolymph derived from pre-pupae and tyrosine as a substrate, suggested that the mechanism involved inhibition of the enzyme tyrosinase or an enzyme downstream of it in the melanin synthesis cascade. However, the precise details of the mechanism were not investigated. However, three years previously, Lerner (1952) had established that mammalian tyrosinase was inhibited by Ag ions and ascertained that this was due to displacement of native Cu ions. Despite similarity in function, mammalian tyrosinase and insect tyrosinase are a result of convergent evolution with different structures and amino acid composition (Sugumaran 2002), and although both contain a catalytic Cu (I) ion, the mechanism of Ag (I) ion inhibition could be different. This has yet to be tested.

Ionic Ag clearly has the potential to act as a developmental toxicant in several species. However, little mechanistic detail is available and acquiring such, especially in mammals is costly and time consuming. The apparent sensitivity of invertebrates means that short lived organisms may be studied providing not only information of direct relevance to the

organism itself but of potential application to higher organisms. With the emergence of engineered AgNPs (Section 1.3) as a mainstream consumer product, exposure of all species to ionic Ag is likely to increase.

1.3. Silver nanoparticles

The above discussion relates to ionic or chemically bound Ag, but elemental Ag is now being mass produced in a form that is both mobile and bioavailable: AgNPs. Silver nanoparticles consist of zero-valent Ag in particulate form with at least one dimension less than 100 nm (Auffan *et al*. 2009). Many AgNPs are spherical although other shapes are possible. (Shervani *et al*. 2008, Tao *et al*. 2008, Cao *et al*. 2011). Objects at the nanoscale often exhibit properties that are absent from the bulk material. These include localised surface plasmon resonance (LSPR) that causes strong optical absorbance at specific wavelengths. These wavelengths can be tuned by altering the shape, size and molecules bound to the particle. The changes in absorbance from molecular binding have made NPs a valuable tool for sensing applications in both biology and chemistry (Willets and Van Duyne 2007, Jain *et al*. 2008). Their vast surface area results in a high surface energy allowing them to excel as catalysts (Tolaymat *et al*. 2010).

Nanoscience is a rapidly expanding field and the physical and inorganic chemistry of AgNPs is only beginning to be unravelled. Two of the most fundamental chemical changes that AgNPs may undergo are agglomeration and dissolution. The terms agglomeration and aggregation are often used interchangeably throughout the literature, sometimes leading to confusion, but each has a specific meaning. Throughout this work any multi-particle assemblage of NPs will be referred to as an agglomerate (see Nichols *et al*. (2002)). Most materials do not remain suspended in nanoparticulate form for any length of time, owing to their high surface energy, agglomerating into larger particles and finally precipitating. For example, when dispersed in a natural water sample, uncapped ~82 nm AgNPs grew to 800 nm within 6 hours and in excess of 2 µm with 24 hours (Li and Lenhart 2012). NanoComposix, a prominent nanomaterial manufacturer, states on their help pages that truly bare AgNPs would be stable for no more than a few seconds (nanoComposix 2012a). To prevent agglomeration and control size during synthesis, a capping agent is bound to the surface of the particle (Poole and Owens 2003). Capping agents may function electrostatically (Russel *et al*. 1989, Poole and Owens 2003),

increasing the zeta potential of the particles such that they repel one another, or may act sterically, preventing physical contact between the particles (Napper 1977, Poole and Owens 2003). Citrate is a commonly used electrostatic capping agent, which also serves as a reducing agent, greatly simplifying the synthesis (e.g. in Smith *et al*. (2008)), whereas a polysaccharide (e.g. in Braydich-Stolle *et al*. (2010)) or polyvinylpyrrolidone (PVP) (e.g. in Foldbjerg *et al*. (2009)) are frequently used to provide steric hindrance.

Capping (or coating as it is also referred to) of AgNPs, whilst inhibiting agglomeration, cannot completely prevent it and the final suspension state is dependent on many factors including the nature of the solvent (Warheit 2008). Furthermore, suspending (or re-suspending) particles from powdered form often results in significantly more agglomeration than found in suspensions that were synthesised *in situ*. Polysaccharide coated NPs were far more prone to agglomeration when placed in a cell culture medium; the agglomerating effect of the medium was attenuated by the addition of foetal calf serum (Murdock *et al*. 2008). As electrostatic capping agents operate through charge-charge interactions, high ionic strength may facilitate agglomeration (MacCuspie 2011).

Silver nanoparticles, rather than agglomerating into a larger single mass may also dissolve, leaching Ag (I) ions into solution. Dissolution occurs with oxidation of Ag on the particle surface followed by proton dependent dissolution yielding, water and Ag (I) ions, although the exact mechanism is likely to be more complex reactive oxygen species (ROS) intermediates (Liu and Hurt 2010). The kinetics of the reaction are dependent on many factors including capping agent (Kittler *et al*. 2010), temperature (Liu and Hurt 2010, MacCuspie 2011), concentration (Lee *et al*. 2012), medium, and particle size (Zhang *et al*. 2011). In general, the reaction is reported to be nearing equilibrium within 4 ~ 10 days under most conditions although timescales as short as 1 day (Lee *et al*. 2012) and as long as ~ 75 days (Kittler *et al*. 2010) have been reported for citrate capped nanoparticles. As so many varieties of NPs are available, it is imperative that the agglomeration and dissolution properties are assessed within the system in which they will be used.

Common methods used for determining the extent of dissolution of AgNPs are dialysis (Kittler *et al*. 2010) and centrifugal ultra-filtration (Lee *et al*. 2012). An aged and partially dissolved AgNP solution is dialyzed or filtered across a membrane with pore size less than the size of the AgNPs. However, it has been shown that the capping agent may provide

potential binding sites for the released ions (Liu and Hurt 2010, Liu *et al*. 2010). For instance, ions released from the surface of citrate capped nanoparticles exist in two phases: free Ag (I) ions and those ligated by citrate (Liu and Hurt 2010). When AgNPs are separated from the solvent, ligated ions are left behind with the particles. Although these ions are bound and not free, they provide a sink of potentially bioavailable ions (Liu *et al*. 2010). Any dialysis or filtration procedure that does not account for this may underestimate the true amount of Ag (I) ions released and hence the amount of bioactive silver. As such dissolution studies are often undertaken to determine the inherent dissolution characteristics of AgNPs, the conditions are chemically well defined and strictly controlled with only fundamental parameters such as temperature, pH, particle size etc. varied. However, more complex biological media would add more variables and, in particular, provide the opportunity for Ag (I) ions to be bound by Cl^- ions, precipitating before traversing the dialysis or filtration membrane.

1.3.1. Toxicokinetics

1.3.1.1. Exposure, absorption and bioavailability

Engineered AgNPs have a multitude of applications, many of which entail the possibility of human exposure or environmental release, either deliberately or inadvertently. One prominent application for AgNPs is as an antimicrobial, and they seem particularly well suited to deodorizing fabrics that come into contact with the body (Project on Emerging Nanotechnologies 2013). Other uses of AgNPs as an antibacterial, such as their use in food containers and de-odorising room sprays, have greater potential for human exposure. Furthermore, medical implants often incorporate AgNPs to prevent infection (Tolaymat *et al*. 2010). Aside from hazards that may arise from direct skin contact with AgNP-impregnated fabrics (Kulthong *et al*. 2010), the potential for release of Ag following their washing or disposal must be considered. Geranio *et al*. (2009) reported that oxidative dissolution of AgNPs during washing of most impregnated fabrics was minimal, especially at alkaline pH as would be found in a normal wash cycle. Depending on the fabric, however, a considerable quantity (up to 45%) of the material's Ag was released as particles, mostly larger than 450 nm. An alternative method for using AgNPs to disinfect fabrics is direct addition to the wash water, which produces an effluent with much smaller particles. When Farkas *et al*. (2011) examined the effluent from a washing machine that dispenses AgNPs during a wash, they found Ag concentrations of 11 µg/l, mostly as

particulates of average diameter 10 nm. In 2010, an estimated 15% of anthropogenic Ag inputs into waterways of the European Union were derived from AgNPs originally incorporated into fabrics and plastics (Tolaymat *et al*. 2010).

The risks of exposure to released, non-dissolved AgNPs are complicated because the subsequent environmental fate of the nanoparticles is poorly understood. Lui and Hurt (2010) contend that the ultimate destiny of citrate capped AgNPs in natural, oxic waters, is dissolution (Liu and Hurt 2010), but that, due to the kinetics of the process, Ag in the form of nanoparticles may persist long enough to exert toxicity through particle specific mechanisms. Other studies suggest that whilst dissolution may be a dominant factor, it does not necessarily proceed to completion (Kittler *et al*. 2010, Zhang *et al*. 2011) and is dependent upon primary particle size (Zhang *et al*. 2011). Agglomeration does not appear to greatly reduce dissolution as the resulting structure only loses minimal surface area. Agglomeration may be so slow in low natural organic matter freshwater environments that singly dispersed AgNPs may remain non-agglomerated for up to 48 hours (Chinnapongse *et al*. 2011). Recent work indicates that sulphidation reactions requiring oxygen may limit the solubility and toxicity of AgNPs although how this affects bioavailability is not discussed (Levard *et al*. 2011, Liu *et al*. 2011).

Studies of the bioavailability of AgNPs are in their infancy, and the relative contributions of discrete particles and dissolved ions are largely unknown. What data are available suggest that a not insignificant fraction of the total Ag from orally administered AgNPs is absorbed in some form; this is important as oral exposure, along with transdermal, is the most likely route for human exposure. It is critical, however, that the forms of Ag transported into the circulation are established. Unfortunately most studies that use oral exposure cannot, or do not attempt to, ascertain whether absorption of discrete particles occurred; however, careless terminology can be seen with phrases such as "AgNPs were found in organ X" meaning no more than that Ag was found in organ X following administration of AgNPs. Many studies are performed using only parenteral administration but, without knowledge of oral absorption and bioavailability parameters, it is difficult to interpret the data generated in a more general context. Indeed, the huge influx of intact AgNPs seen following an intravenous injection may prove toxicologically irrelevant in most exposure situations. Nonetheless, there are applications being developed for AgNPs that may entail deliberate parenteral exposure such as their use as drug carriers

(Loutfy *et al*. 2013) or for *in vivo* bio-imaging (Ravindran *et al*. 2013). The study of AgNP bioavailability is further complicated by the plethora of sizes and shapes of AgNPs available, along with the many different capping agents that are used.

The oral bioavailability of AgNPs may be influenced by the fact that absorption may occur as intact particles or dissolved ions. The contributions of each form are as yet unknown. The oral bioavailability of a single dose of 8 nm citrate capped AgNPs to rats was in the range of 1 – 4%, depending upon the dose (Park *et al*. 2011a). This is equal to or greater than that estimated for Ag (I) in the same species (Furchner *et al*. 1968). However, transfer of only 0.5% of Ag was found across an *in vitro* human intestinal epithelium model, regardless of whether Ag was presented as AgNPs or $AgNO_3$, suggesting that Ag (I) ions are the transported species (Bouwmeester *et al*. 2011). Similarly, van der Zande *et al*. (2012) suggest that, as the accumulation patterns of Ag in rats correlate strongly with the Ag (I) ion content of the AgNP suspension, absorption is dependent on Ag (I) ions. However, Loescner *et al*., (2011) noted that although a considerable ionic component was present in the AgNPs, it could not account for the entire uptake.

The literature surrounding the bioavailability of AgNPs to invertebrates is limited, but hints that the primary form of Ag taken up following AgNP exposure is ionic. For studies involving oral bioavailability, the most studied organisms are worms, both aquatic and terrestrial. *L. variegates*, exposed through sediment to two sizes of uncapped AgNPs (primary diameters of 30 and 80 nm) or $AgNO_3$, accumulated Ag from all three forms, in a concentration-dependent manner. No differences were seen in tissue burdens regardless of the form of Ag or the primary size of the NP with bio-accumulation factors (BAFs) of 0.05 – 0.07 (Coleman *et al*. 2013), which is a similar BAF to that reported by Hirsch (1998) for Ag sulphide in this organism. Schlich *et al*. (2013) reported 10 fold higher BAFs for the terrestrial earthworm *E. fetida*; these BAFs were similar for AgNPs and $AgNO_3$. The levels of Ag (I) ions in the soil pore water were <0.0001% of the nominal Ag concentration regardless of Ag form, suggesting that Ag (I) ions were the bioavailable species (Schlich *et al*. 2013). Shoults-Wilson *et al*. (2011), however, found that more Ag was absorbed from AgNPs than was accountable for by the Ag (I) content of the soil alone.

1.3.1.2. Distribution and accumulation

Some features of AgNP accumulation and distribution are likely to be similar to those of Ag (I) ions, given that it is inevitable that organisms will be exposed to the two simultaneously from synthesis residues, *ex vivo* dissolution, dissolution within the gut and true *in vivo* dissolution. One of the most important issues yet to be resolved is the contributions of ions and particles to the accumulation and distribution of Ag following AgNP exposure. In particular, as the developmental effects of Ag are poorly studied and the likelihood of exposure of children and adults of child-bearing age to AgNPs is increasing, it is necessary to understand if the distribution of Ag (I) ions and AgNPs differ because this could fundamentally alter toxicity.

Parenteral administration can help distinguish between distribution processes that are dependent on dissolution of AgNPs before they reach the circulation and those that are not. Dziendzikowska *et al*. (2012) introduced AgNPs by intravenous bolus to rats, which resulted in Ag rapid distribution to the liver, lungs and spleen with delayed distribution to the brain and kidneys. The relationship of AgNP size to distribution and accumulation is unclear as there are reports of both a direct (Dziendzikowska *et al*. 2012) and an inverse relationship (Lankveld *et al*. 2010). Accumulation within any organ is cause for concern but the presence of Ag in the brain (Dziendzikowska *et al*. 2012) and testes (Lankveld *et al*. 2010) requires further investigation with the latter giving rise to potential reproductive implications. Compared to rats, rabbits preferentially concentrate Ag in the testes, (Lee *et al*. 2013b), suggesting that species differences may be particularly important to AgNP induced reproductive toxicity.

Oral exposure to AgNPs results in different distribution patterns compared to parenteral administration. Following oral exposure of rats to AgNPs, for 28 days, a large deposition of Ag was seen in the wall of all compartments of the alimentary canal (Loeschner *et al*. 2011, van der Zande *et al*. 2012). Aside from accumulation in the gut wall, a similar organ distribution was found following parenteral dosing, with the liver, kidney (van der Zande *et al*. 2012), and spleen accumulating most Ag with a proportion located in other organs including the brain (Loeschner *et al*. 2011, van der Zande *et al*. 2012). Similar distribution patterns were found when Ag (I) ions were administered in place of AgNPs at equivalent doses, and Ag-containing particulates of dimensions comparable to the

administered AgNPs in were seen in *both* AgNP and Ag (I) ion exposed rats (Loeschner *et al*. 2011, van der Zande *et al*. 2012). Whether these were metallic nanoparticles or insoluble Ag salts was not known, but this serves as a caution against premature conclusions of intact NPs being found in tissues.

Tissue distribution in invertebrates following AgNP exposure, by any route, is largely unknown, but some differences from Ag (I) ions are apparent. Two studies examined accumulation of AgNPs in snails, one freshwater (*L. stagnalis*) (Croteau *et al*. 2011) and one estuarine (*Peringia ulvae*) (Khan *et al*. 2012). Although the authors were able to demonstrate significant accumulation within the soft tissues, measurements were not made upon individual organs. The marine gastropod, *Littorina littorea*, however, has been subject to analysis of Ag distribution following AgNP and $AgNO_3$ exposure whether supplied via the food, water column or both. Regardless of administration route, tissue accumulation did not differ from control for AgNPs whereas waterborne, but not dietary, exposure to $AgNO_3$ caused elevated Ag concentrations in the stomach, kidney and visceral mass (Li *et al*. 2013). A ^{110m}Ag tracer study was performed using the Iceland scallop, *Chalmys islandica* with both $AgNO_3$ and poly(allylamine) capped AgNPs of two sizes (Al-Sid-Cheikh *et al*. 2013). Common to all forms of Ag was the presence of high ^{110m}Ag activity in the hepatopancreas, intestine, crystalline stylus, and anus and lower levels in the gills, mantle, gonads, kidney and muscle tissues. Silver was slowly re-distributed to the digestive system, specifically, the crystalline stylus, gastric shield and hepatopancreas. Ionic Ag and the smaller nanoparticles caused Ag accumulation to a much greater degree in the gastric shield than the larger AgNPs. The authors interpret this as resulting from minimal dissolution of the larger particles compared to the smaller ones, which behaved like the Ag (I) ions (Al-Sid-Cheikh *et al*. 2013).

1.3.1.3. Elimination

The elimination kinetics of AgNPs and how these relate to what is known about ionic Ag are poorly studied. Preliminary evidence suggests that intravenously administered AgNPs are excreted more slowly than ionic Ag, potentially allowing for greater accumulation, although this does appear to be dependent on species. How the route of administration affects elimination is unknown. There do appear to be similarities between AgNP and ionic elimination with suggestions of biliary excretion in mammals (Park *et al*. 2011a,

Dziendzikowska *et al*. 2012, Lee *et al*. 2013b) indicating that AgNPs may be excreted as ionic Ag. Following a single intravenous dose of citrate-capped AgNPs in rabbits, a small proportion was excreted in the urine but a far larger component was eliminated in the faeces. In the absence of oral administration this is consistent with excretion in the bile and/or intestinal secretion. Total elimination within seven days was only 12.7% (Lee *et al*. 2013b). A similar route of excretion was suggested by Park *et al*. (2011a), who found that rats dosed intravenously with citrate capped AgNPs faecally eliminated the majority with urinary rates of less than 0.5% of the faecal rate. Absolute elimination rates from the urine in orally exposed rats are similar to injected rats (despite only 1 - 4% oral bioavailability), suggesting that preferential elimination routes may differ depending on the route of administration (Park *et al*. 2011a). Park *et al*. (2011a) estimated an elimination half-life of ~99 hours following an intravenous dose of 1 $mg/kg_{BodyMass}$ AgNPs but larger doses had higher, but inestimable half-lives. Following 10 $mg/kg_{BodyMass}$ orally, the elimination half-life was ~30 hours, but the authors do not report the half-life for the 1 mg/kg dose; the reason is not stated but it appears that a significant decline in plasma levels had not occurred before the end of the experiment, suggesting very protracted elimination (Park *et al*. 2011a). This suggests that the dose-elimination relationship may be different depending upon the route of administration. In another study, rats that received an oral dose of AgNPs over 28 days achieved an estimated 50% clearance in most tissues after 7 days and virtually none remained in these tissues after 84 days. The exceptions were the brain, testes, kidneys, and spleen; the brain retained ~95% of its maximum level after 84 days (van der Zande *et al*. 2012). Overall, the effects of $AgNO_3$ were qualitatively similar, if exaggerated quantitatively (van der Zande *et al*. 2012). Lee *et al*. (2013a) administered 10 and 25 nm AgNPs to rats for 28 days (orally) also found that the brain and testis were the slowest to clear as demonstrated by a significant Ag residue in these tissues four months after the last dose.

Elimination of AgNPs from invertebrates has received little study, but what data there are suggest that AgNPs are handled differently to Ag (I) ions but in a manner that depends upon species. Formal elimination kinetics for AgNPs administered via food to *D. magna* were computed, and half-lives were exposure concentration-dependent with 5 mg/l excreted with $t_{½}$ = 5.3 days and 500 mg/l more rapidly with $t_{½}$ = 2.4 days. These rates were slower than for equivalent exposures to $AgNO_3$ (Zhao and Wang 2011). Elimination from the tissues of the Iceland scallop, *C. islandica*, has been modelled with a fast and

slow eliminating compartment. In this organism half-lives were dependent upon particle size and the slow compartment had $t_{½}$ = 8 – 24 days for the larger nanoparticles but $t_{½}$ = 19 – 136 days for the smaller particles. In both compartments the small AgNPs had slower elimination kinetics than $AgNO_3$ (Al-Sid-Cheikh *et al*. 2013). However, *L. stagnalis* eliminated Ag accumulated from dietary or waterborne AgNPs more rapidly than from $AgNO_3$. A statistically significant rate constant for $AgNO_3$ elimination could not be estimated, suggesting very slow elimination. It was possible to estimate rate constants for waterborne AgNPs, which converted to $t_{½}$ = 12 days, but not for dietary citrate-capped AgNPs. Humic acid capped AgNPs had elimination half-lives of ~9.2 days and ~13.6 days for waterborne and dietary exposures, respectively (Croteau *et al*. 2011). The elimination half-life for citrate capped AgNPs from the estuarine snail, *P. ulvae* was longer than that from *L. stagnalis* at ~25.6 days and was independent of the exposure concentration (Khan *et al*. 2012).

1.3.2. Toxicodynamics

The novelty of AgNPs and the diverse forms that they may take means that toxicological study of these particles is only beginning. The pace of innovation within the nanotechnology industry increases the difficulty of catching up with the latest developments. As soluble Ag-containing entities AgNPs are likely to exert some of their toxicity via similar mechanisms to Ag (I) salts, but the potential for novel toxic properties exists even if only a result of high intracellular Ag (I) concentrations subsequent to the endocytosis of an AgNP that serves as a massive reservoir (Park *et al*. 2010b). Alternatively, particles may induce toxicity through a particle specific mechanism such as surface mediated oxidative stress (Kovacic and Somanathan 2010).

Silver nanoparticles, like Ag (I) ions, are acutely toxic to aquatic organisms but usually at higher concentrations. For instance, the 96 hour LC_{50} of $AgNO_3$ to the fathead minnow *Pimephales promelas* is 15 µg/l whilst in the same organism 35 nm AgNPs have an LC_{50} of 1.25 mg/l (Laban *et al*. 2010). The 48 hour LC_{50} for PVP- capped AgNPs (~75 nm) and $AgNO_3$ to the adult zebrafish *Danio rerio* were reported as 84 and 25 µg/l, respectively (Bilberg *et al*. 2012). Mammals appear particularly resistant to acute toxicity induced by AgNPs with mice having a 14-day LC_{50} recorded as >750 $\mu g/m^3$ for inhalation (four hour exposure; bare nanoparticles) (Sung *et al*. 2011) and >5000 mg/kg (single dose;

starch capped) for oral exposure (Maneewattanapinyo *et al*. 2011). There are no reported LC_{50} values following intravenous administration; however, 120 mg/kg causes no deaths in mice whereas deaths (number unspecified) were reported to occur at 30 hours following 200 mg/kg (Xue *et al*. 2012). Oral exposure (28 days) of rats to 20 nm uncapped and 15 nm PVP-capped AgNPs at a dose of 90 mg/kg/day caused no detrimental effects (van der Zande *et al*. 2012). Likewise no major metabolic or histological disturbances were seen in rats following 28 days inhalational exposure to uncapped 12-15 nm AgNPs (Ji *et al*. 2007). Very high doses (300 mg/kg/day and 1000 mg/kg/day) of 60 nm AgNPs caused a slight disturbance of liver function (Kim *et al*. 2008). However, following intravenous administration in rats, there appears to be evidence of immunotoxicity at the fairly low dose of 6 mg/kg. At this dose a key finding was the profound suppression of natural killer cell function; indeed, the threshold dose for this effect was 0.06 mg/kg. Splenic mass was reduced at doses as low as 0.01 mg/kg (de Jong *et al*. 2013). Changes in inflammatory cytokines and B lymphocytes have been observed following administration of 1 mg/kg AgNPs orally to mice for 28 days (Park *et al*. 2010a). It is clear that some systems are far more sensitive to the effects of AgNPs and it is significant that one of the most sensitive is the immune system as this is a very active system with high cell turnover.

As a burgeoning field NP toxicology has largely been centred on establishing toxicity ranges and general effects in adult animals with little attention, as of yet, to the potential for developmental toxicity. The sensitivity of a highly dynamic system such as the immune system to AgNPs suggests that embryogenesis and foetal growth could also be targets. Interestingly, zebrafish embryos are more sensitive to AgNPs than an equivalent concentration of $AgNO_3$ - the reverse of that seen in adults (Griffitt *et al*. 2008). Although limited, there appears to be evidence of foetal toxicity to rats at fairly low doses. A single intraperitoneal injection of 0.4 mg/kg in pregnant females at gestation day eight or nine resulted in foetus and placentas of reduced mass when measured at day 20 (Mahabady 2012). However, 250 mg/kg administered to rats orally for 52 days, before, during, and after gestation produced no signs of foetal or newborn toxicity (Hong *et al*. 2013).

Administration of AgNPs to various invertebrates induces oxidative stress and alters the expression of antioxidant and stress related genes. *E. fetida* up-regulates the expression of both catalase and HSP70 (heat shock protein) after 3 days exposure to PVP capped AgNPs (Tsyusko *et al*. 2012) and *D. melanogaster* up-regulates HSP70, catalase, SOD, p53

protein, p38 protein and several caspases (Ahamed *et al*. 2010). Both species suffered a depletion of endogenous antioxidants and exhibit higher levels of molecular oxidative lesions (Ahamed *et al*. 2010, Tsyusko *et al*. 2012). Precisely how this oxidative stress relates to developmental anomalies in these species is unclear but both exhibit delayed development following exposure to AgNPs. *C. elegans* develop more slowly following exposure to AgNPs although the magnitude of the developmental delay is influenced by the capping agent. The low toxicity of the citrate-capped particles was attributed to the binding of Ag (I) ions to the capping agent as well as free citrate that may have dissociated from the particle surface in a mechanism similar to that discussed above in Section 1.3 (Yang *et al*. 2012). However, AgNPs were apparently able to affect development via direct induction of oxidative stress, although the extent of this effect was modulated by capping agent and particle size (Yang *et al*. 2012).

Four studies, all published in 2011, have examined the effects of AgNPs, administered via the food on various aspects of *Drosophila* development (Gorth *et al*. 2011, Key *et al*. 2011, Panacek *et al*. 2011, Posgai *et al*. 2011) but did not compare these with the effects of ionic Ag. The most intriguing of these found an apparent association between *increasing* particle size and toxicity (Gorth *et al*. 2011). However, those results seem to indicate a high degree of agglomeration and this size dependency may simply be an artefact of this. Nonetheless, 100 nm AgNPs were shown to concentration-dependently reduce pupation success, with 10 µg/ml showing no change in pupation and 100 µg/ml causing a significant decrement. Panacek *et al*. (2011) found qualitatively similar results using BSA capped 29 nm AgNPs with a toxicity threshold of 80 µg/ml. Significantly, Panacek *et al*. (2011) found trans-generational toxicity. When exposed to a sub-lethal concentration of AgNPs (5 µg/ml) fecundity dropped over two consecutive generations. Although the differences were slight, Posgai *et al*. (2011) demonstrated that smaller particles, particularly those that are uncapped, were more toxic than larger ones (Posgai *et al*. 2011), contradicting the assertion of increasing toxicity with size (Gorth *et al*. 2011). Posgai *et al*. (2011) found a modest recovery of pupation success and a slight amelioration of developmental delay following antioxidant treatment and hypothesised that oxidative stress is critical for toxicity. However, they did not consider any direct effect of the antioxidant on particle oxidation and subsequent dissolution. Key *et al*. (2011) performed similar investigations to those described above but with significantly higher concentrations of AgNPs; their minimum concentration was 830 µM extending up to 83 mM. All concentrations caused a

reduction in the number of eclosing adults. All of the above studies reported de-pigmentation, similar to that described for $AgNO_3$ (Gorth *et al*. 2011, Key *et al*. 2011, Panacek *et al*. 2011, Posgai *et al*. 2011); some hypotheses regarding mechanisms, with varying degrees of plausibility, were proposed. Curiously, none attribute the de-pigmentation to release of Ag (I) ions, even though in some cases their data appear to hint at such a mechanism. One study has suggested that de-pigmentation is an AgNP specific effect through comparison with ionic Ag, but using concentrations that are unlikely to be truly equivalent (Armstrong *et al*. 2013). Further details regarding de-pigmentation can be found in Section 6.1. Clearly, AgNPs are a developmental poison to *Drosophila*, but how much of this toxicity is derived from the particles *per se* and how much from dissolved ions is unknown. None of the studies compared the effects of *equivalent* concentrations of AgNPs and ionic Ag for developmental delay, mortality, or de-pigmentation. This is critical in distinguishing between particle and dissolution mediated effects.

1.4. Aims and Objectives

The developmental toxicity of Al and Ag remains relatively unstudied in terrestrial insects and humans. The primary aim of this thesis is to characterise the developmental toxicity of Al and Ag to *Drosophila* and to understand how the form the metal may influence toxicity. Furthermore, the influences of potentially protective interventions are to be examined. One objective was to ascertain the suitability of *Drosophila* as a model for the toxicity of these metals with a view to understanding the potential for both human and insect toxicity.

It is hypothesised that a primary effect of the toxicity of Al and Ag will be to increase mortality at sensitive developmental stages, specifically larval growth, the larval to pupal transition, and the pupal to adult transition. Furthermore, it is proposed that at lower exposure concentrations, even in the absence of loss of viability, the progression from one stage to the next will be delayed. For this developmental toxicity to occur, it is hypothesised that significant deposition of the metal in the tissues is required but that this may occur the absence of toxicity. The speciation of metals is often critical for toxicity and their uptake. It is therefore possible that different ligands for Al and whether Ag is present in the nanoparticulate form will influence the magnitude or character of toxicity. Although Si is a ligand for Al that may reduce its bioavailability or toxicity when supplied simultaneously, the question that is to be addressed here is whether *Drosophila* are able to

mobilise endogenous Si such that they may detoxify Al. The relatively short lifespan of *Drosophila* will be exploited to examine if any developmental toxicity translates into a reduced lifespan. Since a considerable component of AgNP toxicity probably results from release of Ag (I) ions, it is hypothesised that some developmental parameters affected by $AgNO_3$ will be similarly affected by AgNPs. In particular, the failure to lay down cuticle pigmentation post-eclosion that has been shown to occur following both $AgNO_3$ and AgNP administration is hypothesised to occur via a common mechanism. Considering this, it is suggested that Cu will antagonise the developmental toxicity of both Ag (I) ions and AgNPs. As it is hypothesised that dissolution of AgNPs is critical for any toxic effects it is further hypothesised that aging of AgNPs will cause the release of dissolved Ag in a pH dependent manner. These hypotheses are summarised in Figure 1.

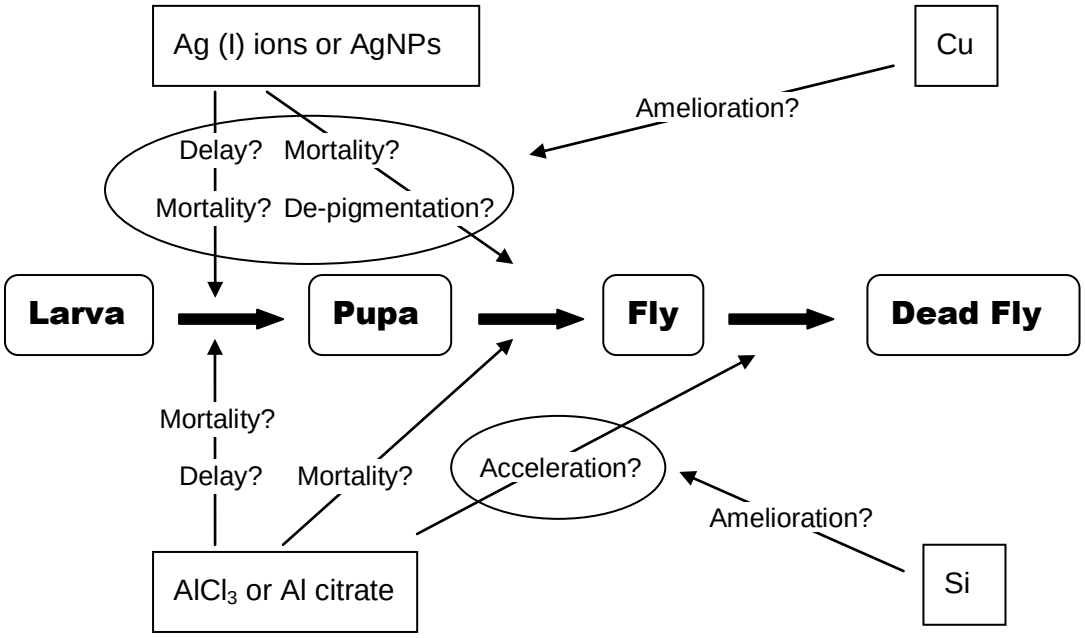

Figure 1. Schematic diagram of questions relating to developmental toxicity of aluminium and silver to *Drosophila*. Developmental stages are shown in rounded rectangles, treatments are shown in rectangles whilst possible toxicity targets for amelioration or shown in oval boxes.

1.4.1. *Drosophila* as a model organism and experimental approach

The use of a short lived organism, such as *Drosophila*, allows the study of developmental toxicity over a fairly short timescale. *Drosophila* is a holometabolous insect and its development is segmented into easily measureable stages. A *Drosophila* egg, when cultured at 25 °C hatches after 21 hours of development. The larva spends approximately the next 100 hours feeding whilst progressing through three larval instars (Demerec 1994). As development progresses, the level of juvenile hormone (JH) decreases and timed pulses

of ecdysone initiate the larval moult that culminates in ecdysis stimulated by eclosion hormone secreted in response to the waning of the ecdysone pulse (Truman 1981, Horodyski 1996). At some time during the third instar (the end of the obligatory feeding phase), and depending on nutrient availability, larvae attain a critical mass and commit to metamorphosis. They continue to feed for a further facultative feeding period after which they enter the wandering phase, in response to another pulse of ecdysone, whilst they select a favourable pupation site (Denlinger and Zaarek 1994). Initiation of pupation is signalled by an ecdysone pulse and co-ordinated by a suite of neuropeptides. During the pupal period the larval body is largely broken down and replaced by adult structures. Eclosion occurs after about 10 days after egg laying (AEL), under similar hormonal control to the larval moults (Denlinger and Zaarek 1994, Horodyski 1996). After eclosion, a short period of maturation occurs, during which the cuticle is sclerotized and acquires its pigmentation under the control of the hormone, bursicon (Fogal and Fraenkel 1969, Truman 1981). After this the fly is able to display its entire repertoire of innate adult behaviour (Demerec 1994). Total lifespan averages 30 – 40 days but can be longer (Demerec 1994, Roberts 1998).

Despite obvious physiological differences between the fruit fly and humans, many similarities exist, and a remarkable number of genes are conserved over a large evolutionary gap; Reiter *et al*. (2001) estimate that up to 75% of human disease genes have a *Drosophila* ortholog (Reiter *et al*. 2001). Conserved genes that are directly relevant to metal metabolism include the ortholog of the human di-valent metal ions transporter, *malvolio* (Bettedi *et al*. 2011); the ortholog of the monovalent Cu transporter Ctr1, Ctr1A (Hua *et al*. 2010); the iron binding proteins transferrin (Yoshiga *et al*. 1999) and ferritin (Charlesworth *et al*. 1997); and a putative ceruloplasmin ortholog, multi-copper oxidase 3 (Bettedi *et al*. 2011). In contrast to rodents the cost of housing flies is minimal and they may be propagated in very large numbers to provide statistical power.

The easy distinction between stages of the *Drosophila* lifecycle provides a good window into toxicological processes that may affect development. Many metrics can be devised for assessing developmental metal toxicity, including numbers of viable individuals at each stage and the rate of progression from one stage to the next. For example, chromium (Cr) toxicity was examined by deriving the ratio of successful events from exposed animals to control animals following a fixed laying period using a fixed number of adults (Hepburn *et al*. 2003). The developmental toxicity of both Al (Wu *et al*. 2012b, Kijak *et al*. 2013) and

AgNPs (Gorth *et al*. 2011, Key *et al*. 2011, Panacek *et al*. 2011, Posgai *et al*. 2011) have been preliminarily investigated using *Drosophila*. Lifespan is another useful endpoint, the value of which is enhanced by the large numbers of flies available for experimentation. *Drosophila* has been successfully used to characterise the toxicity of both Pb and As. Differences across two strains in Pb sensitivity have been exploited by using recombinant inbred lines and have enabled the identification of a quantitative trait locus that mediated the sensitivity (Hirsch *et al*. 2009). Similarly, Ortiz *et al*. (2009) used recombination mapping and site-directed recombination techniques (FLP-FRT recombination) to pinpoint a gene duplication conferring As resistance. The field of nanotoxicology is now starting to exploit *Drosophila*. Vales *et al*. (2013) have demonstrated that cobalt (Co) nanoparticles are genotoxic using this organism. Pandey *et al*. (2013) found that silica nanoparticles caused cellular membrane damage and mitochondrial dysfunction in the midgut cells.

To test hypotheses relating to toxicity throughout the larval and pupal period, *Drosophila* will be exposed to various concentrations of $AlCl_3$ or Al citrate (for Al investigations) or $AgNO_3$ or AgNPs (for Ag investigations). The precisely defined and visually apparent developmental stages of *Drosophila* permit easy analysis of developmental progression times and mortality at each stage. Further studies of Al toxicity will include determination of lifespan. This is proposed as a measure of chronic toxicity, not only for its convenience of determination but also because the experimental period is extended over the entire lifespan allowing temporally separated administration of silicic acid in an attempt to mitigate Al toxicity; such temporal separation is necessary to prevent *ex vivo* interaction. De-pigmentation will be analysed after eclosion, following exposure of larvae to $AgNO_3$ or AgNPs with or without supplemental Cu to act as an Ag (I) ion antagonist, potentially preventing the de-pigmentation. However, as the de-pigmentation phenomenon exhibited by Ag exposed flies occurs only with larval exposure any need for temporal separation between Ag and Cu will require that the medium be changed during the larval period. To ascertain whether developmental toxicity of these metals requires a significant body burden of the metals cohorts of larvae and/or flies will be exposed to concentrations used in the developmental assays and their metal uptake determined. Finally, to assist in determining AgNP specific and Ag (I) ion mediated effects assays will include testing for susceptibility to oxidative stress and growth rates for both forms of Ag and comparing the outcome.

Chapter 2. General methods and materials

2.1. Chemicals

Routine chemicals used throughout this work were all of analytical grade and were obtained from Sigma-Aldrich Ltd., UK, Fisher Scientific UK Ltd., or VWR International Ltd., UK. More specialist chemicals are described as they are introduced. Stable chemicals in regular use were prepared as stock solutions and diluted as required but were never more than four months old. Polyvinylpyrrolidone (PVP) and ascorbic acid solutions and silver nanoparticle (AgNP) suspensions were prepared freshly, as required, due to their instability in aqueous solutions.

2.2. Fly husbandry

Canton-S wild-type flies (laboratory cultured, at the University of Manchester, for over five years; ultimate source: Bloomington *Drosophila* Stock Center, Indiana) were used for the Al investigations. Cultures from a population of wild caught flies, kindly provided by Dr. Frederic Mery (Centre National de la Recherche Scientifique, France), were used throughout the Ag investigations. Cultures from wild caught flies were used for Ag work to provide a less inbred, more environmentally relevant stock. Fly stocks were maintained in 250 ml plastic bottles (~250 flies/bottle) plugged with Droso-Plugs™ (Genessee Scientific Corporation, USA) containing 80 ml of standard *Drosophila* food medium, comprising water (5 l), glucose (390 g), maize (360 g), dried yeast (250 g) and agar (50 g), supplemented with methylparaben (135 ml) and propionic acid (15 ml) as preservatives. This medium was prepared in house by Media Preparation Services, University of Manchester. Flies were reared at 25 °C under at 12/12 hour light/dark cycle and were transferred to fresh medium every 14 days.

2.3. Preparation of media for experimental treatments

Throughout the investigations, all flies received test substances delivered via the standard *Drosophila* medium (as described in Section 2.2) except in a few circumstances (noted as they occur) when live yeast slurry was used as the food vehicle. Slurry was used primarily for ease of harvesting larvae (Section 2.5). Yeast slurry was prepared from live baker's yeast (AB Mauri, UK) (24.9 g) and de-ionised H_2O (5.4 ml), which were mixed until

smooth; test substances were added directly to the slurry when required. For dosing standard *Drosophila* medium, the medium was heated in a plastic vessel using a microwave oven (Sanyo Super Showerwave – 900W), stirring periodically, until completely molten with no observable lumps. Test substances were then added. Eighty millilitres of *Drosophila* medium was decanted into 250 ml *Drosophila* bottles, or 10 ml into 40 ml vials, where used, loosely covered and allowed to set. Once set, condensation was carefully removed from the inside of the bottle or vial using tissue paper. For all test substances the concentrations reported are added concentrations; as no metal chelators were used trace concentrations of bioavailable metals may have been present in control groups.

2.4. Egg collection

Each experiment used flies of a specific age. To achieve age-synchronised populations, carefully timed egg collections were performed. Approximately 800 flies (determined by mass: 1 fly weighs ~1 mg live weight) of 5 – 10 days of age, previously maintained in mass culture, were collected under CO_2 anaesthesia and placed in a polystyrene box ('laying box') with three holes in the lid. The holes were to admit small acrylic dishes (pucks) that were prefilled with plain agar (Melford Laboratories, UK; product number: M1002) (1% in H_2O, 2 ml) supplemented with glacial acetic acid (1%) and ethanol (2%); a small amount of live baker's yeast was placed at the edge of each puck to stimulate oviposition (Figure 2).

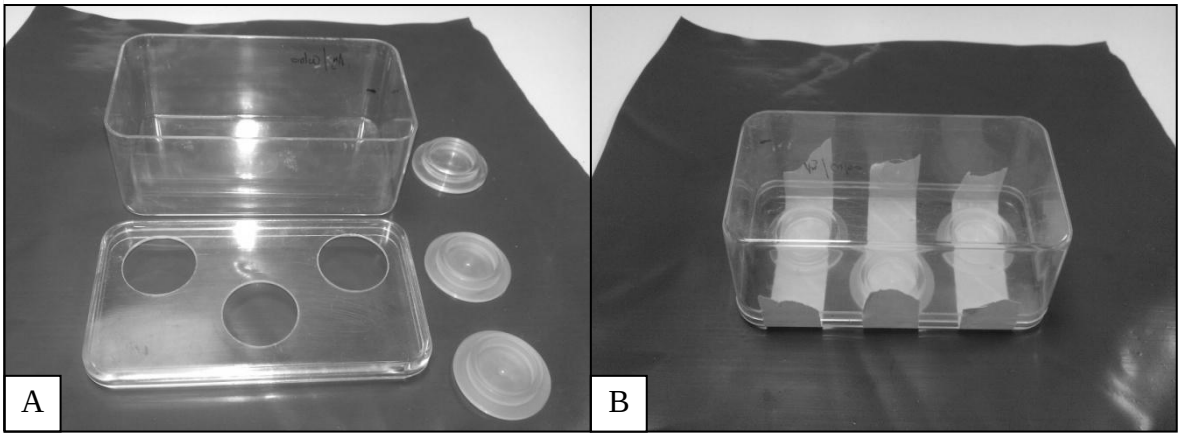

Figure 2. Laying box and pucks. (A) disassembled and (B) assembled

Once the flies had recovered from anaesthesia the boxes were oriented such that the laying surface (the agar) was at the bottom; flies are reluctant to lay in an inverted position. To

ensure eggs were of the same developmental age, the box was incubated undisturbed, in the dark, at 25 °C for one hour, after which time the pucks were removed and replaced with freshly filled ones, and the egg-laden agar was discarded. The boxes were returned to the incubator for a further three hours after which the flies were removed. The egg-laden agar discs were removed from the pucks and placed in Petri dishes. If an experiment required an indeterminate number of eggs the agar discs were used as they were. If a specific number of eggs was needed then individual discs were placed on a Petri dish under a dissecting microscope (Leica MZ75) and counted at x 25 magnification. Using a scalpel a section of agar was cut containing the required number of eggs.

2.5. Feeding and collection of larvae

Yeast slurry (Section 2.3) was used for larval feeding in circumstances that required that larvae were easily separated from the food substrate. Otherwise standard *Drosophila* medium was used. If larvae were to be collected in bulk, eggs were collected as described in Section 2.4 and each complete egg-laden agar disc was placed in a Petri dish containing plain agar (5% in H_2O). A quantity of yeast slurry (described in Section 2.3), sufficient to cover the agar base layer without covering the eggs, was poured into the Petri dish, and the plates were incubated under the conditions described in Section 2.2. When a specific number of larvae was to be cultured for an experiment, eggs were counted and the egg-laden agar slice (Section 2.4) placed in an agar dish, as above, and exactly 10 ml of yeast slurry was dispensed around the eggs using a syringe (Becton Dickenson and Company, UK).

At the appropriate time point for the experiment (described as required), the yeast plates, now containing age-synchronised larvae, were flooded with de-ionised water and the yeast agitated with a soft paintbrush. The resulting mixture was strained using a basket assembly constructed from a 120 μm nylon mesh (Small Parts, Inc., USA) attached to a truncated 50 ml BD Falcon centrifuge tube (Fisher Scientific UK Ltd., UK) and residual yeast was flushed away under running de-ionised H_2O. The mesh was removed from the basket assembly, briefly blotted on the underside with tissue and the cleaned larvae were transferred to a Petri dish prefilled with plain agar (2.5%) prior to use.

To collect larvae from standard *Drosophila* medium, vials or bottles containing larvae embedded in food substrate were filled to about 2 cm above the surface of the medium with 20% sucrose solution. The now submerged surface was gently agitated with a soft paintbrush to disturb the larvae and the vessel topped to the brim with further sucrose solution. These vials/bottles were left to stand for five minutes to allow the larvae to float to the surface of the liquid. Floating larvae were pushed gently to the lip of the vessel with a soft paintbrush and a small amount of sucrose solution containing the larvae was decanted into the collection basket described above. To capture any larvae that may have escaped transfer, the vessel was refilled with sucrose and the process repeated. Larvae in the basket were rinsed with running de-ionised H_2O, blotted and transferred to a plain agar dish as above.

2.6. Gustatory assays

The most practical method for administering metals or other substances to *Drosophila* is via the food medium. However, any such contaminant in the food may elicit a gustatory response, ultimately affecting ingestion. To determine if the taste of the substances used in these investigations resulted in a behavioural change in larvae, a mass positional preference assay was used (Cobb *et al*. 2009), in which the tastant is presented in the agar surface that larvae crawl across. Adult gustatory responses were assessed using an oviposition based preference assay similar to that described by Possidente *et al*. (1999). This test relies upon gustatory receptors found in multiple locations on female *Drosophila* including those on the ovipositor itself. Both these tests are binary choice assays and yield a preference index expressed as a percentage. For both assays, controls were created by formulating a choice test between two identical non-tastants (untreated agar for larvae or medium for oviposition assay) with one choice randomly designated as a "sham-tastant" before the assay commenced. If no additional factors were at play these would yield a mean of 50%.

To make gustation assay plates for larvae, plain agar (5% in H_2O) was poured into Petri dishes and allowed to set. Using a scalpel the agar in each plate was cut into quadrants and two diagonally opposed sections removed and discarded. Agar (5% in H_2O) supplemented with the substance to undergo taste testing (e.g. Al or Ag) was added using a syringe to the now empty quadrants until level with the solid non-supplemented agar quadrants, thus

forming an arena divided into four sections: two plain agar and two agar containing the test substance.

Early 3rd instar larvae (72 hours after egg laying (AEL)) (See Section 2.5) were collected from yeast slurry as described in Section 2.3 and for each replicate (1 plate), 30-40 larvae were placed with a damp paintbrush in the centre of a gustation test plate (flat but otherwise with a random orientation), and permitted to wander freely across the entire surface for five minutes. After this time had elapsed, the number of larvae in each quadrant was counted and the percentage of larvae on the test substrate was calculated. As sharp delineations were present between quadrants, all larvae were assigned to one or other category based upon the position of their mouth hooks.

The oviposition assay was performed using the laying boxes described in Section 2.4. Rather than plain agar as used for larval gustation tests, this assay was performed using standard *Drosophila* medium supplemented with the tastant. For each replicate two pucks were made (both with red food dye (1%) to facilitate egg counting), one consisting of unmodified medium and the other containing the tastant. These pucks were placed in the holes at each end of the box lid and the lid fitted to the box. Approximately 200 adult *Drosophila* (mixed sex) were placed in each box through the centre hole and the hole was plugged with an empty puck. The laying boxes were incubated (laying surface at the bottom but otherwise in a random orientation) at 25 °C for 3 hours. Following this the pucks were removed and the eggs counted under x 25 magnification. The percentage of eggs on the test substrate was then calculated.

2.7. Developmental studies

2.7.1. Assessing the effects of metals on the developmental cycle of *Drosophila*

To characterise the developmental toxicity of the metals under study and how this was modulated by their speciation or particulate nature, *Drosophila* were followed from hatching until eclosion and assessed for viability at various stages: 48 hours AEL (approximately the beginning of the 2nd instar under normal developmental conditions), pupation and eclosion. In addition, having established ranges for which test substances did not induce 100% mortality, the time taken to pupation was assessed; this was to allow greater sensitivity to potentially milder toxicity that may otherwise be obscured using the

blunter measure of viability at pupation (pupation success). Details of individual protocols for each metal can be found in the corresponding chapters (Chapters 3 and 5 for Al and Ag respectively). These developmental studies were performed using either a yeast pre-feeding stage followed by final development on standard *Drosophila* medium (referred to hereafter as yeast pre-feeding - YP) or by feeding solely on medium (referred to hereafter as medium throughout development - MTD). The use of the YP protocol was used to allow the easy determination of larval viability.

Drosophila that were to be reared under the MTD protocol were obtained by collecting the required number of eggs for the experiment (stated at the appropriate point) and transferring the egg-laden agar slice (Section 2.4) directly into a bottle/vial containing standard *Drosophila* medium spiked with the substance under test or unspiked as control. The larvae/flies remained in these containers until the end of the experiment, unless otherwise specified. Under the YP protocol, 100 eggs were counted and placed in a Petri dish as described in Section 2.5. At 96 hours AEL, larvae were collected, counted, and transferred to vials containing standard *Drosophila* medium at the same concentration of test substance. Development was allowed to proceed from this point in exactly the same manner as in larvae that were fed on medium throughout development.

To determine pupation success and the time to pupation, pupae were counted periodically (every 3 – 12 hours), starting at 110 hours AEL, and marked on the outside of the vial to avoid double counting. Larvae were considered to have pupated when they had reached the white puparium stage (stage P1) as defined by Bainbridge and Bownes (1981). This was evident as cessation of larval movement, complete eversion of the anterior spiracles, shortening of the body and adherence to the bottle/vial wall. Counting was continued until pupation was complete: a point considered to have been reached when a period of 48 hours had elapsed with no further pupations. The bottles/vials containing pupae described above were incubated further under the same conditions and the flies were allowed to eclose. When eclosion had finished (a point defined as 48 hours without further eclosions) the number of adult flies was counted. Adult flies were considered as eclosed when they had reached the exarate adult, newly eclosed stage (stage A1), as defined by Bainbridge and Bownes (1981) i.e. adult has fully emerged but has yet to expand its wings.

2.7.2. Assessing the effects of metals on larval length

To complement developmental work experiments were conducted to determine whether physical growth rate was affected by metals. Specific exposure protocols for each metal are described in the relevant chapter (Chapters 3 and 5 for Al and Ag, respectively). Following an exposure period in yeast slurry and/or standard medium, larvae were removed from medium as described in Section 2.5 and killed by placing the collection basket in boiling water. Killing the larvae by this method resulted in a flaccid carcass that was easily extended and laid out. Dead larvae were placed, ventral side down, as straight as possible on a Petri dish and the dish placed on a black filter paper to enhance contrast alongside a scale marker of 100 mm. The Petri dishes were photographed in an uncompressed format (RAW) using a digital SLR camera (Canon EOS 1100D) mounted on a tripod (Slik [*sic*] Compact II) at a height of 400 mm levelled with a spirit level. The camera was zoomed such that the 100 mm scale bar filled approximately $4/5^{th}$ of the field of view. The images of larval corpses were transferred to ImageJ software for analysis. Using the 100 mm scale the software was calibrated and the length of each maggot measured along the midline from anterior to posterior tip in mm to the nearest 0.2 mm. If a slight curvature of the larval body was evident a multi-section line was used, maintaining a track along the midline.

2.8. Metal uptake studies

2.8.1. Metal administration protocols

The toxic effects of many metals emerge only after a period of uptake has occurred and tissue concentrations reached a toxic threshold, and therefore the body concentrations of metals and Si were determined after various periods of exposure. The specifics of each metal exposure experiment are described in the appropriate chapter (Chapters 3 and 4 for Al and Ag respectively). Whichever element is under consideration, an unavoidable consequence of delivering test substances to animals in the food medium is that at any specific time the gut will contain considerable amounts of test substance within the food. In order that amounts of metal absorbed into the tissues could be determined, it was necessary to purge the gut content. To assess the period of time necessary for feeding larvae and adults on non-spiked food to ensure a non-contaminated gut, clearance time was determined for both adults and larvae under control and metal fed conditions.

To determine larval gut clearance time, five replicate plates were prepared for each treatment. For each plate 100 eggs were collected (Section 2.4) and placed on a Petri dish containing plain agar (5%) with 10 ml of yeast slurry (control or $AlCl_3$ (10 mM)) dispensed around them. These Petri dishes were incubated under the conditions described in Section 2.2 for 60 hours (early third instar). After this time larvae were removed from the yeast (Section 2.5) and transferred to a second Petri dish containing 10 ml yeast slurry that had been coloured with 5 mg/ml of erioglaucine, a strongly coloured, blue dye that is not absorbed from the alimentary canal; larvae that were Al exposed also received Al spiked blue yeast. Larvae were allowed to feed freely on the dyed food for 12 hours after which time they were transferred to uncoloured fresh yeast. At 2, 4, 8, 12, and 16 hours, larvae were removed from the yeast and the percentage of animals that had voided their gut of dye determined. A larva was considered clear of dye when no there was no evidence of blue colouration at 20 x magnification using reflected light. After each count, larvae were returned to a fresh dish of undyed yeast.

Adult gut transit time was determined in a similar manner to that described above. One hundred eggs were collected per replicate and placed in 250 ml *Drosophila* bottles on standard *Drosophila* medium and allowed to pupate, eclose and mature for 3 days. At this point, the adult flies were transferred to bottles containing *Drosophila* medium dyed with 5 mg/ml erioglaucine and returned to the incubator for 24 hours to ensure they ingested a sufficient quantity of pigment. Having ingested the dye, flies were transferred to bottles containing plain medium. At 4, 8, 12, 16, 28 and 32 hours flies were examined under 20 x magnification and the percentage of clear animals recorded. After each count flies were transferred to bottles containing fresh medium.

To determine metal and Si levels in *Drosophila* larvae, 100 eggs were collected (Section 2.4) per replicate and each replicate placed on a Petri dish containing plain agar (5%). Yeast slurry (10 ml) containing the appropriate concentration of test substance was dispensed around them (Section 2.5). These Petri dishes were then incubated under the conditions described in Section 2.2. After 72 hours incubation, larvae were collected and transferred to a second Petri dish, identical to the first but containing only control yeast, to allow the spiked yeast to be cleared from the larval gut prior to analysis. The plates were incubated for a further 24 hours after which the larvae were harvested, washed in de-ionised water, placed in pre-weighed, acid washed, 2.0 ml micro-centrifuge tubes (Star-

Labs, UK), capped and transferred to a freezer at -18°C for 24 hours. Larval corpses were dried at 60 °C for 48 hours and their dry mass was determined before digestion.

To administer metals and Si for measurement in adult *Drosophila*, 250 eggs were collected per replicate and placed directly into a 250 ml bottle containing 80 ml spiked *Drosophila* medium. Bottles were incubated under the conditions described in Section 2.2 until all flies had eclosed. Newly eclosed flies were transferred to new bottles containing fresh control medium for 48 hours to allow any residual metal-containing food to be eliminated from the flies' guts. If metal administration was to continue into adulthood (described where it occurred) gut purging was performed only after the exposure period was complete. After gut purging, flies were harvested, washed in de-ionised water, placed in 30 ml universal tubes and transferred to a freezer at -18°C for 24 hours. The flies were then dried in 2.0 ml micro-centrifuge tubes at 60 °C for 48 hours and their dry mass determined before digestion.

2.8.2. Tissue digestion and analysis

All plastic-ware was acid washed by bathing for 48 hours in 10% HNO_3 followed by thorough rinsing in de-ionised water. Tissue digestion protocols for each metal are described in the appropriate chapter. Quantification of metals and Si was accomplished using a Perkin-Elmer Optima 5300 Dual View inductively coupled plasma atomic emission spectrophotometer (ICP-AES) using wavelengths of 396.153 nm, 328.068 nm, 324.752 nm, and 251.611 nm for Al, Ag, Cu and Si respectively. Standard curves for Al, Ag and Cu were prepared by linear regression using serial dilutions (final concentrations: 10, 50, 100, 500, and 1000 μg/l) of a multi-element plasma standard solution 4 (Alfa Aesar, UK) supplied at 1000 mg/l and containing a total of 23 elements in HNO_3 (2%). The standard curve for Si was prepared in the same manner but used a Si standard (Alfa Aesar, UK) supplied at 1000 mg/l in HNO_3 (5%) and trace quantities of hydrofluoric acid (HF). Matrix matching was not possible owing to the unknown composition of the biological digest matrix. Reference blanks were run every 10 samples throughout the analysis. Method detection limits were calculated as three times the standard deviation of three repeat samplings of a blank. Method detection limits were 3, 2, 2 and 13 μg/l for Al, Ag, Cu and Si respectively; unless stated all ICP-AES measurements were above detection

limits. Spike recoveries and analysis of certified reference material are described in the relevant methods sections.

2.9. Statistical analysis

All parametric data are reported as mean +/- 95% confidence interval (CI). All data sets were tested for homoscedasticity (equal variance) using a modified Levene's test (Brown-Forsythe test). This test was chosen as it is considered robust to deviations from normality (Brown and Forsythe 1974). Binomial data (proportions) were arcsine-root transformed before analysis to both normalise the data and stabilise the variance as the latter, especially, can inflate errors rates when using ANOVA (Snedecor and Cochran 1967). Parametric data (including that which had been transformed) with nominal (i.e. categorical) independent variables (including that derived from uptake, gustation, developmental success and, locomotor, negative geotaxis, heart rate, and dissolution experiments) were analysed by one, two or three way ANOVA (with a nested or repeated measures structure where appropriate) followed by a Tukey-Kramer post-hoc test. The Tukey-Kramer test is simply a variant of the Tukey test that is robust to differences in group sizes (Kramer 1956) and was chosen as it provided a good compromise between power and protection against type I error (false positive) (Sokal and Rohlf 2012). The pigmentation data (Chapter 6) was based upon an ordinal scale and was therefore unsuitable for parametric analysis, with or without transformation because of its non-continuous nature. These data were therefore analysed by the non-parametric, Kruskal-Wallis test with Dunn's post-hoc test. Due to the limited number of values on the ordinal scale, multiple ties (subjects with identical scores) were inevitable, making display by box and whisker plot of limited value; data were displayed as a compound bar chart instead, allowing the percentage of subjects with each and every score to be seen.

Time-to-event (also known as survival data) are best analysed through the use of specific survival analysis techniques. This is necessary to overcome the limitations that other techniques possess when confronted with time-to-event data. Such data are rarely normally distributed and, most importantly, are usually subject to censoring. Censored data are incomplete; a death (this could be any event but death will be used throughout this discussion for the purpose of example) may only be known to occur within a specified range of times rather than a precise time point. This leads to three major forms of

censoring: left, right and interval censoring. Left censoring occurs when death can only be said to occur *before* time t; right censoring, only *after* time t and interval censoring, only *between* times t_1 and t_2. In this work all time-to-event data were interval censored with some instances of right censoring. The interval censoring here was *asynchronous* as the intervals between measurements were not identical throughout the experiment. Left censoring did not occur.

Two key functions are related to survival analysis:

- Survival function
- Hazard function

The survival function is defined as:

$$S(t) = \mathrm{P}(T \geq t)$$

This means the probability of the "time to death" as shown by the random variable T is greater than or equal to some time *t*. Stated less mathematically it is the probability that the event has not occurred until at least time *t*.

The hazard function is defined as:

$$h(t) = \lim_{\delta t \to 0} \left\{ \frac{\mathrm{P}(t \leq T < t + \delta t \mid T \geq t)}{\delta t} \right\}$$

The hazard function h(t) is thus a probability per unit time. It is the conditional probability that the event will occur in the interval *t* to $t + \delta t$, given that it has not already occurred per unit time, in the limit as *t* tends zero. That is to say, it is the proportion of subjects that are not already dead but will undergo an event per unit time, when averaged over an infinitesimally small unit of time. A value for the hazard function is thus the hazard rate or instantaneous failure rate. Using the hazard rate it is possible to compare the survival experiences of two or more groups by calculating the hazard ratio (HR), a quantity derived from the two complete curves that can be tested statistically. In this work time-to-event data were analysed non-parametrically, using Kaplan-Meier methods to produce survival

functions and hazard ratios, the latter of which were statistically tested with log-rank tests. These techniques allow survival data to be analysed with the minimum of assumptions about the distribution of the data and the shape of the hazard function curves. Median ratios are shown when useful to data interpretation but cannot be tested statistically and, due to the non-parametric nature of the data, median values can only take specific times at which measurements occurred (no interpolation). As such, even obviously different curves may yield a median ratio of unity (Collett 1994).

When survival data are analysed, rather than using a measure of central tendency (e.g. median or mean) and spread for statistical inference, the survival function is used to generate hazard functions. For this reason survival data are displayed as a cumulative frequency plot of the number of subjects (replicates) that die at each time point rather than, for example, a box and whisker plot. This forms a graphical display of the empirical survival function. The confidence intervals associated with these values therefore represent the 95% confidence interval for the fraction of the population of subjects that would die. As interpolation of the empirical survival function is not permitted in non-parametric analysis, graphic display of these functions is usually a stepped connecting line to indicate that events occurred at specific time points. However, in this work, data points of empirical survival functions have been connected with straight lines, simply for the purpose of aiding readability; multiple superimposed step-functions become very difficult to read especially when confidence intervals are included. These lines must not be interpreted as interpolations.

Linear regression was performed to ascertain the relation between Si and Al accumulation (Section 3.2.3) and for construction of the standard addition curve (Section 4.3.1). Kruskell-Wallis tests and Dunn's post-hoc tests were performed using GraphPad Prism® (Version 3.00) software. All other data analysis was performed using NCSS 2007 (Version 7.1.18) software.

Chapter 3. Aluminium toxicity in *Drosophila melanogaster*

3.1. Introduction

Aluminium is ubiquitous throughout the Earth's crust (Cox 1989) and highly toxic. Despite limited bioavailability under natural conditions, human activity has mobilised significant quantities of Al permitting it to become an important poison to all forms of life (Driscoll and Schecher 1988). Aluminium is a potent neurotoxin in mammalian systems. Aluminium phosphate injected intracerebrally into adult rabbits caused the development of neurofibrillary tangles similar, though not identical, to those seen in Alzheimer's disease, along with seizures and often death within three weeks (Klatzo *et al*. 1965, Terry and Pena 1965).

Despite the profound neurotoxicity of parentally administered Al to rabbits, it took the deaths of many haemodialysis patients that showed histologically similar lesions over a decade later before the Al was identified as the causative factor of DAE (Alfrey *et al*. 1976, Alfrey 1978). Importantly, it was noted that orally administered Al containing drugs produced a similar responses, especially in children. From this point on, the role of Al as a possible causative factor in neurodegenerative diseases, especially Alzheimer's disease has been a source of considerable controversy (Gupta *et al*. 2005, Miu and Benga 2006, Kawahara and Kato-Negishi 2011, Tomljenovic 2011).

Exposure to Al in the general population is considerably less that that experienced by renal patients but does result in bio-accumulation, albeit over a more protracted timescale. Likewise, Alzheimer's disease develops slowly and the pathological hallmarks are quite different suggesting that mechanistic differences exist (Kruck and McLachlan 1988). If Al is causal in Alzheimer's disease, accumulation of Al would occur from conception and throughout development, raising the question that its toxic effects during this period may be critical to its mechanism of action. Even if cleared of involvement with the development of Alzheimer's disease, the overt toxicity of acute exposure to Al leaves open the possibility of subsequent chronic effects even if administration ceases. This is particularly concerning as human infant milk formulations, especially soy-based, contain high levels of Al (Burrell and Exley 2010). With neurodegeneration being the classical pathology of Al exposure, it is not surprising that most mammalian studies have focussed

upon neurological endpoints. However, the possibility that Al exposure during development may result in toxicity indicates that studies should extend to the whole organism and not be confined to the nervous system. Indeed, there is no reason, *a priori*, to restrict whole life studies to this anatomical region.

Both rodent and rabbit models have become well established in investigating Al induced neurodegeneration and have found more limited application in assessing developmental toxicity. Developmental toxicity, physical, biochemical and behavioural, has been reported on several occasions when Al is administered to pregnant and lactating mice (Golub *et al*. 1987, Donald *et al*. 1989) and rats (Gomez *et al*. 1991, Colomina *et al*. 2005). These effects have also been noted in mice (Golub *et al*. 1995) and rats (Paternain *et al*. 1988, Bernuzzi *et al*. 1989, Muller *et al*. 1990) that received Al only trans-placentally. Administration of Al to pregnant rabbits produces a reduction in kit bodyweight and an impaired learning and memory (Yokel 1985). Kits that received milk from exposed females without pre-natal exposure exhibit reduced weight but no other anomalies (Yokel 1984).

Mammalian models, although attractive with respect to physiological similarity to humans, are costly and subject numbers must be minimised for ethical reasons. It is likely that for these reasons, there are no conception-to-death type studies of Al toxicity involving mammals and that developmental studies lack statistical power. The use of an invertebrate organism that completes its life cycle in a matter of weeks is therefore attractive. As a holometabolous insect, *Drosophila* has three distinct life stages that may be used as milestones for the assessment of developmental toxicity, which combined with an approximate lifespan of 60 days makes it a potentially valuable tool. Knowledge regarding the effects of Al on *Drosophila* is minimal, but studies indicate accumulation of the metal and a concentration-dependent reduction in lifespan that is particularly apparent when the animals are fed from hatching until death (Massie *et al*. 1985, Wu *et al*. 2012b). Vacuolar neurodegeneration and behavioural deficits have also been observed in adult flies fed Al (Wu *et al*. 2012b) but the consequences of lifetime Al administration on these parameters is not known.

Studies demonstrating of the toxic effects of Al are, however, not universal, and probably reflect the differences in dosing and, especially, chemical speciation of Al (Yokel 1987).

The toxic properties of metal ions are influenced by the water and lipid solubility, affinity for various transporters, and the co-ordinating ligands of the metal ions. Without alternatives to displace aqua-ligands, Al is poorly soluble at neutral pH, but certain organic acids (and their conjugate bases), in particular citrate, are able to maintain Al in solution across a broad range of pH values (Martin 1992). Another ligand of particular interest is silicic acid as its interaction with Al depends upon both its polymerization status and that of the Al. Oligomeric silicic acid reduces the absorption of dietary Al in humans whereas it is unable to do this in monomeric form (Jugdaohsingh *et al*. 2000). Nonetheless, it has been suggested that monomeric silicic acid in beer may promote renal excretion of Al (Bellia *et al*. 1996, Pena *et al*. 2007), although this has now been questioned as the effect could not be replicated with pure silicic acid (Reffitt *et al*. 1999). Despite this, monomeric silicic acid when pre-loaded into snails ameliorates the behavioural toxicity of subsequently administered Al. The mechanism involves *in vivo* formation of insoluble HAS and their storage in lysosomal granules although the chemical intermediates are unknown (Dobranskyte *et al*. 2004, White *et al*. 2008). This interaction of Al and Si has neither been investigated in *Drosophila* nor its potential as a moderator of Al induced developmental toxicity been determined.

The aims of the chapter were to examine the feasibility of using *Drosophila* as an invertebrate model of Al developmental and chronic toxicity. To determine if *Drosophila* took up Al in significant quantities in parallel with toxicity, body burdens were measured. These studies were performed with both the chloride salt of Al and the citrate chelate to ascertain if speciation affected either uptake or toxicity of the metal. Total lifespan studies were performed, not only for direct examination of chronic toxicity to this organism, but also as they presented an opportunity to test the ameliorating capacity of silicic acid by administering it separately from Al using developmental stages as convenient break-points. Finally, preliminary screens were made into behavioural toxicity such that they may form the foundation of future study.

3.2. Aluminium specific methods

3.2.1. Chemicals and media preparation

Spiking of food (yeast slurry or standard medium) throughout the Al studies was achieved using either $AlCl_3$ or Al citrate. $AlCl_3 \cdot 6H_2O$ was made to a 1 M stock solution and Al

citrate stock solution (1 M) (equimolar Al and citrate) was made by first preparing a $AlCl_3$ (2 M, 250 ml) solution to which citric acid (4 M, 125 ml) solution was added. The resulting solution was adjusted to pH 7 by adding sodium hydroxide (NaOH; 5 M reducing to 1 M as pH approached target) drop-wise whilst monitoring pH using a glass electrode pH meter (Denver Instruments, NY, USA). The volume of this solution was made up to 500 ml and the pH confirmed. As a control for the effects of the citrate ion, Na citrate was used. This was prepared as a 1 M stock solution adjusted to pH 7 using hydrochloric acid (HCl), supplied at >= 37% (Sigma Aldrich, UK: TraceSELECT®). Sodium orthosilicate (Na_4SiO_4) solution was prepared as a 250 mM stock solution from Na_4SiO_4 powder (VWR International Ltd., UK) consisting of sodium metasilicate (Na_2O_3) plus NaOH that yields Na_4SiO_4 in solution.

Aluminium chloride, Al citrate and Na citrate spiked medium at 10 mM or 100 mM was produced by adding 1 ml or 10 ml respectively to 84.9 g (90 ml) melted standard *Drosophila* medium (Section 2.3) plus the volume of de-ionised water required to make 100 ml (if necessary) and allowed to set as described in Section 2.3. Aluminium ion induced hydrolysis caused an Al concentration-dependent drop in pH (from pH 5.5 in control) in the medium to which it was added. To control for this, pH controls were made at pH 4 and pH 3 for 10 mM and 100 mM $AlCl_3$ respectively, by adding HCl (37%) drop-wise to melted standard *Drosophila* medium until the correct pH was attained, determined using pH indicator strips (resolution: 0.5 pH units) dipped into the food substrate and quickly rinsed with de-ionised water; the indicated colour was not influenced by this brief rinse. The volume was then made up to 100 ml using de-ionised H_2O. Despite the greater resolution of a glass electrode, its use was not appropriate due to potential damage to the glass membrane by agar based medium and because cooling of the medium and its subsequent setting would have prevented equilibrium between the medium and the electrode being reached. Sodium orthosilicate spiked medium was prepared in a similar manner as the above to a concentration of 20 mM, requiring 8 ml Na_4SiO_4 stock solution and no additional de-ionised H_2O. This resulted in a basic medium that was returned to control pH (5.5) using HCl (37%) and subsequently made to 100 ml with de-ionised H_2O. Aluminium chloride at 1 mM, along with all concentrations of other chemicals, had no discernible effect on medium pH. Yeast slurry (Section 2.3) was spiked in the same manner as the medium with the test substance replacing an appropriate volume of de-

ionised water. For example $AlCl_3$ (10 mM) yeast slurry was prepared by adding 300 µl $AlCl_3$ stock solution to 24.9 g yeast plus 5.1 ml de-ionised H_2O.

3.2.2. Gustatory responses to aluminium and silicic acid

To determine if a *Drosophila* exhibited a behavioural response to the presence of Al salts, a mass positional assay was performed. Larvae were tested and their responses recorded as a preference percentage, as described in Section 2.6. Aluminium chloride and Al citrate were tested at 10 mM alongside the pH 4 and Na citrate control. In later exposure experiments, Al was used at concentrations of both 10 mM and 100 mM but it was not possible to perform the gustatory assay at the higher concentration as the agar required to produce the plate would not set at this Al concentration. Silicic acid was tested at 20 mM, the maximal concentration used for any exposure experiments later. Al and silicic acid experiments were performed separately, as making direct comparisons between these substances would not have been useful and would have reduced statistical power for the more relevant comparisons.

3.2.3. Aluminium uptake in whole *Drosophila*

As toxicity of Al was likely to occur only after a period of uptake, the amount of Al in both whole larvae and adult flies was determined following Al exposure. As Si is known to affect the toxicity of Al and has a strong chemical affinity for it, the change in body concentration of one element was measured alongside that of the other. To determine Al and Si tissue concentrations in larvae, five Petri dishes each containing 100 eggs were collected and exposed in yeast slurry for 72 hours before larvae were purged and killed (Section 2.8.1). Two experiments were performed, one at each of two Al exposure concentrations: 10 mM based upon the study of Massie *et al*. (1985) and a logarithmic increase to 100 mM as 10 mM failed to result in significant tissue concentrations. The 10 mM experiment consisted of the following experimental groups in addition to control: $AlCl_3$, Al citrate, pH 4 and Na citrate. The 100 mM experiment was performed with only control, Al citrate and Na citrate groups because of the lethality of $AlCl_3$ at this higher concentration. Adult uptake experiments were only conducted at 10 mM Al as this resulted in a significant body burden of metal. The same experimental groups as used for larval testing at 10 mM were used for adults; five bottles of 250 eggs were collected and exposed for each condition in *Drosophila* medium until pupation (Section 2.8.1). Once the flies

had eclosed they were transferred to new bottles containing freshly spiked medium every three days until 16 days after eclosion. At this point their guts were purged and the flies processed as described in section 2.8.1. A further experiment was performed to determine the uptake of Si following its administration. In addition to control, the experiment comprised of two experimental groups that received silicic acid: 10 mM and 20 mM. These concentrations were chosen so that in later lifespan experiments (Section 3.2.5) Si would be administered in equal or double molar equivalents to Al.

To digest larvae or adults, 750 µL ultrapure HNO_3 (~69%) was added to ~40mg desiccated larval or adult tissue in a micro-centrifuge tube and placed, uncapped, at room temperature, in a fume cupboard for 48 hours. Following this period 750 µL ultrapure H_2O_2 (~30%) was added and the mixture was left, uncapped, for a further 48 hours or until the solution was no longer evolving gas. The vial was then capped, mixed using a vortexer (VWR) for 10-15 seconds to dislodge any adherent material, centrifuged for 5 seconds at 13000 rpm and returned to the fume cupboard for further 48 hours to allow dislodged material to digest. The resulting digest was diluted with de-ionised water to 10 ml (the minimum volume required for analysis), and placed in acid washed sample tubes (VWR, UK) (compatible with the ICP-AES machine) for ICP-AES analysis (Section 2.9.3). In addition to experimental digests, five replicates of reference material, DOLT-4 (Dogfish Liver Certified Reference Material for Trace Metals; National Research Council Canada) containing 200 $\mu g_{Al}/g_{DryTissue}$, and control *Drosophila* spiked with Al to a concentration of 200 $\mu g_{Al}/g_{DryTissue}$ were digested to assess recovery. Recovery from reference material was 91.6 (+/- 20.9: 95% CI) $\mu g_{Al}/g_{DryTissue}$ (~ 46%). Recovery from spiked *Drosophila* was 141.4 (+/- 13.8: 95% CI) $\mu g_{Al}/g_{DryTissue}$ (~71%). As the *Drosophila* digest is the actual matrix of the samples in this assay, the recovery from these spiked samples is likely to be more representative of the experimental data. Despite this recovery not being ideal, the primary purpose of the analyses was comparison with control levels. Recovery was sufficient to make such comparisons valid as the relative differences would remain.

The baseline concentrations of Al and Si in yeast and standard *Drosophil*a medium were also characterised. Five samples of each type of food source, collected from independent batches, were digested in HNO_3 and H_2O_2 using the protocol described above and analysed by ICP-AES. Figure 3 shows that low quantities of Al were found in both medium and

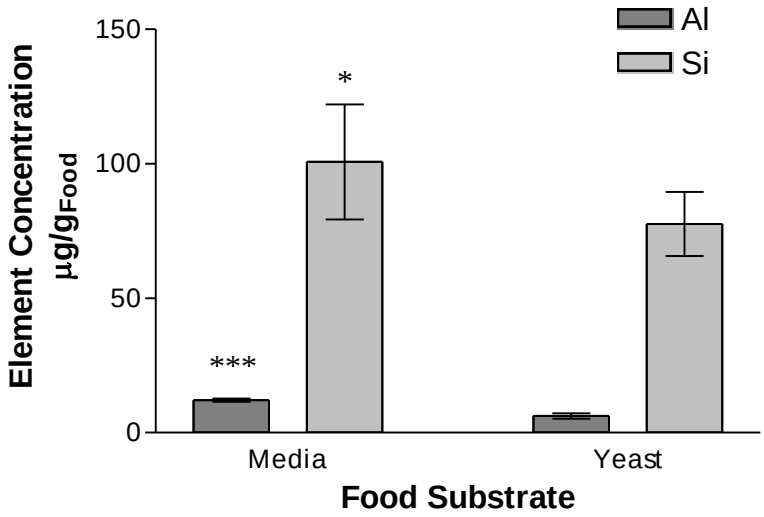

Figure 3. Concentrations of Al and Si in Standard *Drosophila* medium and live baker's yeast. Data are presented as mean +/- 95% CI. n = 5 sampled from independent batches of food. Key to significance values (unpaired *t*-test): *** $p < 0.0001$, * $p < 0.05$ relative to yeast concentration of same element.

yeast; medium contained ~12 $\mu g_{Al}/g_{DryFood}$ (approximately equivalent to 133 µM in hydrated medium); this was significantly ($p < 0.0001$; unpaired *t*-test) more than yeast at ~6 $\mu g_{Al}/g_{DryFood}$ (approximately equivalent to 65 µM in live yeast). Si levels were overall much higher in both substrates (Figure 3) being around 80 $\mu g_{Al}/g_{DryFood}$ in yeast (equating to ~860 µM in live yeast) and ~25% higher (~1.1 mM) in the cornmeal based medium ($p < 0.05$; unpaired *t*-test).

3.2.4. Developmental toxicity of aluminium and silicon

Pupation and eclosion success, and time to pupation (Section 2.7.1) were first studied using the YP protocol, in which larvae are fed for the first half of their larval period with yeast slurry to facilitate easy determination of viability at this stage. For reasons of practicality, three concentrations of Al were tested in separate experiments, in which eggs were collected and exposed to a logarithmic scale of concentrations based upon those used by Massie *et al*. (1985): 1, 10, and 100 mM Al (both chloride and citrate), along with the corresponding pH or citrate controls (Section 3.2.1). Five plates of 100 eggs per treatment were collected and their development followed as described in Section 2.7.1. Following these YP protocol experiments, a repeat experiment using the MTD protocol, in which larvae were fed with medium until pupation, was performed at 10 mM to compare the effects of the medium on Al toxicity. Only $AlCl_3$ and a pH control as experimental groups were used; Al citrate was not studied because the citrate ion itself had proven to be

bioactive, and it was therefore difficult to uncouple the effects of the citrate ion from those of Al (see Sections 3.3.6.2 and 3.3.6.3). Five vials of 100 eggs were collected for each of the control, pH 4 and $AlCl_3$ groups. As Si was hypothesised to ameliorate Al toxicity, its inherent developmental toxicity was also tested using this method for which 15 vials of 100 eggs were used.

To dissociate the effects of Al upon maturation and physical growth, further assays were performed to determine larval mass. One hundred *Drosophila* eggs were collected per replicate and placed on a plain agar (5%) filled Petri dish and surrounded with yeast slurry (10 ml) containing the test substance. In the first experiment larvae were exposed to $AlCl_3$ (10 mM), Al citrate (10 mM), pH 4 medium or Na citrate (10 mM) and in the second, either 100 mM Al citrate or Na citrate (Section 3.2.1). The dishes were incubated at 25 °C for 96 hours after which larvae were harvested from the yeast (Section 2.5), blotted dry, counted and placed in pre-weighed micro-centrifuge tubes. The tubes were weighed and the mass per larva determined (averaged over 100 larvae minus the attrition on that plate).

3.2.5. Effect of Al upon lifespan and its modulation by Si

Having established the toxic threshold for Al (10 mM) and the food substrate that best expressed that toxicity (MTD), Al toxicity to *Drosophila* was further studied by examining its effects on adult lifespan. Aluminium was only presented as $AlCl_3$ and not Al citrate as the pupation rate response to the Na citrate control (see Sections 3.3.6.2 and 3.3.6.3) would have made interpretation difficult, and the additional groups would have created an unmanageably large experiment. The discrete larval and adult stages provided the opportunity to test the efficacy of Si in ameliorating Al toxicity, without chemical interaction between Si and Al in the medium. This was achieved using a pre/post-load design: that is, one treatment was administered during the larval period and the other during the period following eclosion. Treatments were therefore designated in the test as **Larval Treatment/Adult Treatment** to signify the order of Al and Si exposure. As Al displayed developmental delay at 10 mM this concentration was used for the lifespan experiments. Tissue concentrations of Si were raised following administration at silicic acid (20 mM) without evidence of toxicity and to maximize the possibility of a response was used at this concentration (double the administered Al). For each experiment, silicic acid administration was compared against no silicic acid (control) for all Al treatment

groups and controls (control, pH 4 and $AlCl_3$). For comparison a third experiment was performed with Al administered throughout life. Groups within each experiment were:

1. Control/Control, Control/pH 4, Control/Al, Si/Control, Si/pH4 and Si/Al
2. Control/Control, pH 4/Control, Al/Control, Control/Si, pH 4/Si and Al/Si
3. Control/Control, pH 4/pH 4 and Al/Al

To measure lifespan, 100 eggs were collected for 5 bottles per experimental condition and allowed to develop to eclosion. Following eclosion, males and females were separated under a dissection microscope at x 10 magnification, to limit variance due to the potential differential longevity of the sexes (Lints *et al*. 1983) and to prevent multiple matings, which shorten female lifespan (Chapman *et al*. 1995) and might vary in number across treatment groups. These single sex groups were transferred to bottles containing the appropriate medium for the experimental condition. Flies were transferred to new bottles with fresh medium, with the same composition as their previous bottle, every two days. During changing dead flies were removed and counted. Any escapees or damaged flies were right-censored at this point (recorded as having died at an undetermined point *after* this time) (See section 2.9). Fly deaths were recorded until the entire population had died.

3.2.6. Measuring behavioural toxicity of aluminium

To test for functional deficits that may emerge in *Drosophila* following exposure to Al, two behavioural assays were chosen: larval locomotor activity and negative geotaxis in adults. Larvae for the locomotor assay were cultured using the YP protocol, to facilitate harvesting, and were exposed during their development to either control conditions, $AlCl_3$ (10 mM), Al citrate (10 mM), Na citrate (10 mM), or pH 4 medium. A high concentration (100 mM Al) experiment was also used but limited to Al citrate and Na citrate as experimental groups; $AlCl_3$ is lethal at this concentration. Larvae were harvested at 96 hours AEL (mid 3rd instar under normal developmental conditions). For each test (replicate) a larva was placed in the centre of a plain agar (5%) filled Petri dish covered with its lid and illuminated from above. After a 60 s period of acclimation, the path of the larva was traced on the lid with a fine marker pen with marks made for each 30 s period, for a total of 3 minutes. Twenty larvae were tested for each experimental condition after which the plate lids with tracing were scanned into an image file using an HP Scanjet

G4210. The lengths of the paths were determined using the segmented line measuring tool of imageJ software, using the width of each Petri dish for calibration. The slowed development of the Al citrate (100 mM) exposed animals allowed a further set of measurements to be made at 120 hours for this group. It was not possible to perform age-matched controls (120 hour control larvae) as these animals had pupated at this point.

Whilst the locomotor assay represents spontaneous activity, it is possible that Al may alter responses to external stimuli. The negative geotaxis assay measures reactivity to stimuli by exploiting the natural escape behaviour of the adult fly. As such, it combines aspects of both sensory and motor function. To test negative geotactic behaviour, 50 eggs per replicate were collected and cultured to adults using the MTD protocol (Section 2.5). Experimental groups were limited to $AlCl_3$, and pH 4 medium to reduce the experimental workload to practical limits. Ten bottles per experimental group were collected and, following eclosion, adults were sexed and 20 male flies placed in new vials with fresh medium of the same composition as that used for their larval life. Flies were given 5 days to fully mature before experimentation.

For the test, the 20 flies were transferred to an empty *Drosophila* vial, circumscribed with a line 8 cm from the bottom, and the vial was capped. Under overhead illumination the vial was tapped sharply three times to dislodge all flies to the bottom of the vial. On the impact of the third tap the timer was started. The number of flies to reach the top of vial in 10 s was counted and calculated as a percentage of totals.

3.3. Results

3.3.1. Gustatory responses to aluminium

Neither the pH 4 nor the Na citrate control resulted in change in positional preference relative to the no tastant control (Figure 4A). Similarly, $AlCl_3$ was neither attractive nor repellent to *Drosophila* larvae; however, Al citrate was significantly repellent ($p < 0.05$) (Figure 4A). In contrast to the results obtained from larvae, both forms of Al and their respective controls (pH 4 and Na citrate) deterred oviposition in adults ($p < 0.05$) (Figure 4B) suggesting that adults can detect these substances and find all of them distasteful. Silicic acid at 20 mM produced no behavioural response in *Drosophila* larvae or adults ($p > 0.05$; student's *t*-test (unpaired) in both instances; data not shown).

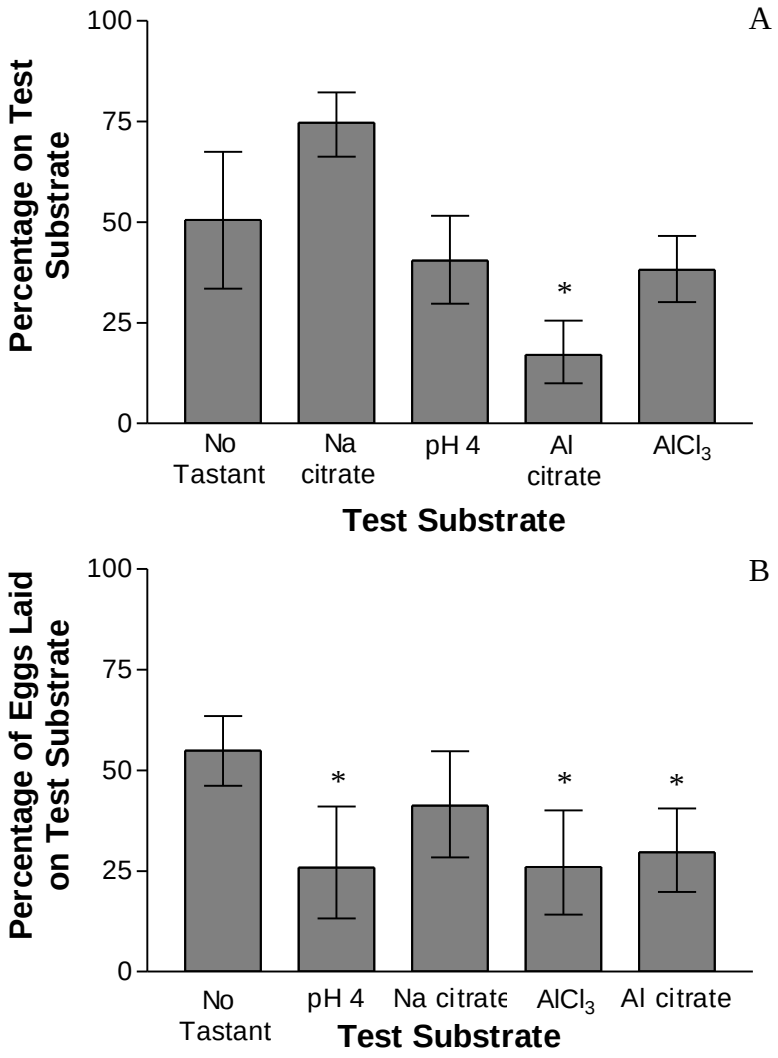

Figure 4. Gustatory responses of *Drosophila* to aluminium. (A) Percentage of larvae on test substrate after 5 minutes. (B) Percentage of eggs laid by adult females upon the test substrate after 2 hours. Test substrates were: $AlCl_3$ (10 mM), Al citrate (10 mM), pH 4 and Na citrate (10 mM). n = 8 plates (~40 larvae/plate) for all groups. Data are presented as mean +/- 95% CI. Key to Statistical significance: * $p < 0.05$ (Tukey-Kramer test) relative to no tastant control in that graph.

3.3.2. Elimination of food from the larval and adult gut

The adequacy of the gut purging regimen, to be used for metal analysis experiments, was assessed by following erioglaucine dye transit through the gut. At 12 hours, 100% of lavae had cleared the dyed food altogether regardless of whether they had received Al before the purging commenced (Figure 5A). No significant differences (repeated measures ANOVA) were seen, at any time point, between the control and metal fed larva. Figure 5B shows that the adult elimination pattern was similar although slightly more protracted with a short lag phase at the beginning. Nonetheless, all flies were clear of dye at 32 hours whether

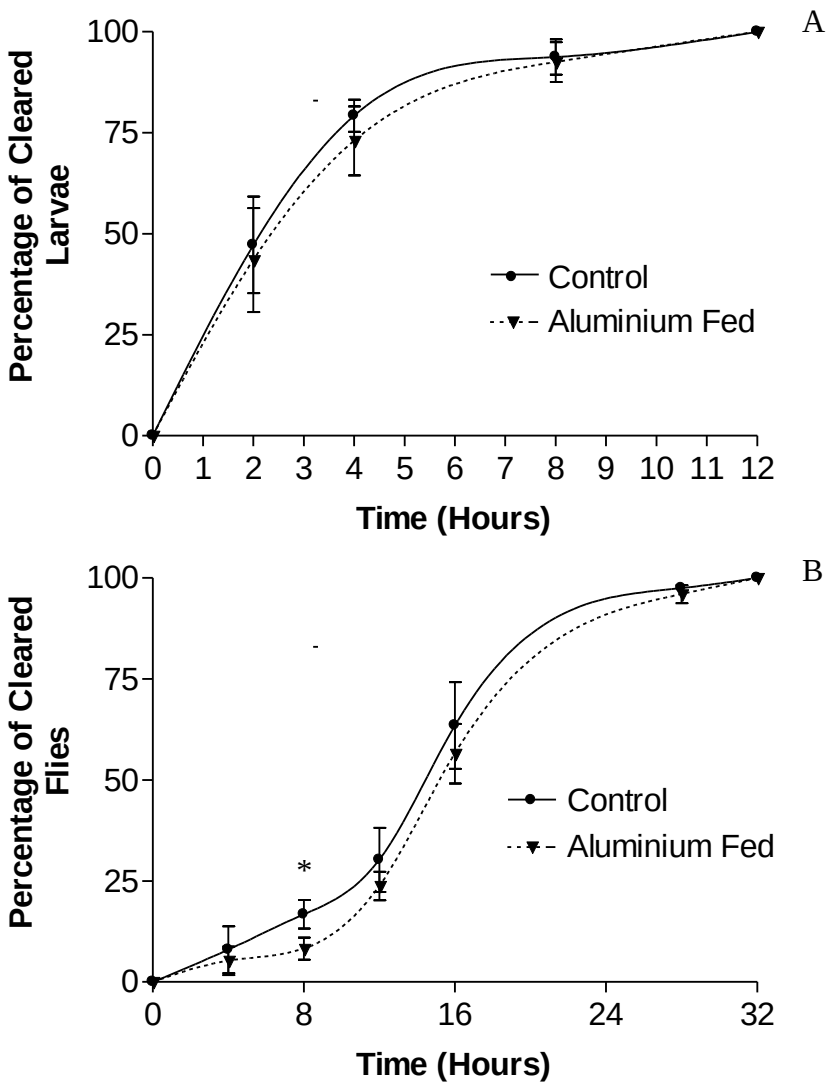

Figure 5. Clearance of erioglaucine dye from gut. Aluminium fed animals received Al (10 mM) until the point that they were purged. (A) Larval clearance (B) Adult clearance. Each data point represents mean percentage (+/- 95% CI) of animals that were totally clear of dye. n = 5 plates or bottles (100 animals/replicate). Key to significance values: $p < 0.05$ Al fed relative to control at that time point (two-factor Tukey-Kramer test following repeated measured ANOVA).

control or metal fed. The only significant difference ($p < 0.05$) between the control and Al fed groups was at eight hours with the latter having a lower percentage of flies clear. This difference disappeared by the next time point.

3.3.3. Whole body tissue concentrations of aluminium and silicon in larval *Drosophila*

Larval uptake of two forms of Al (chloride and citrate) was determined after 48 hours feeding in yeast followed by 24 hours on uncontaminated yeast. Two concentrations of

each were used but body loads were determined in separate experiments, to limit the size of each individual experiment, so results are not directly comparable. In the 10 mM experiment (Figure 6A) neither the pH 4 control (to mimic the native acidity of the $AlCl_3$ medium) nor the Na citrate control (to control for the citrate counter-ion at pH 5.5) affected the concentration of Al found within the *Drosophila* tissues ($p > 0.05$; ANOVA - main effect). Furthermore, there was no significant ($p > 0.05$; ANOVA - main effect) change in larval Al concentration relative to control (pH 5.5) in those that had received medium spiked with either $AlCl_3$ (pH 4) or Al citrate (pH 5.5) at 10 mM. A concentration of 100 mM Al in the medium was used in an attempt to force some accumulation in the short time available. At this concentration, however, $AlCl_3$ resulted in 100% mortality within 12 hours of hatching. The 100 mM data were logarithmically transformed to stabilize the variance as the raw data did not meet homoscedacity assumptions ($p < 0.05$; modified Levene's test). Al citrate (100 mM; pH 5.5) led to a small but significant accumulation, relative to both the absolute and citrate controls (Figure 6B).Tissue concentrations of Si were measured in tandem with Al. In the 10 mM Al exposure experiment (Figure 7A) no significant difference was seen in the Si concentrations in any group ($p > 0.05$; ANOVA). When Al citrate was administered at 100 mM (Figure 7B) the Si tissue concentration data showed evidence of heteroscedasticity ($p < 0.05$; modified Levene's test) but were unsuitable for variance stabilizing transformation and were therefore compared using *t*-tests with Welch's correction for unequal variance. Three comparisons were made giving a significance threshold of 0.017 after Bonferroni correction ($\alpha = 0.05$). No significant difference was seen between the Al exposed group and either of the controls ($p > 0.05$; Tukey-Kramer test). However, a small but significant increase in Si concentration was seen in larvae exposed to Na citrate. Overall, these data do not suggest that Al administered in either form to larvae cause a concomitant increase in Si uptake.

3.3.4. Whole body tissue concentrations of aluminium and silicon in adult *Drosophila* following aluminium administration

In addition to determining the uptake of Al and Si in *Drosophila* larvae the concentrations were measured in the tissues of adult *Drosophila* after administration of Al throughout the larval period (~5 days) plus 16 days post-eclosion. The modified Levene's test revealed significant heteroscedasticity in the Al tissue concentrations data that was mitigated by

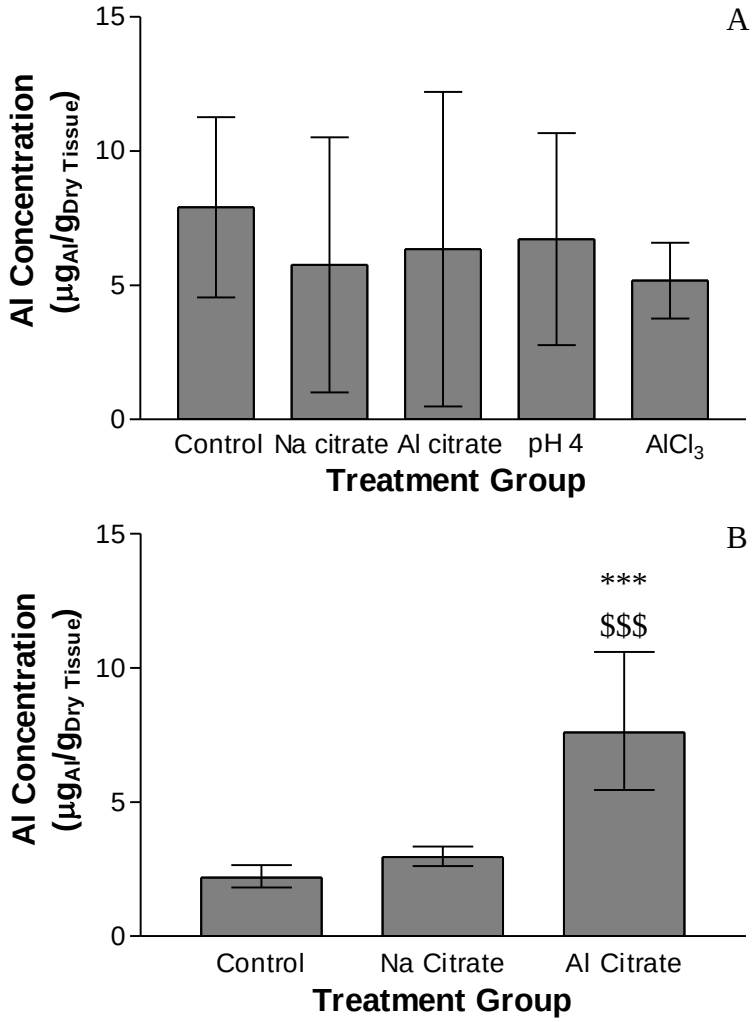

Figure 6. Whole body concentrations of aluminium in *Drosophila* larvae. (A) Following administration of $AlCl_3$, Al citrate Na citrate 10 mM or pH 4 for 72 hours AEL. (B) Following Na and Al citrate administration at 100 mM for 72 hours AEL. Error bars represents +/- 95% CI. n = 5. 100 mM data (B) are back transformed following logarithmic transformation for statistical analysis. Key to significance values: *** $p < 0.001$ relative to control in that graph; $$$ $p < 0.001$ relative to Na citrate in that graph.

logarithmic transformation; data were therefore analysed to this scale. As with the larval experiments no significant differences ($p > 0.05$; Tukey-Kramer test) were found between control and either Na citrate or pH 4 medium. However, Figure 8A shows that after ~26 days of Al exposure (including ~5 days as pupae), adult flies had significantly higher body concentrations of Al with respect to control for both $AlCl_3$ ($p < 0.01$) and Al citrate ($p < 0.001$) containing food. $AlCl_3$ exposure resulted in significant whole body tissue concentrations ($p < 0.001$) relative the pH 4 control; likewise Al citrate exposed flies contained higher concentrations of Al ($p < 0.001$). A significant difference between the two forms of Al was seen with Al citrate achieving higher tissue concentrations than $AlCl_3$,

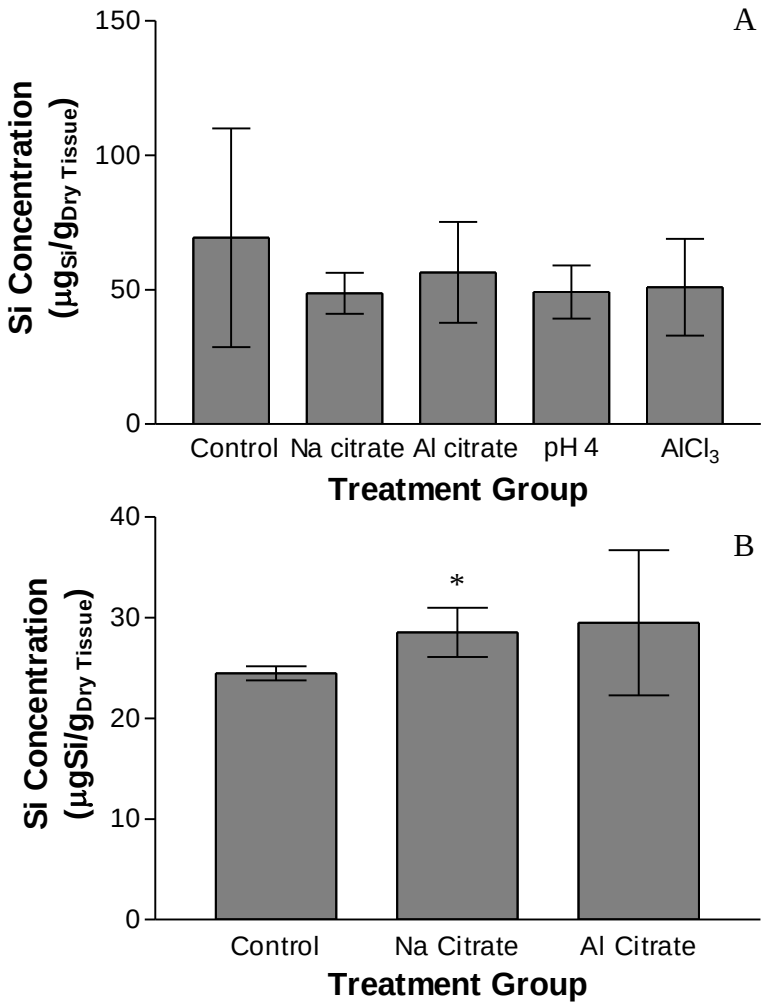

Figure 7. Whole body concentrations of silicon in *Drosophila* larvae following administration of aluminium. (A) Si tissue levels after exposure to control, $AlCl_3$ (10 mM), Al citrate (10 mM), Na citrate (10 mM) or pH 4 medium for 72 hours AEL. No significant differences were found between any groups ($p > 0.05$); ANOVA – main effect) (B) Si tissue levels following exposure to control, Al citrate (100 mM) or Na citrate (100 mM). Key to significance values: * $p < 0.017$ (unpaired *t*-test with Welch's correction for unequal variance) relative to control in that graph. In both graphs data are presented as mean +/- 95% CI. n = 5.

resulting in a mean ratio of tissue concentrations ($AlCl_3$:Al citrate) of 2.590 (95% CI 1.004 – 6.667; $p < 0.001$). For comparison, the mean ratio of $AlCl_3$ to control was 11.940 (95% CI 4.911 – 29.107). Overall, administration of Al at 10 mM caused an increase in Al body burden in adult *Drosophila* in either form. No significant differences ($p > 0.05$; Tukey-Kramer test) were seen in the tissue concentrations of Si, taken up from from basal levels found in the medium, except for a small reduction in $AlCl_3$ exposed flies relative to control ($p < 0.001$) (Figure 8B).

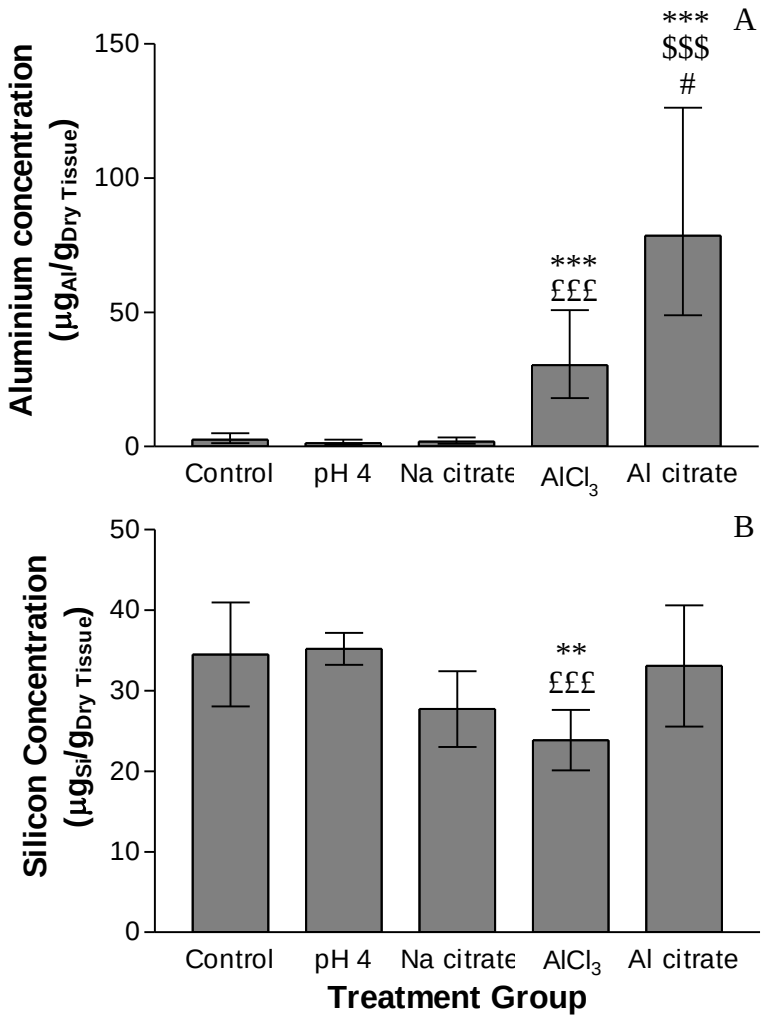

Figure 8. Whole body concentrations of aluminium and silicon in aluminium exposed *Drosophila* adults. Body aluminium (A) and silicon (B) concentration following exposure to control, $AlCl_3$ (10 mM), Al citrate, (10 mM), Na citrate (10 mM) or pH 4 medium throughout larval life and 16 days post eclosion. Data in (A) are back transformed following logarithmic transformation for statistical analysis. All data are expressed as mean +/- 95% CI. n = 5. Key to significance values: ** $p < 0.01$ and *** $p < 0.001$ relative to control; $$$ $p < 0.001$ relative to Na citrate; ££ $p < 0.01$ and £££ $p < 0.001$ relative to pH 4 medium; # $p < 0.05$ relative to $AlCl_3$.

3.3.5. Whole body tissue concentrations of silicon and aluminium in *Drosophila* following silicon administration

To determine the uptake of Si in *Drosophila* and its possible effects upon the uptake of Al found naturally in the medium, *Drosophila* were exposed to two concentrations of silicic acid (plus control). Medium-concentration-dependent Si tissue levels were seen in newly eclosed adult *Drosophila* (Figure 9A). A linear regression line was fitted to the data producing $r^2 = 0.8485$ ($p < 0.0001$; linear regression). The concentration of Si in control flies was 34.51 $\mu g_{Si}/g_{DryTissue}$ (95% CI 20.05 – 40.97 $\mu g_{Si}/g_{DryTissue}$) increasing to 100.70

$\mu g_{Al}/g_{DryTissue}$ (95% CI 79.34 - 122.10 $\mu g_{Si}/g_{DryTissue}$) for 20 mM Si exposed flies. Unlike the weak effect of Al administration upon Si uptake, Al uptake from basal levels in the medium was strongly dependent upon administered Si (Figure 9B). Tissue levels of Al in the absence of added Si were consistent with previous experiments (c.f. Figure 8A). Linear regression revealed a strong relationship ($r^2 = 0.9132$; $p < 0.0001$) between the concentration of administered silicic acid and the concentrations of Al found in the tissues.

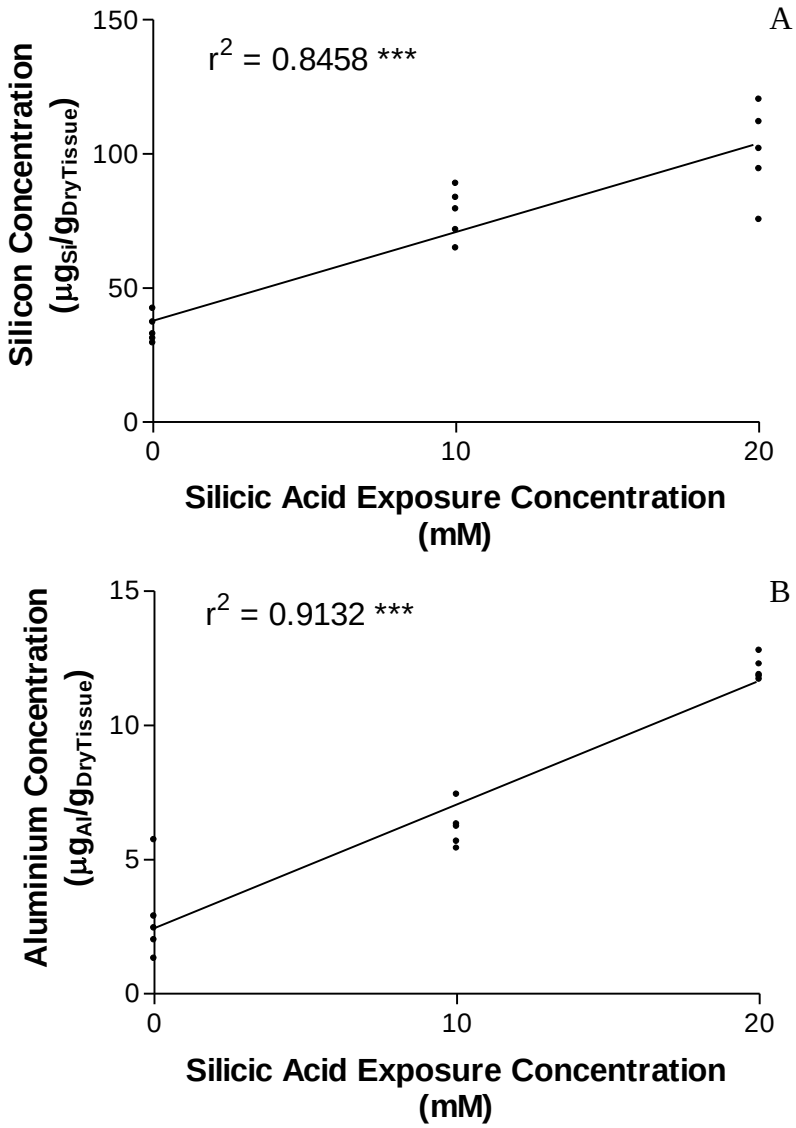

Figure 9. Whole body concentrations of Al and Si in *Drosophila* adults following administration of silicic acid. Tissue concentrations of (A) Si and (B) Al following exposure silicic acid for the entire larval period; analyses were made on emerged adults. Linear regression lines are fitted to the data. n = 5 for each concentration. Key to significance values: *** $p < 0.0001$ relative to $r^2 = 0$.

3.3.6. Developmental toxicity of aluminium and silicon compounds

3.3.6.1. Effects of aluminium on larval viability, and pupation and eclosion

The toxicity of Al to *Drosophila* in early life stages was assessed by determining the number of surviving larvae following two days exposure to 1, 10 and 100 mM added Al. As the three concentrations were tested at different times the experiments are not directly comparable. Indeed, considerable variation between the control groups was observed, suggesting that larval viability, between experiments, was affected by uncontrolled (and unknown) factors. Viability of control larvae was between 60 and 80%. There was no change in larval viability for any treatment group, save 100 mM $AlCl_3$, which resulted in 100% percent mortality ($p < 0.05$ relative to control; Tukey-Kramer test) (data not shown). Pupation success was determined in the same experiment by observing larval behaviour using the yeast pre-feeding (YP) protocol and an additional experiment using the medium throughout development (MTD) protocol. Figure 10A and B show there is no significant difference ($p > 0.05$; ANOVA - main effect) in pupation success for any treatment at 1 or 10 mM, respectively. At 100 mM the only significant difference relative to control ($p < 0.05$) was for $AlCl_3$ (100 mM) (Figure 10C). This was a result of no larvae having survived to two days. A lack of significance was seen whether the percentage of pupae was calculated using viable larvae or eggs as the denominator.

To understand how the food substrate may affect the expression of Al toxicity, developmental toxicity was examined using MTD protocol in addition to the YP protocol. In this experiment only $AlCl_3$ was used due to complications arising from effects of the citrate counter-ion (see below and Section and 3.3.6.3). No differences ($p > 0.05$; ANOVA - main effect) in pupation success were seen for $AlCl_3$ (or pH 4) when administered through the MTD protocol (data not shown). Only pupae as a fraction of eggs could be determined as extracting larvae from the medium at two days was not practicable.

Eclosion success was examined from both of the above cohorts of pupae (those from the YP and the MTD protocol). In contrast to the negative results seen at 10 mM (YP protocol) for pupation success, a small, but significant reduction in eclosion success, when calculated as a percentage of eggs, was recorded for Al treated flies, whether exposed to the Cl^- salt at pH 4 ($p < 0.01$) or the citrate chelate at pH 5.5 ($p < 0.01$) (Figure 11A). Neither the pH 4 control nor the Na citrate control media produced significantly different

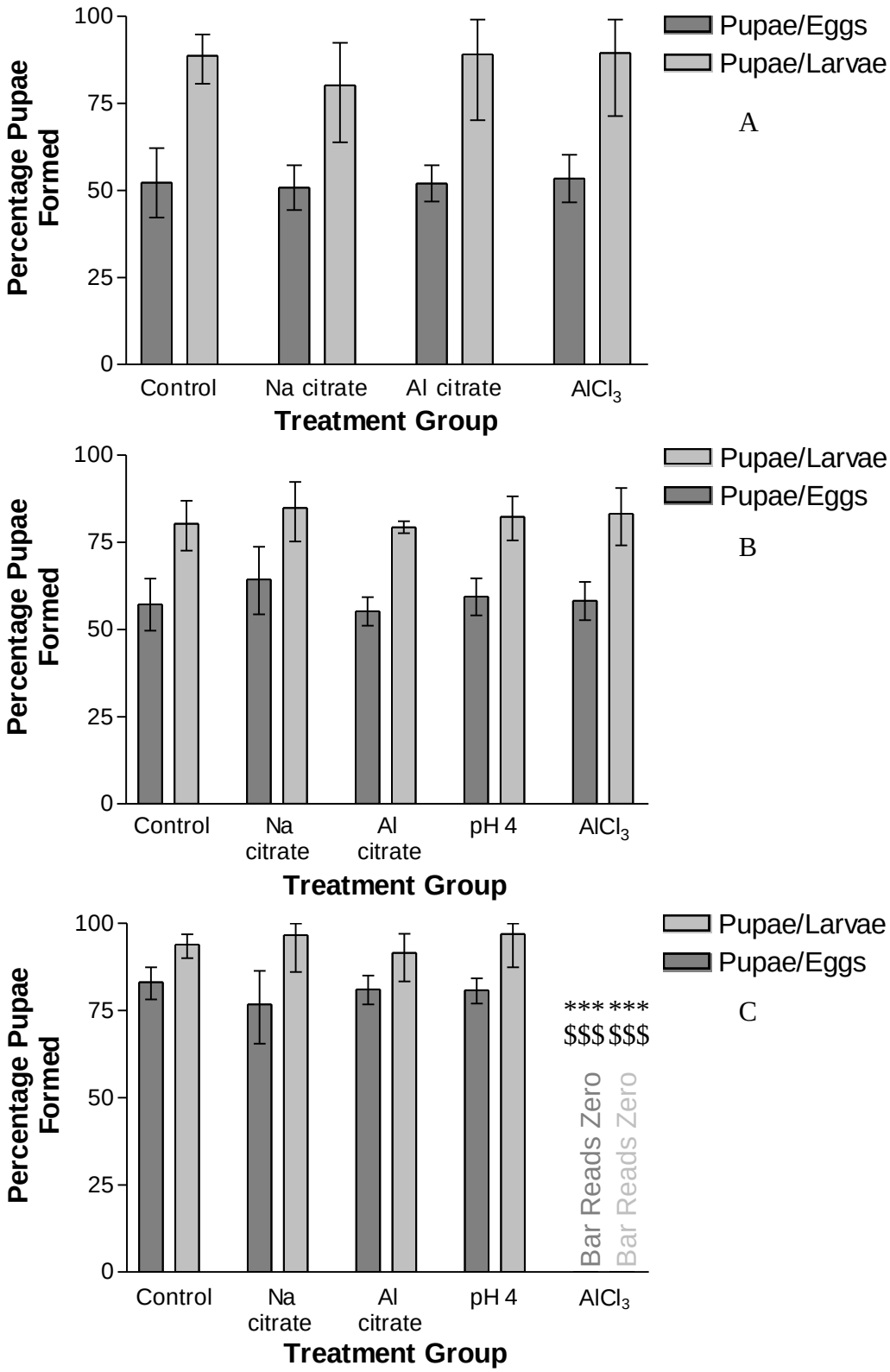

Figure 10. Pupations success following exposure to Al. Mean percentage successful pupations +/- 95% CI following exposure to (A) 1 mM, (B) 10 mM and (C) 100 mM $AlCl_3$, Al citrate, Na citrate or a pH matched control for $AlCl_3$ (10 mM and 100 mM were matched to pH 4 and pH 3 respectively whilst no pH control was made for 1 mM as medium pH was unaffected). All treatments were administered from hatching. n = 5. Key to statistical significance: *** $p < 0.001$ relative to control in respective graphs; $$$ $p < 0.001$ relative to pH 4 or 3 in panels B and C respectively.

levels of eclosion measured as a proportion of eggs. When measured as a proportion of larvae or pupae, however, in addition to the significant differences already described for the Al groups, Na citrate treatment resulted in a significant reduction in successful eclosions ($p < 0.05$ and $p < 0.001$ for larval and pupal numbers as denominators). Furthermore, Na citrate treatment did not produce significantly different numbers of eclosions to Al citrate. The pH 4 control was not significantly different from control, no matter which fraction was used. There was no significant difference between the proportions of successful eclosions, regardless of the denominator, between the two forms of Al (Tukey-Kramer test). The MTD protocol (used to administer Al at 10 mM) also produced a significant drop in the percentage of eggs ($p < 0.01$; Tukey-Kramer test) and pupae ($p < 0.01$; Tukey-Kramer test) eclosing (Figure 11B).

3.3.6.2. Pupation and eclosion success following administration of silicic acid

As Si was to be administered as a potential antidote to the toxic effects of Al, it was necessary to determine if any toxicity could be attributed the Si itself. Using the MTD protocol pupation and eclosion success were assessed following administration of 20 mM Si. No significant differences were found for pupation success (data not shown) or in the proportions of *Drosophila* eclosing following Si administration whether the fraction was calculated using eggs or pupae as the denominator (data not shown).

3.3.6.3. Time to pupation following administration of aluminium or silicon

The three concentrations of Al (1, 10 and 100 mM) that were examined for pupation success were analysed for developmental delay. For 1 mM exposure, five hazard ratio (HR) comparisons were made yielding a significance threshold of 0.01 after Bonferroni correction at a family-wise error rate (α) of 0.05. The seven HR comparisons (log-rank tests) for 10 mM Al and three for 100 mM resulted in significance thresholds of 0.0071 and 0.017, respectively. Consistent with a lack of effect upon pupation success, 1 mM Al administration in either form did not result in any significant delay in pupation. At this concentration no pH control was needed and Na citrate caused no significant change in the pupation rate (Figure 12A). When administered at 10 mM, neither form of Al resulted in a significant difference in pupation rate relative to control ($p > 0.0071$; log-rank test). However, Na citrate caused an unexpected acceleration of pupation rate relative to control with $HR_{NaCitrate:Control} = 1.43$ (95% CI of 1.19 – 1.71; $p = 0.00010$; log-rank test); stated in

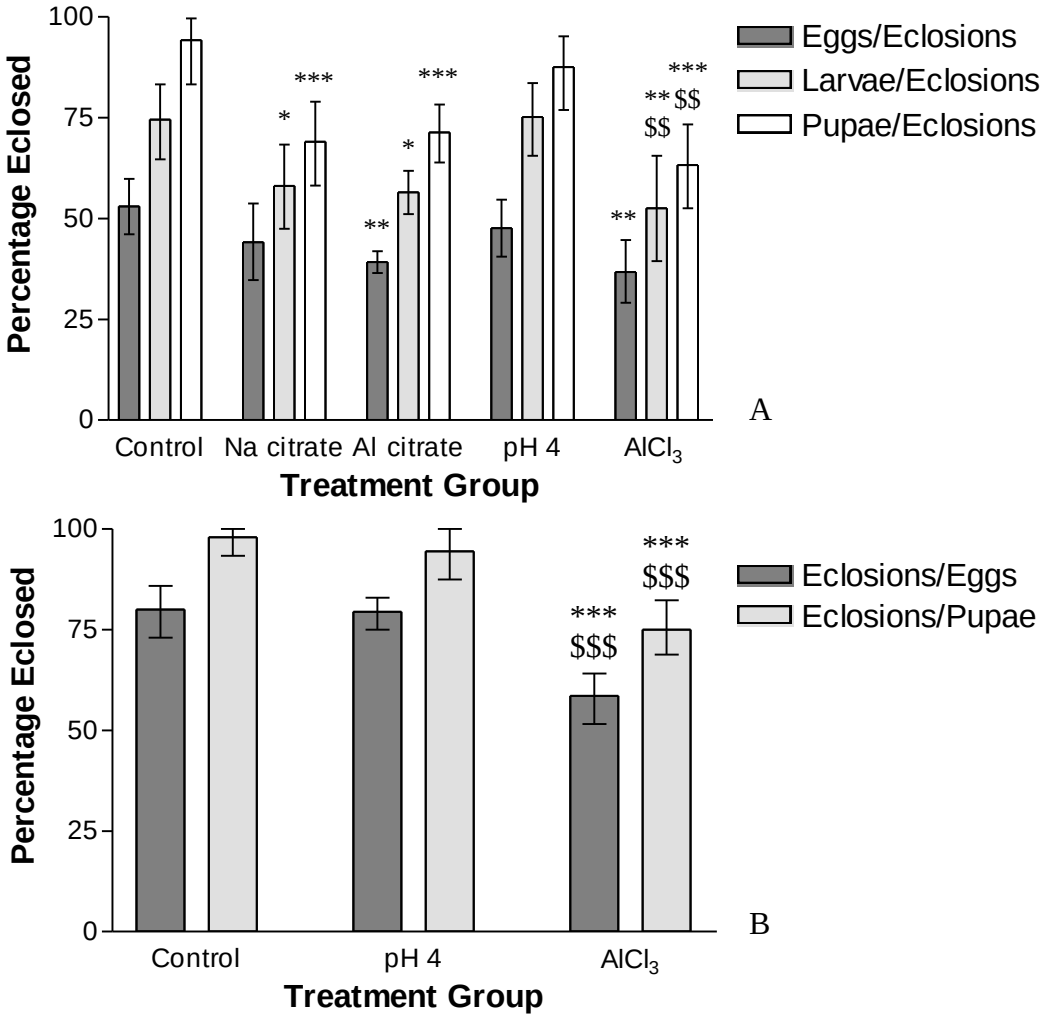

Figure 11. Eclosion success following Al exposure. (A) Mean percentage of eggs, larvae or pupae (+/- 95% CI) after administration, using YP, of 10 mM $AlCl_3$, Al citrate, Na citrate or pH 4 medium. n = 5. (B) Mean percentage of eggs and pupae (+/- 95% CI) after administration, using MTD, of $AlCl_3$ (10 mM) or pH 4 medium from hatching. n = 10. Key to significance values: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ relative to control in that graph; $$ $p < 0.01$ and $$$ $p < 0.001$ relative to respective pH 4 in that graph.

words, this means that at any given time point, the fraction of Na citrate exposed larvae that will pupate per unit time (at the limit as time approaches zero) will 1.43 times greater that the fraction of control larvae that will pupate per unit time (Figure 12B). As a consequence of the significant increase in pupation hazard rate, relative to control, following Na citrate administration, the pupation HR of Al citrate relative to Na citrate was significant i.e. $HR \neq 1$ ($p = 9.8 \times 10^{-4}$).

At 100 mM $AlCl_3$ could not be tested for its effects on pupation time due to its lethality. At this concentration Al citrate produced a significant delay in pupation relative to both

control, with a $HR_{AlCitrate:Control}$ = 0.50 (95% CI of 0.39 – 0.64; p = 2.5 x 10^{-7}; log-rank test), and Na citrate with a $HR_{AlCitrate:NaCitrate}$ = 0.56 (95% CI of 0.39 – 0.79; p = 3.4 x 10^{-4}; log-rank test) (Figure 12C).At this concentration, unlike 10 mM, no difference (p = 0.13; log-rank test) was seen between control and Na citrate groups.

As with pupation and eclosion success, pupation rate at 10 mM Al, using the MTD protocol, was examined to ascertain if any interaction with the medium occurred. Three hazard ratio comparisons were made using a significance threshold of 0.016. Figure 13 shows that administration of $AlCl_3$ in this manner produced different results to those seen in Figure 12B that used the YP protocol. Aluminium chloride led to significantly slower pupation with $HR_{Al:Control}$ = 0.58(95% CI of 0.55 – 0.61; p = 0). The pH 4 control was not significantly different from the neutral control. To corroborate the lack of toxicity of silicic acid to *Drosophila*, the time to pupation in larvae exposed to 20 mM for their entire larval period was measured. Consistent with the previous pupation and eclosion success experiments no significant difference was seen in the rate of pupation following exposure to 20 mM Si (data not shown).

3.3.6.4. Larval mass following aluminium exposure

To address whether any relationship exists between the effects of Al (under the YP protocol) on physical growth and physiological maturation, the mass of larvae was determined at 96 hours AEL. Exposure at 10 mM to either form of Al or Na citrate, or pH 4 medium caused no significant ($p > 0.05$; ANOVA – main effect) alteration in wet body mass (Figure 14A) whereas larvae exposed to 100 mM Al citrate had significantly lower mass than control animals ($p < 0.001$) and those exposed to Na citrate ($p < 0.001$) (Figure 14B). At this concentration, Na citrate caused a moderate, but significant, decrease in body weight ($p < 0.01$) compared to control.

3.3.7. Lifespan analyses of aluminium exposed *Drosophila* and the effect of supplementary silicic acid

To further characterise Al toxicity to *Drosophila*, its effect upon total lifespan was determined using the MTD protocol for simplicity and to maximise the likelihood of a robust response. The notation used from treatments is: **Larval Treatment/Adult Treatment.** That is if the treatment is described as Control/Aluminium then the animal

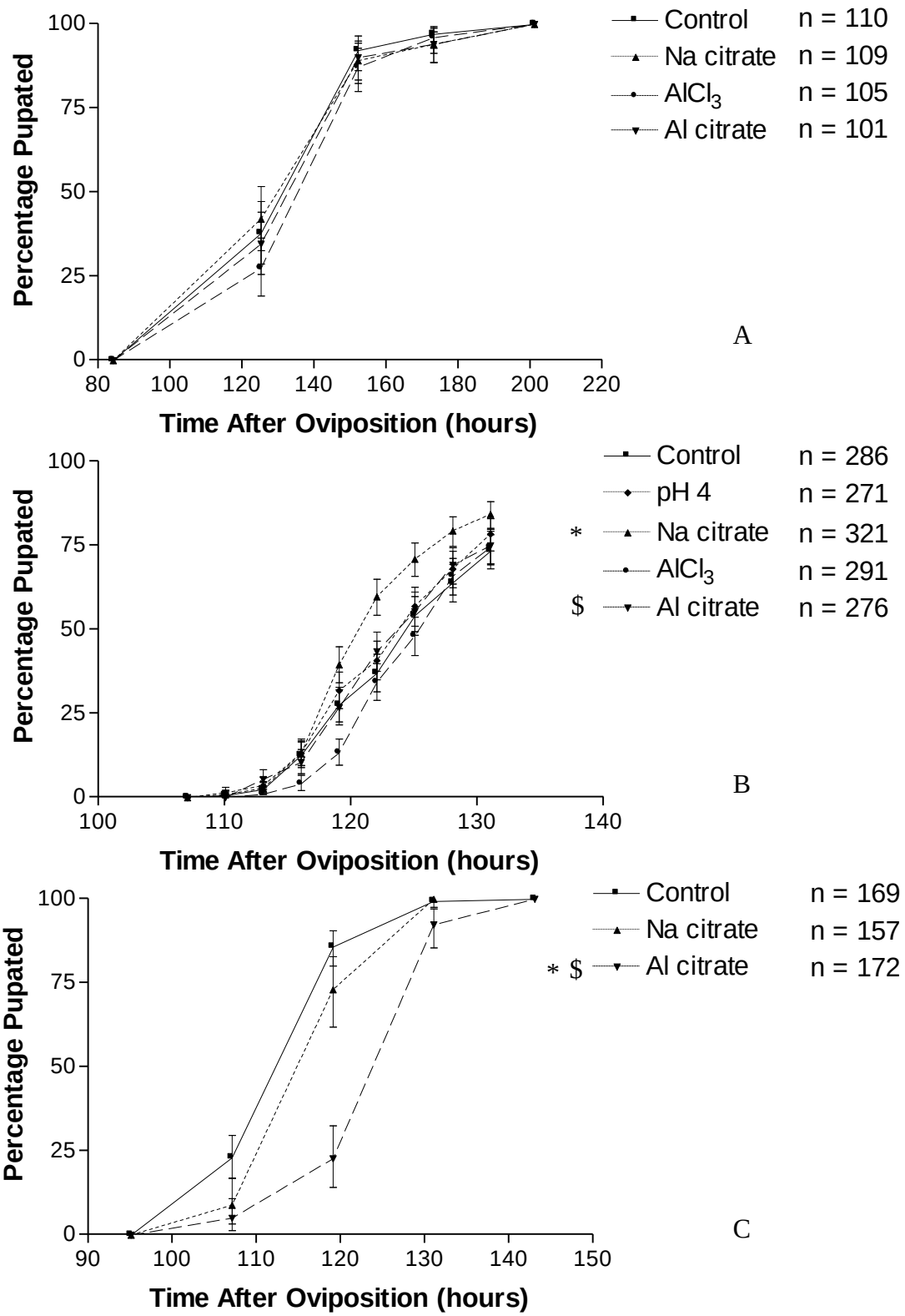

Figure 12. Time to pupation following administration of Al throughout larval development using yeast pre-feeding. After exposure to (A) $AlCl_3$, Al citrate or Na citrate (all 1 mM; no pH control necessary) (B) $AlCl_3$, Al citrate, Na citrate (all 10 mM),or pH 4 medium and (C) Al citrate or Na citrate (both 100 mM). Replicates for each group are shown in the treatment key. Error bars represent +/- 95% CI. Key to statistical significance: * p < Bonferroni corrected significance threshold ($\alpha = 0.05$) relative to control in that panel; $ p < Bonferroni corrected significance threshold ($\alpha = 0.05$) relative to Na citrate in that panel. See text for actual significance thresholds.

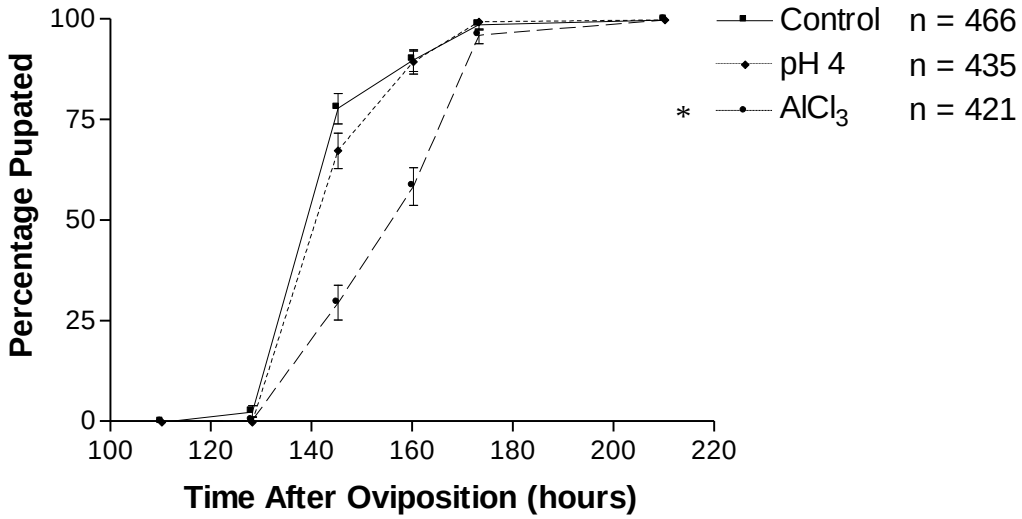

Figure 13. Time to pupation following administration of $AlCl_3$ (10 mM) throughout larval development using the MTD protocol. Error bars represent +/- 95% CI. Replicates for each group are shown in the treatment key. Key to statistical significance: * $p < 0.016$ relative to control (log-rank test).

received control medium throughout its larval period and Al following eclosion until death. The effect on adult lifespan of Al administered to larval *Drosophila* was determined. Administration of silicic acid was administered throughout the adult period to ascertain if this could ameliorate any effects of Al. Males and females were analysed separately to minimise variability due to their different life expectancies. A total of 11 HR comparisons (log-rank tests) were made, which with a family-wise error rate (α) = 0.05 yielded a significance threshold of 0.0045 after Bonferroni correction. Figure 15 shows the survival data for *Drosophila* exposed to Al as larvae and Si as adults. No significant differences ($p > 0.0045$; log-rank test) were found relative to control of either males or females (Figure 15A and B respectively). Male flies that had received pH 4 medium as larvae and Si as adults had a significantly shorter lifespan that controls with a $HR_{pH4/Si:Control/Control} = 1.75$ (95% CI of 1.51 - 2.02; $p < 5 \times 10^{-10}$; log-rank test) whereas the lifespan of similarly exposed female flies was not significantly different from control ($p = 0.056$; log-rank test). Conversely, females exposed to control conditions as larvae and Si as adults, pH 4 as larvae and control as adults, or Al as adults had increased life expectancy ($p < 0.0045$; log-rank test) relative to control but males in these treatment groups were not significantly different from control. The result that stood out from the rest in the male experiment was the pH 4/Si group which had a significantly reduced lifespan compared to all groups.

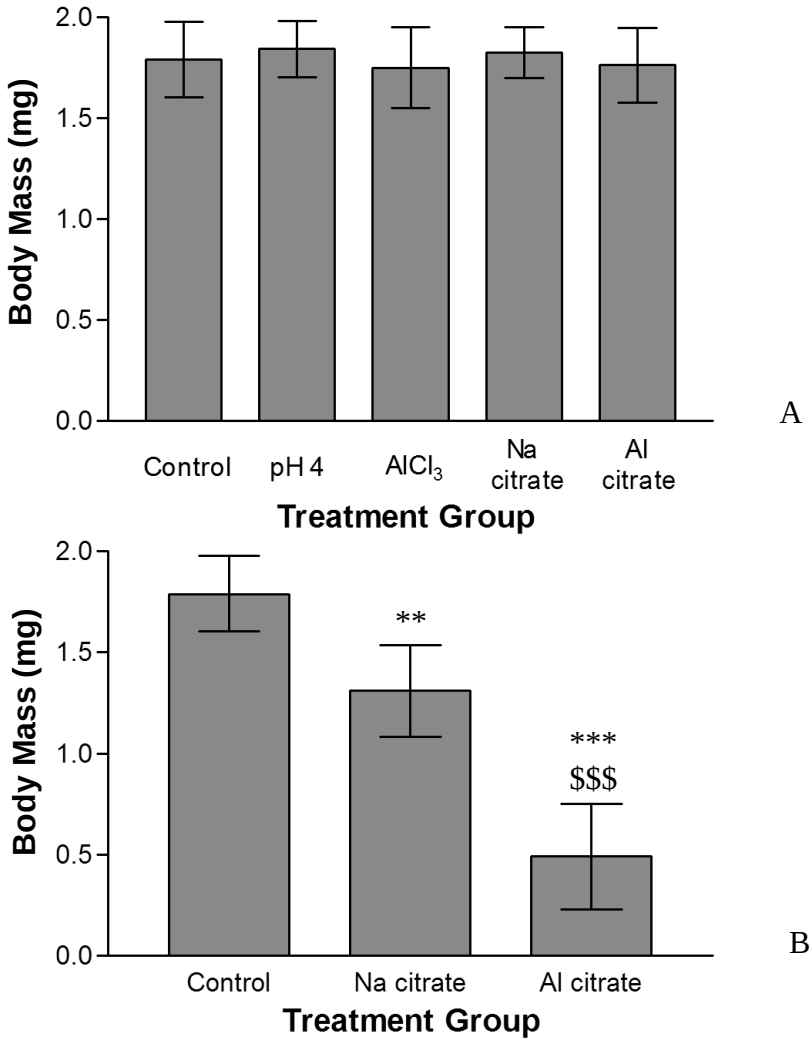

Figure 14. Larval mass (wet) following aluminium exposure for 96 hours after egg laying. Mass was determined from groups of 50 larvae, following exposure to (A) $AlCl_3$, Al citrate, Na citrate (all 10 mM) or pH 4 medium and (B) Al citrate or Na citrate (100 mM). Data are presented as mean +/- 95% CI. Key to significance values: ** $p < 0.01$ and *** $p < 0.001$ relative to control within that panel; $$$ $p < 0.05$ relative to Na citrate within that panel.

Significantly reduced female lifespan relative to all other treatment groups, except control, was seen. No other clear patterns emerged. For example, the only significant difference in males, that did not involve the pH 4/Si group, was that the life span of Al exposed flies was longer when they had *not* received Si as adults. Likewise, the significantly greater lifespan ($p < 0.0045$) in females of Al exposed larvae that received Si as adults compared to those that only received Al is not evidence for an ameliorating effect as these latter animals were not significantly impacted by Al relative to control. The Al/Si treated group had also showed improved survival relative to both control/control and pH 4/Si treated groups. There is a thus an indication that, in combination, Al and Si may extend lifespan

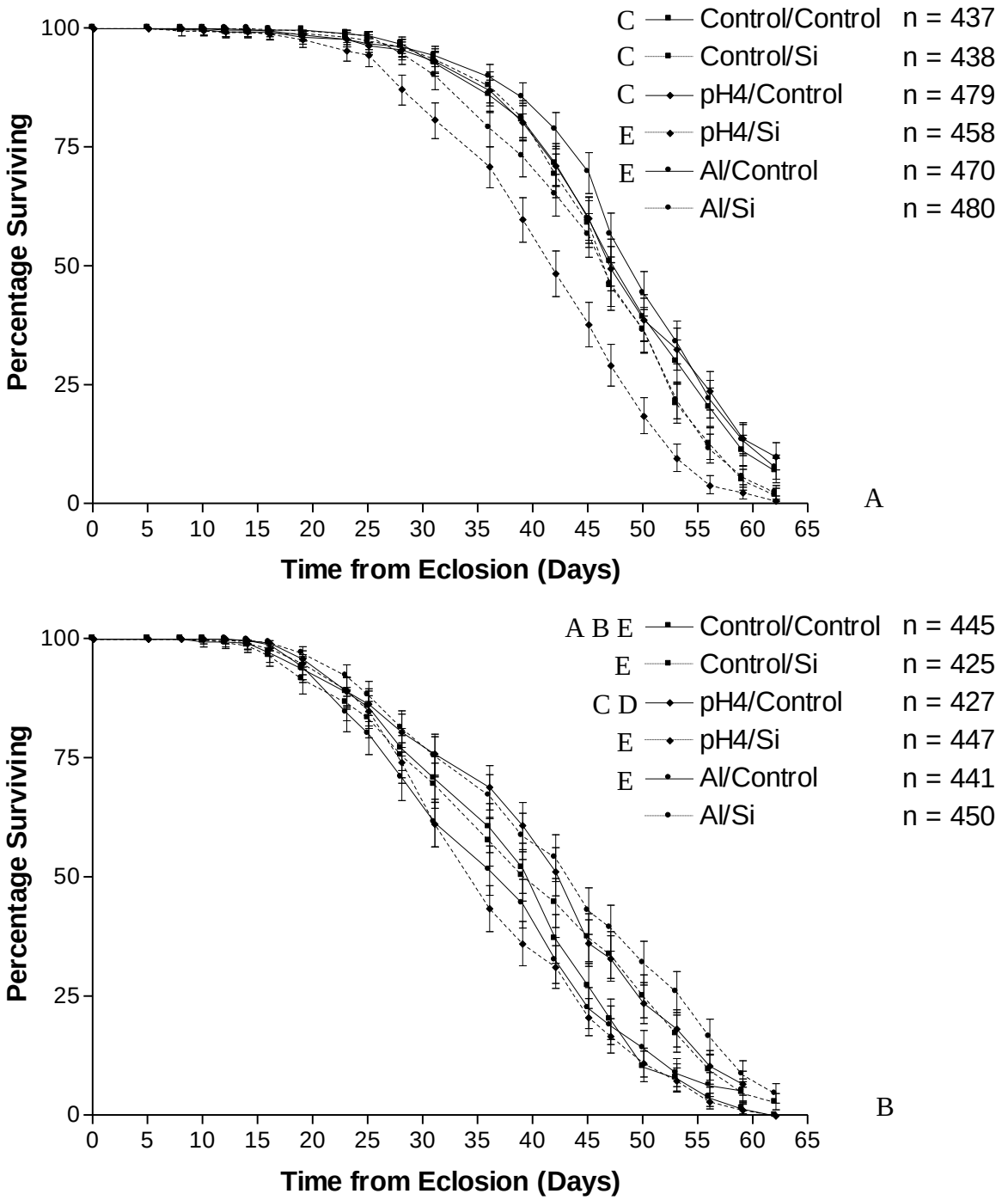

Figure 15. Lifespan of *Drosophila* following larval administration of Al and silicic acid exposure as adults. Survival curves, measured from eclosion, of (A) males and (B) females following exposure to $AlCl_3$ (10 mM) or pH 4 control medium as larvae with and without silicic acid (Si) (20 mM) exposure throughout adulthood. Error bars represent +/- 95% CI of fraction surviving. Replicates for each group are shown in the treatment group key. Key to statistical significance: A, B, C, D and E $p < 0.0045$ relative to Control/Si, pH 4/Control, pH 4/Si, Al/Control and Al/Si respectively.

in females. As a reduction in lifespan by Al was not demonstrated when administered throughout larval life, the potential for Si to ameliorate Al toxicity could not be assessed using this protocol.

To determine if the sequence of Al versus Si administration impacted upon toxicity the test substances were provided in reverse order. As found with the reciprocal protocol, no significant differences in lifespan were found, for males or females, following Al administration (Figure 16A and B respectively). In male flies, the only group that showed a significantly reduced lifespan was control/pH 4 and this was relative to all groups tested. In females, control/pH 4 was significantly different from control/control ($p = 0.0017$; log-rank test), and control/Al ($p = 6.2 \times 10^{-6}$), but the effect was to increase lifespan. The shortest lifespan in females was found in the Si/control group and was significantly different ($p < 0.0045$) from all groups tested. The Si/Control group in males was not statistically different from any other treatment group. Finally, survival was assessed for $AlCl_3$ and a pH 4 control that were administered throughout life, that is, from the moment of hatching until death. Three HR comparisons were performed resulting in a significance threshold of 0.016 after Bonferroni correction.

Again only minor differences were seen between treatment groups and those that there were often apparently contradictory. Although $AlCl_3$ significantly shortened lifespan compared to controls, this only occurred in males (compare Figure 17A (males) and B (females)) and the change in HR was small and the significance borderline: $HR_{Al:Control}$ = 1.20 (95% CI of 1.04 – 1.39; $p = 0.015$). The median $ratio_{Control:Al}$ was 1.07. Similarly, the effects of the pH 4 control were inconsistent across the sexes as exposed females had a significantly shorter lifespan compared to control with a $HR_{pH4:Control} = 1.31$ (95% CI of 1.12 – 1.53; $p = 0.00068$) and a median $ratio_{pH4:Control} = 0.91$; male lifespan, by contrast significantly *increased* with a $HR_{pH4:Control} = 0.77$ (95% CI of 0.66 – 0.90; $p = 0.00083$) and a median $ratio_{Control:pH\ 4} = 1.07$. The net result of these effects were that Al resulted in a significantly ($p = 2.1 \times 10^{-7}$) shorter lifespan compared to the pH 4 group in males but a slight extension ($p = 0.0029$) in females. Overall, whichever sequence of Al administration is used the alterations in lifespan seen, if any, are only of small magnitude.

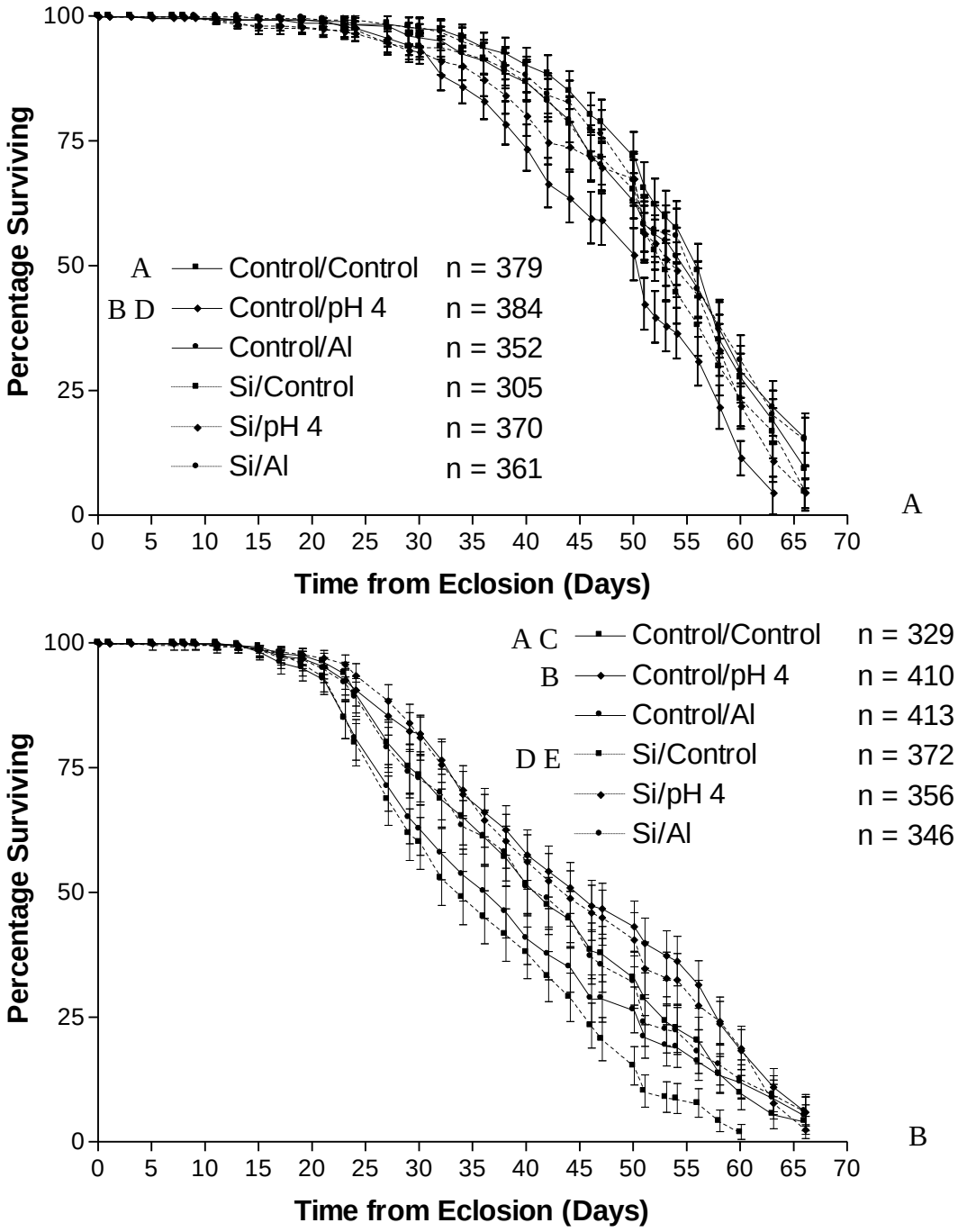

Figure 16. Lifespan of *Drosophila* following larval administration of silicic acid and Al as adults. Survival curves, measured from eclosion, of (A) males and (B) females following exposure to $AlCl_3$ (10 mM) or pH 4 control medium as adults with and without silicic acid (Si) (20 mM) exposure throughout larval life. Error bars represent +/- 95% CI of fraction surviving. Replicates for each group are shown in the treatment group key. Key to statistical significance: A, B, C, D and E $p < 0.0045$ relative to Control/pH 4, Control/Al, Si/Control, Si/pH 4 and Si/Al respectively.

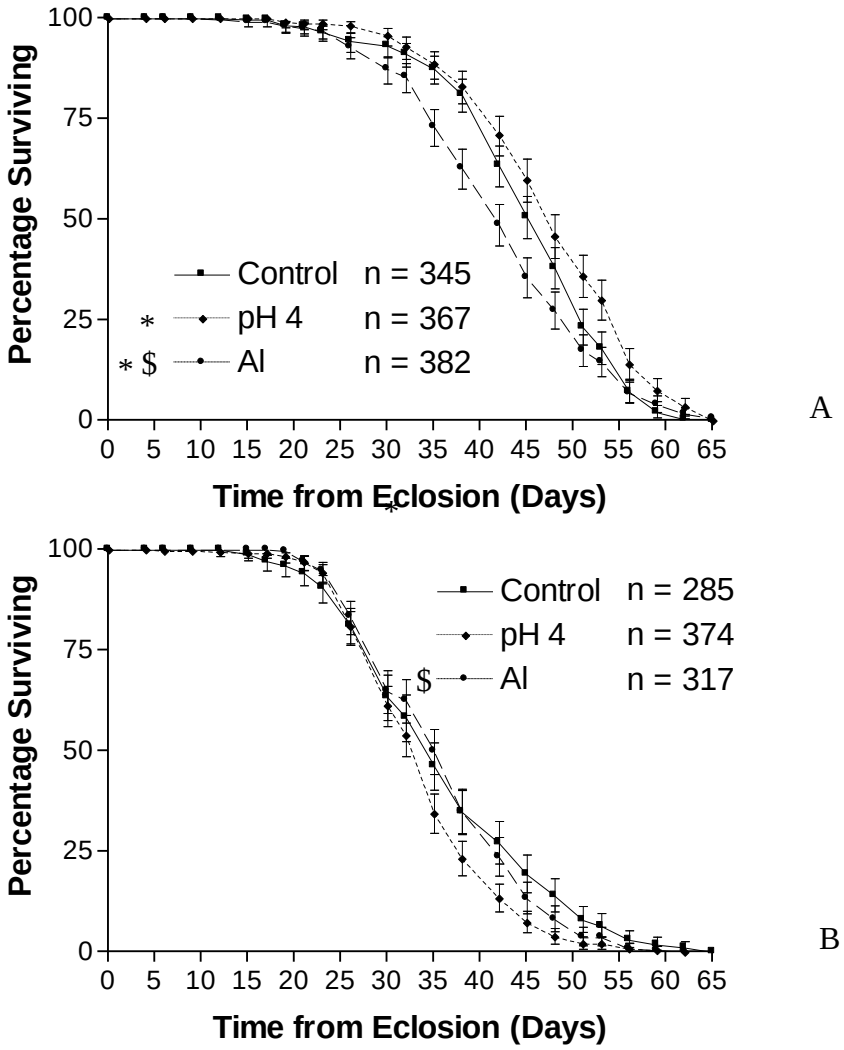

Figure 17. Lifespan of *Drosophila* following administration of $AlCl_3$ (10 mM) and pH 4 control throughout life. Survival curves following Al exposure in (A) males and (B) females Error bars represent +/- 95% CI of fraction pupated. Replicates for each group are shown in the treatment group key. Key to significance values: * $p < 0.016$ relative to control; $ $p < 0.016$ relative to pH 4 in that panel.

3.3.8. Behavioural indices of aluminium toxicity

To determine if functional, especially behavioural, deficiencies were induced following Al exposure, locomotor activity was tested in larvae and reactivity in adults was assessed though a negative geotaxis assay. Exposure to 10 mM Al, either as the chloride or the citrate in yeast, did not significantly affect (two-way repeated measures ANOVA – main effects) locomotor activity of larvae aged 96 hours (3^{rd} instar) (Figure 18A). It was not possible to expose larvae to 100 mM $AlCl_3$ due to its lethality, but 100 mM Al citrate significantly reduced the distance that larvae travelled in each 30 s interval ($p < 0.05$) (Figure 18B). All larvae (exposed or unexposed) moved more quickly over the initial 30s

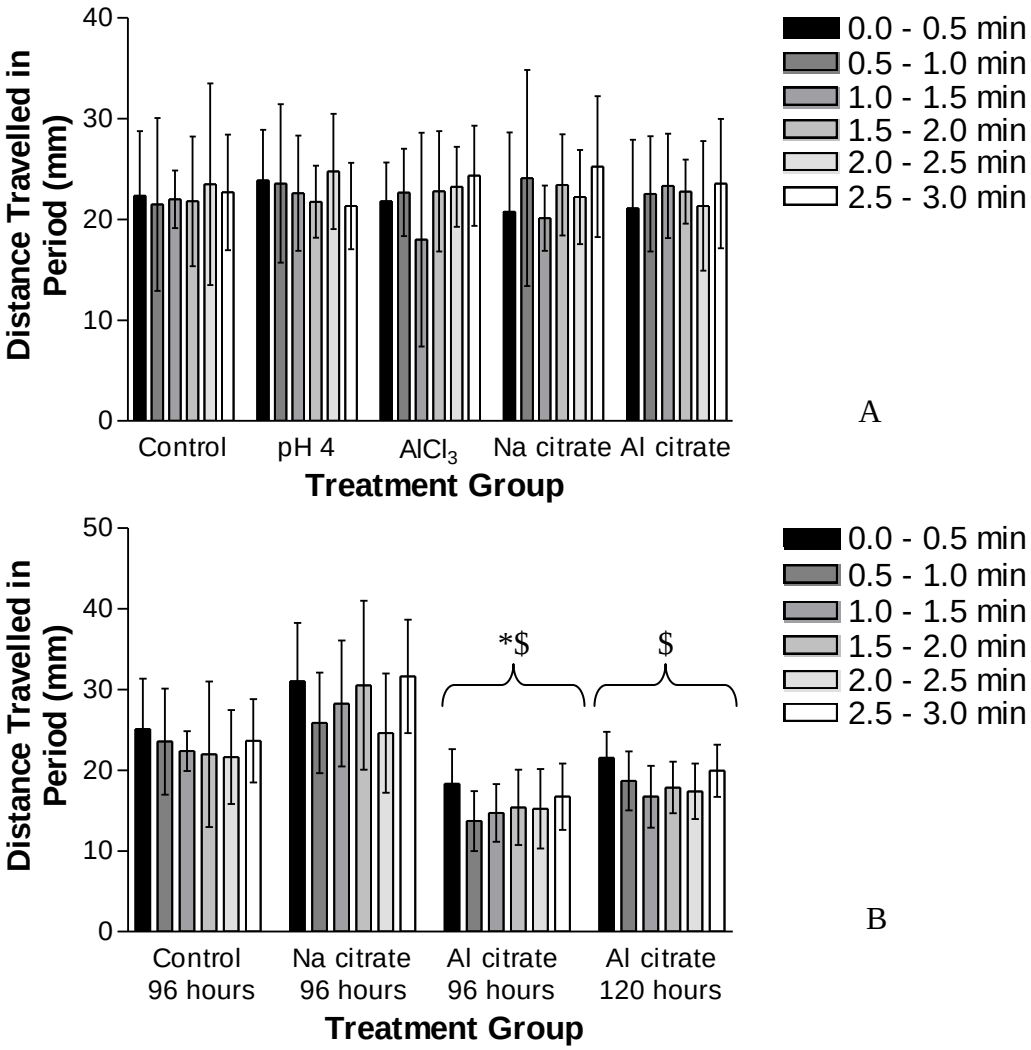

Figure 18. Locomotor activity of *Drosophila* larvae exposed to aluminium. Bars denote mean distance travelled (+/- 95% CI) of a single larva in successive 30 s intervals after exposure to, for 96 hours AEL, (A) $AlCl_3$, Al citrate, Na citrate (all 10 mM) or pH 4 medium and (B) Al citrate, Na citrate (both 100 mM) in addition to a further Al citrate group (100 mM) for 120 hours (a 120 hour control was not possible because of normal pupation behaviour) n = 20. Key to significance values: (A) no significant differences (2-way repeated measures ANOVA – main effects) (B) * $p < 0.05$ relative to control; $ $p < 0.05$ relative to Na citrate (Tukey-Kramer test following 2-way repeated measures ANOVA).

period ($p < 0.05$) than all subsequent time intervals except for the final 2.5-3.0 minute period. The distance travelled in this latter period was significantly different only from the 2.0-2.5 minute period.

Larvae exposed to 100 mM Al citrate were smaller than their unexposed counterparts (consistent with the lower mass seen in Section 3.3.6.4) (Figure 18B) suggesting that the difference in locomotion could be a result of smaller body size rather than a functional disturbance. To test this hypothesis, these larvae were allowed to continue developing for

a further 24 hours and the rate of locomotion measured again. This second measurement was only possible due to the delay in pupation seen with this concentration of Al; the normal pupation behaviour of control animals precluded age-matched controls. Following this extra period of development, locomotor activity relative to younger exposed larvae was not significantly increased ($p > 0.05$; Tukey-Kramer test following two-way repeated measures ANOVA) (Figure 18B). However, the older exposed group was no longer significantly different from control. Although not significantly different from control, the Na citrate group showed a trend towards increased locomotor activity. This increase accounts for the older exposed Al group having significantly lower locomotor activity relative to the Na citrate group ($p < 0.05$) but not the control ($p > 0.05$). Five day post-eclosion adult flies, when exposed to $AlCl_3$ (10 mM) or pH 4 medium for their entire life from egg hatching, showed no significant differences in the negative geotaxis assay ($p > 0.05$; ANOVA – main effect) (data not shown).

3.4. Discussion

Drosophila larvae, when exposed to $AlCl_3$ or Al citrate (10 mM) from hatching until 16 days post-eclosion showed significant body burdens of Al and with a higher tissue concentration resulting from Al citrate exposure. The uptake of Si following silicic acid exposure was found to be concentration-dependent and caused a concentration-dependent uptake of Al from the background levels found in the medium; the converse was not seen. Despite uptake of Al, no Al treatment except $AlCl_3$ (100 mM) caused a reduction in the number of larvae successfully pupating. Pupation was delayed at 10 mM but this depended upon the medium in which Al was administered although the deterioration in eclosion success was similar in both types of medium. Lifespan following eclosion was unaffected by Al and so it was not possible to examine the effects of Si on Al toxicity. The counter-ion associated with Al is able to modulate the uptake of Al and potentially affect its overall toxicity. Overall, the effects of Al appear dependent upon the medium and the results here do not concur with previous studies of uptake and toxicity of Al to *Drosophila* (Massie *et al*. 1985, Wu *et al*. 2012b, Kijak *et al*. 2013).

3.4.1. Experimental limitations

The concentration dependence of Al uptake was determined across two separate experiments largely because the lack of effect at 10 mM was unexpected, considering the

results of Massie *et al*. (1985), who found substantial accumulation following much lower concentrations (0.1 – 1 mM). Unfortunately, across the two experiments a discrepancy between control values was noted. Despite precluding *direct* comparison between tissue concentrations, these differences in control body burdens do not prevent *indirect* comparison of exposed groups by assessing changes relative to control values. So long as internal controls are provided, such comparison is a valid method for determining relative changes in accumulation (or any other metric that may be affected by variable controls) following exposure.

An unfortunate oversight in the formulation of the controls has resulted in a second, more serious, limitation in the tissue concentration data and to the later experiments involving larval development. To control for the possible effects of the citrate ion in Al citrate group, another group of flies was exposed to Na citrate. In itself, this was not an ideal control as the citrate ion in the latter would be completely free, rather than a sizable proportion being in the form of metal chelate. However, the use of Na citrate was better than omitting such a control altogether. Nevertheless, several instances of this control exerting an effect can be seen (Figure 11A, Figure 12B and Figure 14B) making interpretation of the Al citrate data difficult. For this reason, plus that $AlCl_3$ was more toxic, Al citrate was omitted from several experiments. When no toxicity could be demonstrated at 10 mM $AlCl_3$, the concentration was increased to 100 mM and the chloride salt replaced with Al citrate. However, it is now evident that a preferable solution to increasing toxicity, without conferring total lethality, would have been to use $AlCl_3$ at an intermediate concentration.

The main issue relating to the use of a Na citrate control however arose from extending the logic of a counter-ion control to a pH control for the $AlCl_3$ groups. Aluminium chloride acidified the medium and a comparable pH medium was used as a control. However, as pH can be manipulated independently of the $AlCl_3$ concentration it would have been better to compare both Al groups at the same pH as it is impossible to assign an effect to the form rather than the pH. The dependence of pH upon $AlCl_3$ concentration compounded this problem. Nonetheless, although care is needed when comparing $AlCl_3$ and Al citrate, so long as these limitations are respected, such comparisons can still be informative as both are soluble but of differing lipophilicity. Of course, to perform the analyses at identical pH levels would result in Al complexes of different solubilities and/or reduced ligand affinity (Martin 1986), so an alternative disparity is introduced as the cost of removing another.

Whichever path is chosen it difficult to entirely isolate any of these factors, and the best future option would be to perform the experiments using as many combinations as possible, to infer which factors are liable to be most important.

3.4.2. Uptake of Al by *Drosophila*

Drosophila medium and yeast contained Al (Section 3.2.1), but each contained concentrations below the threshold for toxicity previously observed in *Drosophila* (Massie *et al*. 1985, Wu *et al*. 2012b); the baseline concentrations were well below the concentrations added. Several mechanisms exist by which nominal medium values may not concur with measured values, such as precipitation and adsorption to vessel walls. In this study Al was administered in a solid gel matrix that would not allow free diffusion of metal ions and hence minimising losses by adsorption to vessel walls. Any change in speciation resulting in precipitation could not result in Al being lost from the feeding surface as it would be unable to descend through the gel. The final potential source of metal loss is that due to processing by the test organism itself. However, aside from that which is taken up by the organism metal will be returned to the food substrate as excreta. Aluminium, so excreted, may be of different speciation, but this would have no greater impact than speciation changes resulting from interactions with the medium. Considering all these points, especially that speciation changes, regardless of their effect upon solubility, will have no impact on the measured Al concentration of the medium, it was decided that it would be uninformative to compare nominal versus measured concentrations in the *Drosophila* medium. Indeed, although the reasoning is not stated, the practice of relying upon nominal measurements of metal concentration in *Drosophila* studies is prevalent (Maroni *et al*. 1995, Yepiskoposyan *et al*. 2006, Balamurugan *et al*. 2007, Steiger *et al*. 2010, Mishra *et al*. 2011, Wu *et al*. 2012b)

To the knowledge of the author, this is only the third study to examine the amount of Al in the tissues of whole *Drosophila* following Al exposure. This study has demonstrated that larval exposure to 10 mM Al did not result in detectable tissue concentrations whether administered as $AlCl_3$ or Al citrate (Figure 6A). However, when administered during adulthood also, 10 mM Al exposure resulted in elevated tissue concentrations relative to control with higher concentrations seen in Al citrate exposed flies (Figure 8). Citrate has been found consistently to elevate the concentrations of Al accumulated in diverse

organisms. For instance, rats only accumulate appreciable quantities of Al in the bone and brain when Al is co-administered with citrate (Slanina *et al*. 1985, Wu *et al*. 2012a). Massie *et al*. (1985) analysed Al accumulation in whole *Drosophila* using three different concentrations of Al at various periods over 60 days. For their main study, they did not supplement the medium with Al, instead relying upon the baseline level of ~190 μM as the sole supply. At 16 days, those flies were equivalent to the control flies in this study. Under these conditions their flies had accumulated ~49 $\mu g_{Al}/g_{DryTissue}$, a tissue concentration 10 times that of the control flies (7 times after correction for recovery in this study (Section 3.2.3)). At 10 mM Al exposure their flies had accumulated 226.4 $\mu g_{Al}/g_{DryTissue}$ at 16 days. Kijak *et al*. (2013) recorded uptake of Al by *Drosophila* but, in contrast to Massie *et al*. (1985), in minute quantities. Background Al concentrations after a "natural" lifespan were 13 $ng_{Al}/g_{DryTissue}$ and remained unchanged following ~0.75 mM Al exposure. Significant accumulation was observed after ~4.5 mM but only to a level of 47 $ng_{Al}/g_{DryTissue}$, approximately 2 orders of magnitude lower than the background levels seen in this study.

3.4.3. Developmental and lifespan toxicity of aluminium and the effects of silicon

With the developmental toxicity data there was variability amongst control data across the different experiments so medium concentration dependency must be inferred by comparison with the internal control (Section 3.2.4). Only 100 mM $AlCl_3$ reduced larval viability or pupation success. Exposure into adulthood resulted in elevated Al tissue concentrations with Al citrate exceeding $AlCl_3$ and it is possible that differential accumulation was occurring during the larval period also but at levels below detection. This suggests that despite a higher uptake of Al citrate, it is much less toxic than $AlCl_3$, dissociating toxicity from bioavailability. Protective effects of citrate on Al toxicity have been observed both in fish (Driscoll *et al*. 1980, Lacroix *et al*. 1993) and aquatic invertebrates (McCahon and Pascoe 1989) whilst citrate has been shown to increase bioavailability (Priest *et al*. 1996) and facilitate transport to sensitive targets within organisms (Berthon 2002).

Time to pupation was delayed when Al (10 mM) was administered through the MTD protocol (Figure 13) but not using the YP protocol (Figure 12); both methods of administration reduced eclosion success. Kijak *et al*. (2013) reported delayed pupation in *Drosophila* at concentrations of Al above 6 mM and reduction in pupation and eclosion

success at 7.5 mM. This is broadly consistent with the results here. The present data are insufficient to completely elucidate the mechanism behind the developmental delay and increased pre-eclosion mortality. However, as body mass remained unchanged at developmentally toxic Al concentrations, reduced food intake can reasonably be excluded. Furthermore, the gustatory assay found a repellent effect for Al citrate but not $AlCl_3$, despite the reverse pattern of toxicity. As Wu *et al*. (2012b) found distinctive neuropathology following Al administration to flies, it is suggested that the developmental effects here may be a result of neurotoxicity. The increase in time to pupation may be explainable by the fact that neurogenesis is highly active throughout the larval period (Truman and Bate 1988, Ito and Hotta 1992). Furthermore, any disruption to ecdysone (Baehrecke 1996) or JH (Jindra *et al*. 2013) secretion will disturb developmental progression, especially as they interact with various neurotransmitters (Hiruma and Kaneko 2013). The loss of viable animals during metamorphosis suggests that this period is the most vulnerable, again possibly through interaction with the extensive neurological re-modelling that occurs during this transition (Truman 1990). The pupal period, being characterised by rapid cell growth and proliferation has similarities to the Al sensitive embryonic and foetal period in mammals. Although the primary purpose of this work was to investigate Al toxicity to *Drosophila* with its use as a model for human pathology in mind, these results indicate that Al could represent a significant hazard to free-living *Drosophila*. However, the concentrations used here were high and it is improbable that any natural population of flies would encounter such levels. If these findings can be extrapolated to other Dipterans then the possibility for exposure may be increased, depending upon life habits of the organism involved. For example, high concentrations of metals are found in contaminated sediments and result in genotoxicity in midge larvae (Michailova *et al*. 2003, Michailova *et al*. 2006)

No demonstrable reduction of post-eclosion lifespan was seen when *Drosophila* were exposed either as larvae or adults (Section 3.3.7). A slight but significant reduction in the lifespan of males (see Figure 17) was noted following continuous Al exposure although this difference was probably too small (HR = 1.2 at borderline significance; median ratio = 0.93) to be of biological significance. This contrasts with published studies (Massie *et al*. 1985, Wu *et al*. 2012b) of Al in toxicity in *Drosophila* that show premature death as a consequence of Al administration. Massie *et al*. (1985) found significantly reduced lifespan following 1 mM Al exposure whether administered throughout life or only post-

eclosion but no concentration dependence was noted. Wu *et al*. (2012b) found a concentration-dependent decrease in longevity with a toxic threshold of 5 mM Al for adult only exposure and 0.5 mM for lifelong administration. Kijak *et al*. (2013) found a reduction in female, but not male, lifespan following lifelong Al (~1.5 mM). These studies therefore differ markedly from the results presented here.

3.4.4. Suitability of *Drosophila* as a model for Al toxicity and its amelioration by silicic acid

Considerable differences can be seen between the values of parameters measured here and their counterparts in the literature, including metal uptake and lifespan. Furthermore, a dependency of toxicity upon the medium has been noted. Indeed, some of the differences between the work here and the literature may ultimately derive from the medium. Additionally, there is likely to be a genetic component. Many wild type strains of *Drosophila* exist and Ortiz *et al*. (2009) found marked differences in susceptibility to arsenic across 35 different *Drosophila* strains. At the extremes were strains that failed to produce a single eclosing adult at a larval exposure concentration of 0.25 mM such as PVM and KCA 1 to one strain that was completely unaffected by concentrations of 1 mM (Luminy) and one (Oregon-R 1970) that maintained 80% viability at 1 mM. This inherent variability complicates the use of *Drosophila* as a model organism for Al toxicity but does not preclude it. Indeed, rather than a hindrance the differing sensitivities can be harnessed to further understand the mechanism of toxicity. This technique has already been used to dissect Pb toxicity in *Drosophila*, and so far has located a quantitative trait locus containing 125 candidate genes that confer resistance to Pb.

The value of the fruit fly model for understanding Si biology in relation to Al remains to be adequately tested; whatever the reason for the insensitivity of the *Drosophila* lifespan to Al here, it has nullified the utility of the assay for determining the effects of silicic acid. Nonetheless, the work here has shown that silicic acid is taken up in a concentration dependent fashion by *Drosophila*. To achieve stochiometric equivalents (or greater ratios) required the use of high concentrations of silicic acid. It therefore is likely that silicic acid received by *Drosophila* was polymeric (Tarutani 1989) rather than the monomeric. As uptake was seen here in *Drosophila* and by Dobranskyte *et al*. (2004) in *L. stagnalis*, but oligomeric silica is not absorbed by humans (Jugdaohsingh *et al*. 2000), it is possible that this is fundamental difference between invertebrate and vertebrate physiology.

Screening for behavioural anomalies yielded only negative results. If *Drosophila* is to be a successful model for Al induced developmental toxicity, evidence of behavioural impairment (of neurological origin) is important as this is a primary mechanism that appears to operate in mammals. However, as with the negative lifespan experiments it seems that this lack of effect may be derived from the additional factors discussed above and that these can be studied separately given that behavioural toxicity has been observed previously in *Drosophila* (Wu *et al*. 2012b, Kijak *et al*. 2013).

3.4.5. Conclusions

In this work developmental changes in *Drosophila* have been investigated to assess its vulnerability to Al and the feasibility for use as a model of Al toxicity. Under the conditions used in this work it has only been possible to demonstrate with certainty that Al retards developmental progression and reduces viability at eclosion. It is therefore suggested that the principal effect of Al on *Drosophila* occurs during periods of rapid growth, re-modelling and high cell turnover such as characterizes metamorphosis. This assertion is reinforced by noting that exposure throughout the entire adult period did not reduce lifespan. These effects, however, seems highly dependent upon the medium through which Al is administered. Furthermore, comparison to other studies reveals that uncontrolled variables may significantly impact the outcome of Al exposure, such as strain or food medium composition. *Drosophila* certainly has potential to serve as a model for Al toxicity, but it is essential that the sources of variability be identified and quantified such that they may be accounted for and the underlying mechanisms elucidated. Experimental limitations mean that the potential role of Si in Al toxicity in *Drosophila* has yet to be established.

Chapter 4. Physical properties of nanoparticles and uptake of ionic and nanoparticulate silver by *Drosophila melanogaster*

4.1. Introduction

Silver has been well studied in aquatic systems and is known to be highly toxic (Ratte 1999). Despite some studies that indicate acute toxicity to terrestrial vertebrates and invertebrates, a thorough understanding of the severity and extent of the risk of Ag exposure to terrestrial biota is lacking. There are many anthropogenic sources of environmental Ag. Until recently, the predominant producer of Ag-containing effluent was the photographic industry (Purcell and Peters 1998). A more recent and rapidly expanding source of Ag pollution is the increasing use of AgNPs for various purposes including, but not limited to antimicrobials (Jain *et al*. 2008). Study of the behaviour of this form of Ag is still in its infancy, and much remains to be determined about the toxic effects of the particles themselves, their tendency to release the toxic Ag (I) ion, and the relative importance of these properties (Stensberg *et al*. 2011).

The following three chapters examined the comparative toxicity of ionic Ag and AgNPs to *D. melanogaster* but, before toxicity studies using nanoparticles can be performed, it is necessary to characterise them physically. This can be achieved by a number of means, including transmission and scanning electron microscopy (Warheit 2008). Agglomeration is a common, although not universal, finding that depends upon many factors, especially the dispersion of NPs in media other than water (Murdock *et al*. 2008). Particles capped electrostatically are more vulnerable to agglomeration in biological media, owing to charge interactions with the electrolytes; sterically stabilised particles are more robust in this respect (Fritz *et al*. 2002, MacCuspie 2011, El Badawy *et al*. 2012). Although different to biological media, characterisation of AgNPs in de-ionised H_2O does assist in understanding their behaviour whilst minimising interference from external variables; it also allows measurement of individual particles. However, it is only when within the medium in which AgNPs are to be administered that their behaviour becomes relevant to their toxicity. In mammalian studies, NPs can be given as an aqueous suspension, but to administer AgNPs to *Drosophila* in food requires their addition to standard *Drosophila* medium, which is distinct from most biological media examined in the literature. The

behaviour of AgNPs in *Drosophila* medium has not been reported, but citrate capped gold nanoparticles (AuNPs) show no discernible agglomeration in similar medium (Vecchio *et al*. 2012), despite the sensitivity of electrostatically capped NPs to ionic strength (El Badawy *et al*. 2012).

In addition to agglomeration, AgNPs are also susceptible to dissolution to the ionic form. This occurs through oxidation of Ag atoms on the particle surface followed by proton mediated dissolution (Liu and Hurt 2010). The kinetics and extent of the reaction are dictated by many factors including the capping agent (Kittler *et al*. 2010), temperature (Liu and Hurt 2010, MacCuspie 2011), pH (Liu and Hurt 2010), medium, particle size (Zhang *et al*. 2011) and concentration (Lee *et al*. 2012). The reaction reaches equilibrium, within 4 –75 days (Liu and Hurt 2010, Kittler *et al*. 2010, MacCuspie 2011, Zhang *et al*. 2011, Lee *et al*. 2012). The enormous variety of AgNPs, coupled with the sensitivity of their behaviour to subtle changes in any of several parameters, necessitates that each brand or synthetic method is considered unique.

The dissolution of AgNPs, when molecular oxygen is the presumed, ultimate oxidant, occurs over a period of days. However, a far more rapid degradation of AgNPs was observed during this study in an experiment (See section 5.2.1.2) involving simultaneous administration of Cu and Ag to investigate the potential antagonism between the two metals. When added to fly food medium AgNPs usually stain it a light chocolate brown colour, but in the presence of $Cu(NO_3)_2$ the colour faded after about two hours indicating a change in the chemical speciation of the Ag. The loss of colour suggested oxidative dissolution; AgNPs are coloured, Ag (I) ions are not. The mechanism is unclear as electrochemical theory suggests that simple oxidation of Ag(s) by Cu^{2+} ions is not energetically favourable (Vanýsek 2012). However, the nanoparticulate nature of the Ag (Ivanova and Zamborini 2010), the presence of other ions, and the non-standard conditions of the medium could shift this equilibrium and possibly reverse it (Hamann *et al*. 2007). It is hypothesised by the author that the ubiquitous Cl^- ion may play a role in Cu mediated oxidation of AgNP but only empirical testing can help elucidate what other ions and in what concentrations drive this apparent oxidation. It is further hypothesised that given its larger standard electrode potential Fe may also engage in this reaction, perhaps without the need for Cl^- ions.

A common prerequisite for metals to exert toxicity is their accumulation, in a biologically active form, above a certain threshold (Rainbow 2002). Silver accumulates in many aquatic organisms (Rouleau *et al*. 2000, Mann *et al*. 2004, Bianchini *et al*. 2007, Wood *et al.* 2010, Croteau *et al*. 2011, Li *et al*. 2013), but data on terrestrial vertebrates is considerably more limited and for invertebrates virtually absent (Ratte 1999). The toxicodynamic and toxicokinetic behaviour of ionic Ag within living systems is governed, in part, by its mimicry of Cu, and Ag is able to competitively inhibit the Cu transport Ctr1 (Bertinato *et al.* 2010). AgNPs are unlikely to conform to the same rules that govern soluble Ag species, resulting in an altered bioavailability profile. Furthermore, because the particles are subject to dissolution, bio-accumulation will comprise two components. Following oral administration of $AgNO_3$, mice accumulate Ag in various tissues including the spleen, skeletal and cardiac muscle, and the cerebellum (Pelkonen *et al*. 2003). Similarly, female rats rapidly assimilate Ag in the liver, spleen and reproductive organs following intraperitoneal administration of $AgNO_3$ (Hanson *et al*. 2001). Although, evidence has been presented that ionic Ag is able to cross the blood-brain barrier (Rungby and Danscher 1983a) this has been disputed (Lansdown 2007). Oral exposure of rodents to AgNPs results in a considerable deposition in the wall of the alimentary canal, along with lesser amounts being found in liver, kidney, and spleen and a minor fraction in the brain. Two rodent studies that compared AgNPs with $AgNO_3$ found qualitatively similar patterns of distribution of Ag, which was often in particulate form and had the appearance of AgNPs (Loeschner *et al*. 2011, van der Zande *et al*. 2012).

With regard to terrestrial invertebrate accumulation, earthworms and *C. elegans* have proved to be valuable models for both forms of Ag. *C. elegans* accumulates discrete AgNPs intracellularly (Meyer *et al*. 2010). The earthworms *E. fetida* and *E. andrei* accumulated Ag from AgNPs and $AgNO_3$; *E. fetida* accumulated more from $AgNO_3$ (Shoults-Wilson *et al*. 2011) whereas *E. andrei* accumulated more from AgNPs (Schlich *et al*. 2013). Although accumulation in *Drosophila* of Ag from AgNPs has been demonstrated (Gorth *et al*. 2011), the potential for differential accumulation of $AgNO_3$ and AgNPs in *Drosophila* has received almost no attention. Armstrong *et al*. (2013) compared the accumulation of Ag from citrate capped AgNPs and $AgNO_3$ and no differences in accumulation were found. The comparability of these values is, however, questionable as the concentration of $AgNO_3$ was not corrected for its larger molar mass, leading to only 0.6

times the amount of Ag in the $AgNO_3$ group the AgNP group. No comparison between the uptakes of PVP capped AgNPs and $AgNO_3$ has been made in *Drosophila*.
The first aim of this chapter was to examine the physicochemical properties AgNPs. Transmission electron microscopy (TEM) was chosen to assess primary particle size as it gives an indication of possible agglomeration although it cannot provide definitive answers regarding agglomeration in aqueous suspension because of the processing needed for TEM; the solid matrix of fly food medium and the fixation methods used should prevent processing induced agglomeration. Dissolution at differing pH values, but no oxidant other than atmospheric oxygen, and its inhibition by antioxidants was investigated by dialyzing AgNP suspensions. The transition metals Fe and Cu were also tested as oxidants in the presence of and absence of Cl^- ions as these appear to greatly accelerate dissolution. The final part of this chapter aims to characterise the uptake of both forms of Ag from food medium in *Drosophila*, and how this interacts with the uptake of Cu.

4.2. Methods

4.2.1. Stock preparation and physical characterisation of nanoparticles

All investigations using Ag exposure and digestions were conducted in darkness to prevent photoreduction of potentially photosensitive Ag halides (Fujita 2004). The AgNPs used throughout this study were supplied by SkySpring Nanomaterials, USA and were described by the manufacturer as "silver nanopowder self-dispersing 15 nm 10%". The particles were 10% by mass Ag and 90% polyvinylpyrroline (PVP) coating to facilitate dispersion in aqueous media. The Ag component was quoted with a purity of 99.9% with six impurities listed: aluminium (31 ppm), silicon (10 ppm), bismuth (18 ppm), copper (21 ppm), iron (26 ppm) and nickel (22 ppm). The specific surface area was not reported. The description "self-dispersing" was used to indicate that only a minimal preparation procedure was required to create a suspension and that no sonication was needed.
Diamond nanoparticles (Sigma-Aldrich, UK) were supplied uncoated in aqueous suspension (1% (w/v)) and had a nominal particle size of <10 nm. The main impurities of these nanoparticles were barium (< 15 ppm), calcium (< 500 ppm), iron (< 30 ppm), potassium (< 30 ppm), magnesium (< 100 ppm), sodium (< 100 ppm), sulphur (< 100 ppm). Data concerning specific surface area were not available.

Aqueous stock suspensions of AgNPs were prepared freshly for each experiment in 7 ml bijou tubes to a concentration of 10 mM on a metal basis by method of difference as follows. Using an analytical balance, a bijou tube, including the cap, was weighed. Approximately 54 mg AgNPs were added to this tube and the total weight recorded (mass A). Fifty-four milligrams was chosen as 10% of this mass was elemental Ag which equated to 50 μmol, the quantity necessary to produce 5 ml of a 10 mM solution. The AgNPs were then transferred to a second (unweighed) bijou tube and the original tube re-capped and reweighed (mass B). Mass B was subtracted from mass A to yield the mass of AgNPs now in the second tube. This mass was expressed as a fraction of 54 mg and multiplied by 5 to give the volume of water necessary to make a 10 mM suspension in 5 ml. This suspension was shaken briefly to loosen any clumps adhering to the tube wall and then vortexed on full speed for 30 s to fully disperse the particles. The Ag content of this suspension was confirmed by ICP-AES after digestion in hot (90 °C) aqua regia (3:1 molar ratio HCl:HNO_3); the measured Ag content of 54 mg powder was 46.4 μmol (95% CI of 42.1 – 50.7 μmol). A 10 mM working stock solution of diamond nanoparticles was prepared freshly for each experiment by adding diamond nanoparticle stock suspension (60 μl) to de-ionised water (4940 μl).

As part of the investigation to assess the monodispersity of the AgNPs, their absorbance spectrum was measured using a spectrophotometer with a scan width of 340 – 500 nm. From this spectrum the peak absorbance was determined and used for later investigations involving AgNP dissolution. An absorbance spectrum of 100 μM (10.8 μg/ml) 15 nm AgNPs dispersed in dH_2O was obtained Figure 19. Metal nanoparticles exhibit a phenomenon called LSPR due to the interaction of electromagnetic radiation with the conduction band electrons on the NP's surface. Strong optical extinction, from a combination of scattering and absorbance, is seen when the incident wavelength corresponds to the frequency of electronic oscillation, exciting the electrons into a resonant condition (Willets and Van Duyne 2007, Jain *et al*. 2008). The spectrum produced by the AgNPs used here contained a single peak at ~409 nm. This is consistent with the extinction peak due to LSPR associated with non-aggregated silver nanoparticles in the size range 10 – 30 nm (Solomon *et al*. 2007, nanoComposix 2012b,).

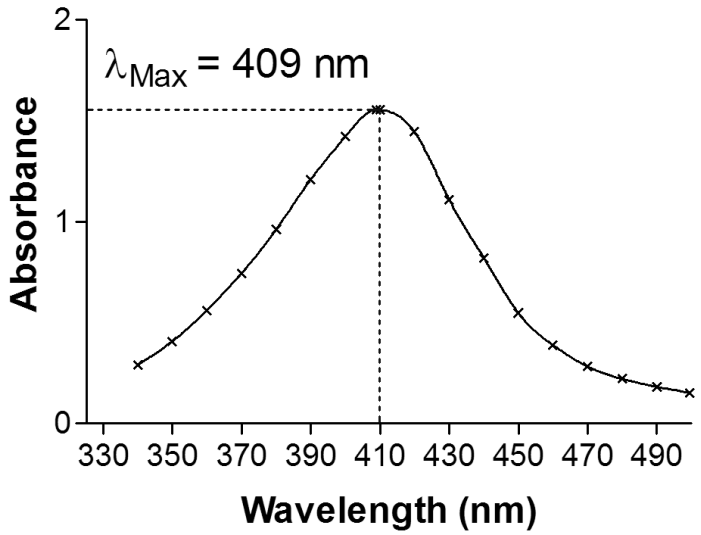

Figure 19. Absorbance spectrum of 100 µM 15 nm AgNPs dispersed in dH_20. A single peak was seen at 409 nm.

To determine primary particle size and assess agglomeration state (after drying and therefore may show artefactual agglomeration), AgNPs were imaged using TEM. Five samples of AgNPs (2 µl – as prepared below) in dH_2O were pipetted onto carbon-coated TEM grids and completely air dried in a fume cupboard. The suspension had been prepared, as described below, such that when 2 µl was pipetted onto a carbon coated TEM grid and visualised, the particle density in the image would be equivalent to that seen if examining a 80 nm section of AgNP containing standard *Drosophila* medium prepared for electron microscopy (See Sections 4.2.4 and 4.3.4. That is, the applied suspension was at a concentration that would achieve the approximate concentration of the food medium (medium equivalent concentration) when the volume (of the applied suspension) was reduced to that of an 80 nm section. This was done so as to allow a direct, if approximate, comparison to be made with the latter. The applied concentration ($C_{Applied}$) of AgNPs pipetted onto the grid was calculated using the following formula:

$$C_{Applied} = \frac{\pi \cdot r^2 \cdot d \cdot C_{MediumEquiv}}{V_{Applied}}$$

Where $V_{Applied}$ (m^3) = final volume, r (m) = grid radius, d (m) = section depth and $C_{MediumEquiv}$ (µM) = equivalent concentration in medium

Thus for $C_{MediumEquiv}$ = 100 μM,

$$C_{Applied} = \frac{\pi \times (0.5 \times 3.05 \times 10^{-3})^2 \times (80 \times 10^{-9}) \times (100 \times 10^{-6})}{(2 \times 10^{-9})}$$

$$= 4.59\ \mu M$$

Medium equivalent concentrations of AgNPs that were tested were 100 μM, 1 mM and 10 mM. A PVP control stock was made to 9.72 mg/ml, the equivalent concentration of PVP found in 10 mM AgNPs. 9.72 mg/ml, medium equivalent concentration, PVP only grids were prepared in the same manner as described above as were suspensions of diamond NPs having been diluted from the working stock concentration (10 mM) described above. These were tested at the same adjusted concentrations as the AgNPs. Samples were visualised using a FEI Tecnia12 Biotwin TEM with tungsten filament at an accelerating voltage of 100 kV. Although this visualisation method provided an indication of the particle density to be expected upon sectioning of spiked media, it is only approximate and certainly cannot be used to determine metal concentration. There are too many variables that cannot be adequately controlled for this to be reliable, including changes in particle distribution during drying, due to effects such as surface tension and NP agglomeration and the inhomogeneity of the suspension. Nonetheless, it provided some insight for judging the later medium based images.

Figure 20 shows TEM images of various medium equivalent concentrations of nanoparticles dispersed in water. Without X-ray microanalysis capabilities it is not possible to state with absolute certainly that the electron dense objects observed are AgNPs but the absence of these entities in the PVP only control (Figure 20H) suggests strongly that they are indeed AgNPs. Even at 10 mM (Figure 20A and B), there was no evidence of agglomeration with all apparent particles showing distinct boundaries from their neighbours, although there were areas of varying particle density that may have resulted from changes in surface tension during evaporation. Progressing down through the concentration range (Figure 20C - G) shows an increasing scarcity of particles within the field of view. At all concentrations, the apparent AgNPs always remained non-agglomerated. In some images particles that were clearly not nanoparticles also visible; these could be residual salts that crystallized out of the water during drying.

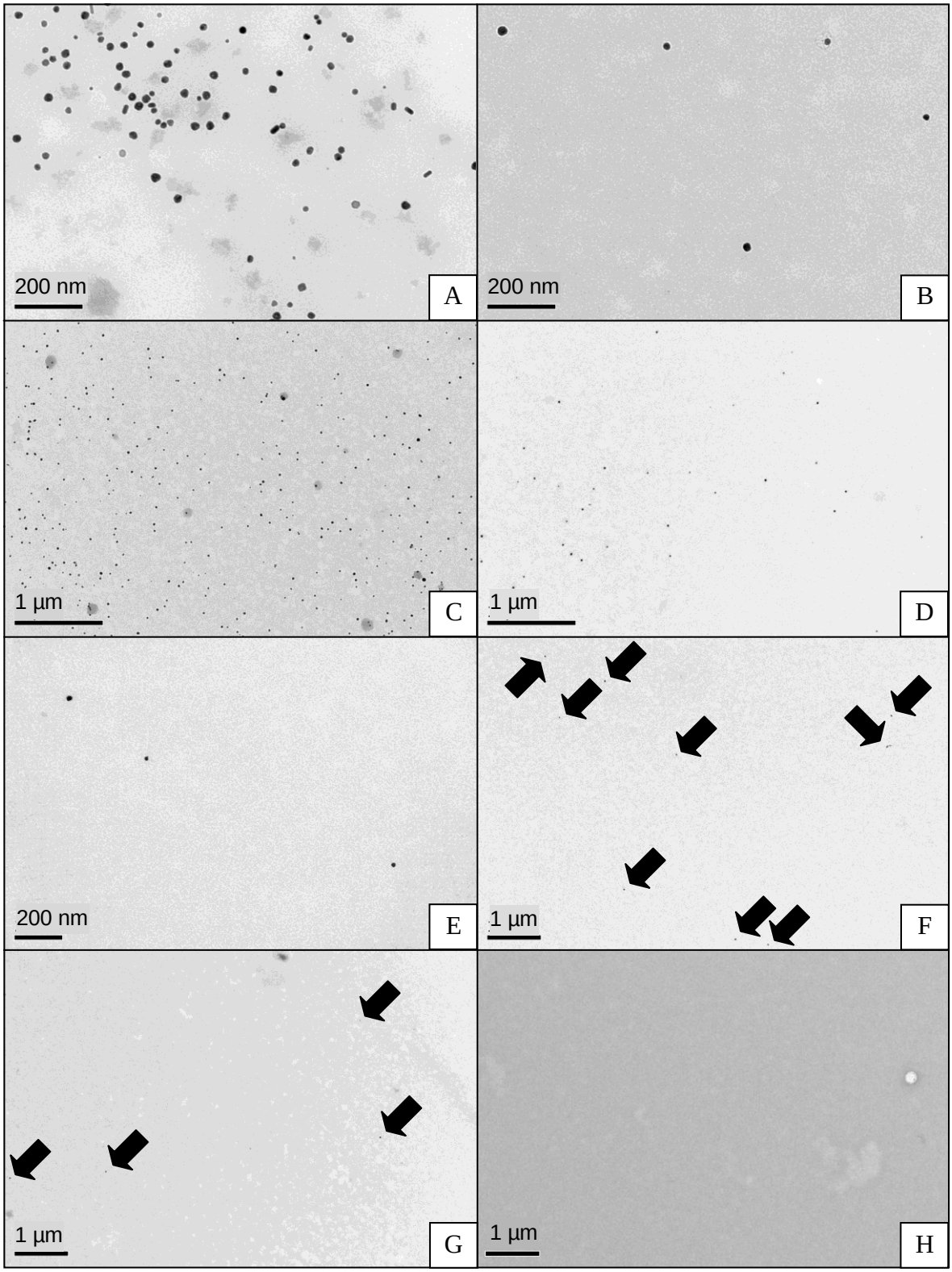

Figure 20. TEM images of commercially supplied 15nm AgNPs and PVP only in water (at food equivalent concentration particle densities). (A) and (B) 10 mM AgNPs at x 13000 magnification, (C) and (D) at x 4800 magnification and (E) 1 mM AgNPs at x 13000 magnification and (F) at x 2900 magnification, (G) at 100 µM particles were scarce (x 2900 magnification), and (H) PVP dispersant only control (No particles). All concentrations show good AgNP dispersal with particles of the correct primary size. Arrows highlight apparent particles where they are not immediately obvious.

The mean primary diameter of the apparent AgNPs was determined by measuring a total of 175 particles, randomly selected from five TEM grids, using ImageJ calibrated to the scale bar superimposed upon the image at the time of capture. The primary particle diameter was 19.4 nm (95% CI 18.5 – 20.3 nm) with a modal class of 19 – 20 nm. The distribution appears to be Gaussian, and a frequency histogram is shown in Figure 21 (n = 175).

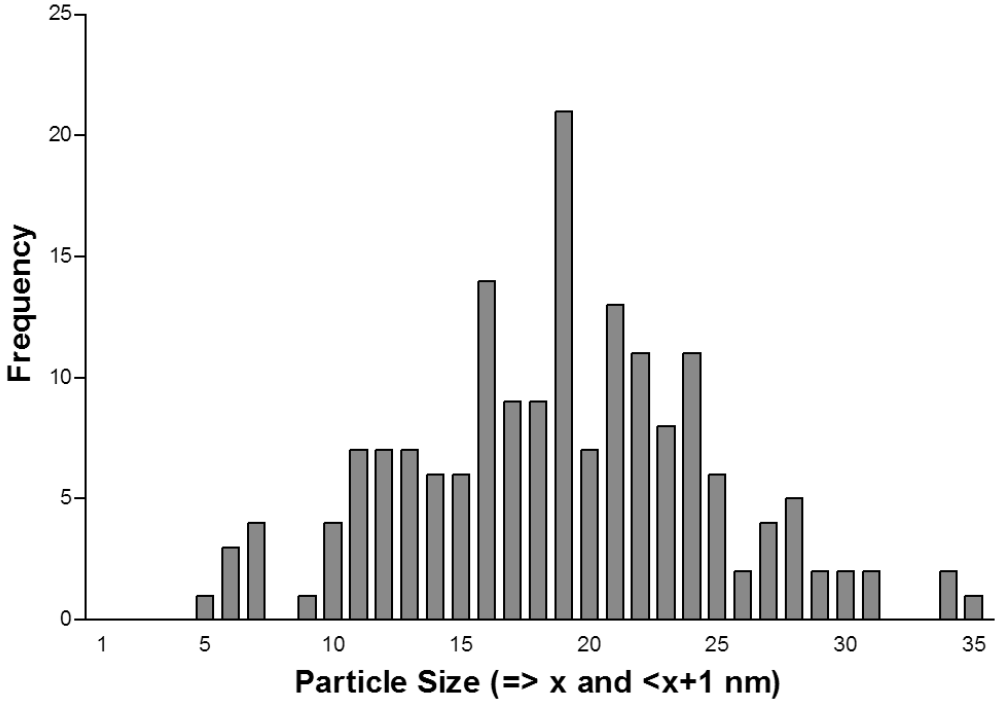

Figure 21. Frequency histogram of primary apparent AgNP sizes. The distribution was approximately normal and the mean particle diameter is 19.4 nm (95% CI = 18.5 – 20.3 nm). n = 175

As with AgNPs the agglomeration state of diamond nanoparticles (in the dried state) was established. With their very different surface chemistry, these behaved totally unlike the AgNPs. When diamond nanoparticles were examined at a concentration of 10 mM (medium equivalent concentration), particle agglomeration was widespread and some areas were devoid of any particles (Figure 22). At higher magnification these agglomerates consisted of many hundreds, possibly thousands, of diamond nanoparticles of approximately the nominal size range (<10 nm). At lower concentrations it was not possible to localise the nanoparticles within the image, presumably due to the scarcity of the aggregates and their correspondingly reduced size. As these particles were supplied in a stock solution and subsequently diluted to the working concentration there is no reason to expect that higher dilutions would exhibit less agglomeration under TEM. However, this

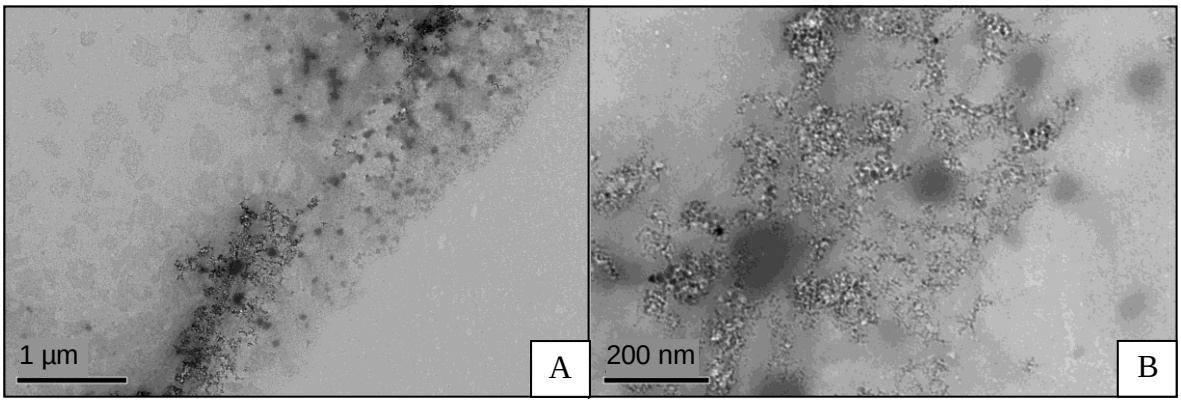

Figure 22. Transmission electron micrographs of diamond nanoparticles. Commercially supplied diamond nanoparticles (<10nm) at 10 mM (medium equivalent concentration) demonstrate a tendency to agglomerate. (A) x 4800. (B) At x 23000 the detail of the agglomerate can be seen. It contains many (100s, possibly 1000s) of particles, some very small.

is not an indication that the particles were necessarily agglomerated in suspension before the drying process for TEM induced any artefacts

4.2.2. Aging of silver nanoparticles at different pH

To determine the effects of time on the release of dialyzable Ag from AgNPs at different pH levels, AgNP suspensions (5 ml) were prepared at 100 µM in either HEPES buffer (pH 7.4) or acetate buffer (pH 4.5) (buffer concentration - 10 mM) and placed in 7 ml bijou tubes, in which a 2 mm hole had been drilled into the lid to allow free diffusion of air. HEPES buffer was prepared as a 100 mM stock solution by adding 11.92 g HEPES (basic) to 400 ml de-ionised H_2O and mixing thoroughly with a magnetic stirrer. Under constant stirring, the pH of the HEPES solution was titrated to down to 7.4 using HCl (37%). When the desired pH had been attained, the volume was increased to 490 ml, the pH confirmed and a final 10 ml of H_2O added. Sodium acetate buffer was prepared as a 100 mM stock by adding glacial acetic acid (2.862 ml; 3.00 g) to 400 ml of de-ionised water and mixing thoroughly. Under constant stirring, the pH was titrated up to 5.5 using NaOH (5 M in de-ionised water). At the correct pH, the volume was increased to a final value of 500 ml and the pH confirmed using the same method as with the HEPES. For each experiment 10 ml of buffer solution was diluted 1:10 and used (instead of de-ionised H_2O) to suspend AgNPs at 10 mM as described in Section 4.2.1. All further dilutions were made using the appropriate buffer. A serial dilution of the 10 mM working stock solution was made to produce 5 ml aliquots of 100 µM AgNP solutions in buffer (10 mM).

The tubes were placed in a humidity chamber and left to age for periods ranging from 1 to 28 days at 25°C. To confirm an oxidative mechanism, additional samples were prepared, supplemented with ascorbic acid (50 mM) and aged for 28 days. The humidity chamber was constructed from a 1 l plastic box suspended by tape inside a 5 l plastic box that was filled 3 cm from the bottom with water. The samples to be aged were placed in the inner box and secured with tape and the lid of the 5 l box, drilled with 12 x 3 mm holes, was secured. This chamber provided high humidity to prevent significant evaporation of the samples whilst still allowing circulation of air. The chamber was loosely covered with aluminium foil (SLS, UK) placed inside a large cardboard box placed in an incubator at 25 °C.

Samples of the desired age were removed from the humidity chamber and 1.5 ml of sample was dialyzed against 1.5 ml plain buffer (HEPES or acetate) using a 1.5 ml Fast Micro-Equilibrium dialyzer (Harvard Apparatus Ltd, UK) and a 5 kD molecular weight cut off (MWCO), equivalent to a pore diameter of 1.5 nm, cellulose acetate membrane (Harvard Apparatus Ltd, UK) for 4 hours at 25°C. Following dialysis, 1.3 ml of dialysate was removed from the chamber and placed in a 10 ml tube. Finally, 700 µl of HNO_3 (70%) and 8 ml of dH_2O were added to the tube before the contents were analysed by ICP-AES for Ag content.

The PVP capping agent coating the particles may represent potential sites for binding of Ag (I) ions rendering them non-dialyzable (Liu *et al*. 2010, Liu and Hurt 2010). To circumvent this difficulty and obtain a true concentration of Ag released from the particles (and not just that which is dialyzable), a standard addition curve was constructed permitting total Ag concentration (adsorbed/complexed and free) to be determined. To construct the standard addition curve, freshly suspended AgNPs (100 µM) (in both pH buffers) were spiked with a series of concentrations of Ag (I) ions, added as $AgNO_3$. The suspensions were immediately dialyzed as described above and analysed exactly as the experimental samples. The measured silver concentration from the spiked samples was plotted against the spike concentration and linear regression performed to obtain the relationship between the two. The point at which the curve met the x-axis corresponded to the true zero concentration (i.e. where the measured concentration would equal zero if a dialyzable-Ag free AgNP suspension were possible), and thus the amount it is below the nominal zero corresponded to the concentration of the totality of Ag (I) ions irrespective of

their adsorption. Furthermore, this plot allowed any concentration of measured Ag (I) to be converted to total Ag (I) relative to the nominal zero (or if added to the true value of the nominal zero, the absolute total quantity of dissolved Ag).

4.2.3. Effects of the transition metal ions, copper and iron, on silver nanoparticles and the influence of chloride ions and ascorbate

To determine the decline in AgNP concentration, hypothesised to be caused by dissolution, in response to addition of Cu and Fe, the optical extinction of an AgNP suspension was monitored using a spectrophotometer by following absorbance at 409 nm (peak extinction wavelength for these AgNPs; Section 4.2.1) with respect to time at 20 °C. Suspensions of AgNPs were made such that the addition of other reagents would yield a final concentration of 100 μM; 4.4 ml suspensions were prepared by adding 500 μl AgNPs (1 mM) suspension to 3900 μl de-ionised H_2O. Sodium chloride (NaCl), copper (II) nitrate ($Cu(NO_3)_2$), iron (III) nitrate ($Fe(NO_3)_3$) and ascorbic acid were made to stock concentrations of 2 M, 10 mM, 10 mM and 1 M respectively. Volumes of $Cu(NO_3)_2$ and $Fe(NO_3)_3$, with and without NaCl, were added to a 1.5 ml semi-micro cuvette (Fisher Scientific UK Ltd, UK) necessary to create the correct concentrations of metal (ranging from 50 – 200 μM) and Cl^- (100 mM) for a 1 ml solution; the volume of these reagents was increased with de-ionised water, as necessary, to 120 μl. For example, target concentrations of 200 μM for Cu^{2+} and 50 mM for Cl^- required $Cu(NO_3)_2$ (20 μl), NaCl (25 μl) and H_2O (75 μl). The cuvette was next placed in the spectrophotometer and 880 μl of AgNP suspension was pipetted into the cuvette, the resulting mixture pipetted up and down once, and the lid of the spectrophotometer closed rapidly. It was possible to perform this operation within 10-12 s and thus the first reading was made at 15 s. Absorbance readings were then taken at 30 s and at successive 30 s intervals until no further appreciable change in absorbance was seen. Some experiments were repeated with the addition of 50 mM ascorbic acid by adding 50 μl ascorbic acid solution to the 880 μl of AgNPs before the final mixing in the spectrophotometer. An $AgNO_3$ only control was performed to establish the optical extinction of dissolved Ag at 409 nm. Silver nitrate is capable of oxidising PVP over a period of approximately one week (Hoppe *et al*. 2006) but cannot cause a net oxidation of zero-valent Ag. $AgNO_3$ was tested for its potential to affect the optical extinction of AgNPs and to determine if the PVP capping agent was involved in the reduction of optical extinction of AgNPs. The hydrolysing nature of Cu

and Fe ions inevitably affects the pH of the resultant solution. To ensure that the factor mediating any observed effects was not pH a control was performed at pH 3.5; this is just below the measured pH value for 200 μM of Fe (III) ions. Likewise, a test with 100 mM NaCl only, added to AgNPs was performed to control for the effects of ionic strength.

4.2.4. Media preparation

Silver-containing test medium used throughout was spiked with either AgNPs or $AgNO_3$. AgNPs were made freshly to a 10 mM stock for each experiment whereas $AgNO_3$ was prepared as a 1 M stock and kept in the dark. To control for the effects of the capping agent of the NPs and the nitrate anion, PVP and $NaNO_3$ controls were used. PVP (molecular weight: 40 000) (Sigma-Aldrich, UK) was made, freshly when needed, as a stock of 9.72 mg/ml, the equivalent concentration that would be found in the 10 mM (by metal content) AgNP suspension, and $NaNO_3$ was prepared at a 1 M stock solution. Silver nitrate, AgNP, $NaNO_3$ and PVP spiked medium were prepared by adding the appropriate volume of stock solution to 84.9 g (90 ml) melted *Drosophila* medium to yield the correct concentrations at 100 ml and stirring briskly until mixed; de-ionised water was added to make up the volume to 100 ml.

To examine the dispersion of AgNPs suspended in *Drosophila* medium, 2-3 mm cubes of *Drosophila* medium, spiked with AgNPs at 100 μM, 1 mM or 10 mM, were fixed with gluteraldehyde (2%) in 0.1 M sodium cacodylate buffer (pH 7.2) for two hours, followed by a wash in the cacodylate buffer. The material was then dehydrated through a graded ethanol series of 25%, 50%, 70%, 80%, 90%, and 100% for 10 minutes each, with 100% repeated 3 times. The dehydrated samples were placed in LR White resin / absolute ethanol mixes of ratios 1:3, 1:1, 3:1 for a minimum of 1 hour and up to 4 hours followed by 100% LR White over the course of a day. Finally, the samples were embedded in gelatine capsules and polymerised at 40 °C for 5 days.

Eighty nanometre sections were cut using a Riechart Jung Ultracut E microtome and placed on formvar coated TEM grids. Samples were visualised using a FEI Tecnia12 Biotwin TEM with tungsten filament at an accelerating voltage of 100 kV.

4.2.5. Gustatory assay

To test the behavioural response to the taste of Ag salts and AgNPs, the gustatory assay was performed as described in Section 2.6. Plain agar plates were made containing $AgNO_3$ or AgNPs at concentrations ranging from 100 to 500 μM. These were produced using a stock concentration of 10 mM for AgNPs and 1 M for $AgNO_3$. To ascertain the effect of Cl^- ions upon the gustatory response, additional $AgNO_3$ plates were made but, immediately before pouring, an equimolar concentration of NaCl was added, the mixture stirred briskly and poured.

4.2.6. Administration of silver nitrate and silver nanoparticles and tissue digestion for determining whole body tissue concentrations

To determine the how much Ag was taken up from the diet relative to the concentration in the standard *Drosophila* medium, how this was affected by aging of the medium, and this interacted with Cu status, *Drosophila* larvae were exposed to $AgNO_3$, AgNPs or $NaNO_3$ at concentrations of 10 μM, 50 μM, and 100 μM or PVP - the equivalent concentration for each concentration of AgNPs as described in Section 4.2.4. Additional groups at 200 μM for AgNPs and the equivalent PVP concentration were also performed; the toxicity of $AgNO_3$ precluded its use at this concentration. To ascertain whether aging contributed to the amount of Ag taken up, an identical set of experimental bottles were prepared and aged, in the dark, for 14 days before eggs were added. Nonetheless, all exposures were performed simultaneously. For each experimental group, five sets of 250 eggs were collected and placed into bottles spiked with the appropriate concentration of test substance. These were incubated and flies collected, purged immediately after eclosion, killed and dried as described in Section 2.8.1. In addition to the experimental groups, to determine Ag recovery, five replicates of unexposed dried flies, supplemented with 200 $\mu g_{Ag}/g_{DryTissue}$ before digestion, were prepared along with five replicates of certified reference material

To prevent the precipitation of Ag in the presence of trace amounts of Cl^- ions during digestion, aqua regia was used for all Ag digests (Daskalakis *et al*. 1997). Micro-centrifuge tubes containing desiccated flies were preheated to 70 °C in a dry bath heating block and 125 μl ultrapure HNO_3 (~69%) was added immediately, followed by 375 μl ultrapure HCl (~37%). As the temperature of the solution increased it began to foam and a

scum formed on the surface. To prevent overflow the scum was pierced and the foam pushed back down the tube using an acid washed pipette tip (Star Labs, UK). Once the solution had settled it was left to evaporate to dryness over ~6 hours and the residual material, consisting largely of lipids, was dissolved/re-suspended in ultrapure ethanol (1 ml 70% in dH_2O) at 70 °C by vortexing. The resulting liquid was centrifuged at 13000 RPM for 5 minutes before evaporation to dryness overnight at 70°C. The now concentrated residue was re-digested using the aqua regia process described above. The entire cycle was repeated five times, using less ethanol for solubilisation as required in each cycle. When the residue had disappeared a final aliquot of aqua regia was added at room temperature and left to settle for 1 week. Finally the sample was vortexed to dissolve any Ag that may have been adsorbed to the tube wall and briefly centrifuged before being diluted to 10 ml for ICP-AES analysis.

In addition to experimental digests, five replicates of reference material, DOLT-4 (Dogfish Liver Certified Reference Material for Trace Metals; National Research Council Canada) containing 0.93 $\mu g_{Ag}/g_{DryTissue}$, and control *Drosophila* spiked with $AgNO_3$ or AgNPs to a concentration of 200 $\mu g_{Ag}/g_{DryTissue}$ were digested to assess recovery. The Ag concentration of the reference material was very low and the results of ICP-AES were below detection. However, recovery from spiked *Drosophila* was 159.6 (+/- 39.5: 95% CI) $\mu g_{Ag}/g_{DryTissue}$ (~80%) for $AgNO_3$ and 164.5 (+/- 31.5: 95% CI) $\mu g_{Ag}/g_{DryTissue}$ (~82%). As the *Drosophila* digest is the actual matrix of the samples in this assay, the recovery from these spiked samples is likely to be most representative of the experimental data. Despite this recovery not being ideal, it was sufficient to allow comparison with control. More importantly, however, no significant difference was seen between recovery for $AgNO_3$ and AgNPs ($p = 0.8247$; unpaired *t*-test), meaning that tissue concentrations of Ag could be reliably compared between $AgNO_3$ and AgNPs. The recovered concentration of Cu from the reference material was 28.3 (+/- 8.5: 95% CI) $\mu g_{Cu}/g_{DryTissue}$, which was not significantly different ($p = 0.2790$; one sample *t*-test) from value stated for the reference material (31.2 $\mu g_{Cu}/g_{DryTissue}$).

4.3. Results

4.3.1. Effects of age and pH upon dissolution of aqueous silver nanoparticles

To examine how AgNPs dissolve with age, the formation of dialyzable Ag from PVP-capped AgNPs was monitored over time at pH 4.5 and 7.4. A standard addition plot was produced for each pH, to assist in distinguishing between Ag that was dialyzable and the total amount that had been released from the AgNPs. The standard addition plots for both pH 7.4 and 4.5 are shown in Figure 23. Neither relationship showed significant deviation from linearity ($p > 0.1$; runs test). Extrapolation back to the x intercept yielded estimates of the initial ionic Ag concentrations in the AgNP suspensions of 3.1 µM and 6.2 µM for pH 7.4 and 4.5 respectively.

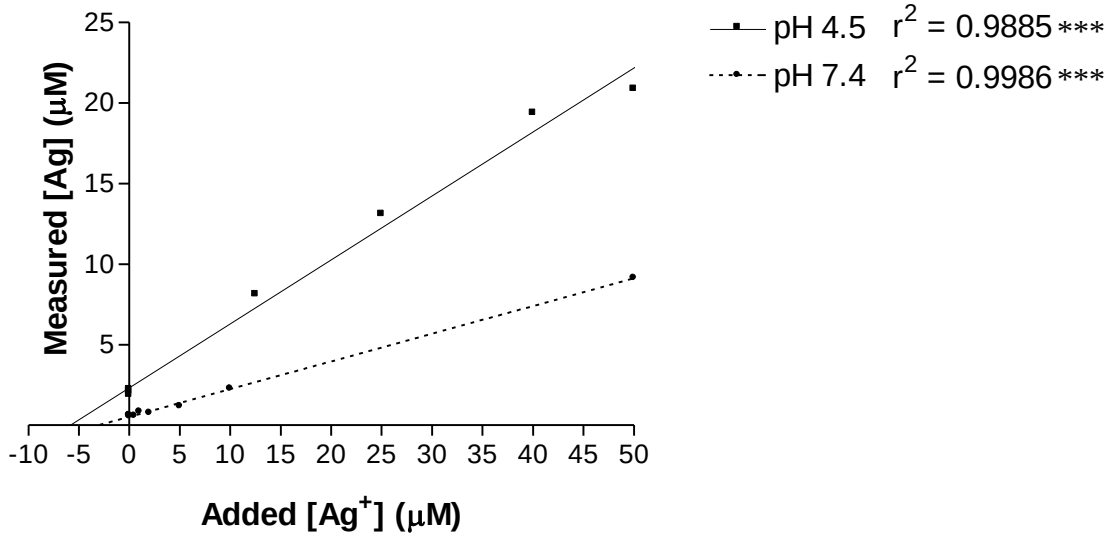

Figure 23. Standard addition plots of dialyzed, $AgNO_3$ (0 – 50 µM) spiked, AgNP (100 µM) suspensions. Linear regression lines are shown for pH 4.5 and pH 7.4 and extrapolated to the x axis intercept. Key to significance values: *** $p < 0.001$

Using the standard addition plots, the raw data obtained from dialyzing aged AgNP solutions were converted to concentrations relative to the initial suspension (Figure 24). As the starting concentration of AgNPs was 100 µM, all reported values for dialyzable Ag are interchangeable with percentages. At pH 7.4, there was no significant difference, relative to day 0, for any day up to, and including, day 11 (Tukey-Kramer test). Beyond day 11, all time-points had concentrations of dialyzable Ag that were significantly different from day 0 ($p < 0.05$). From around day 20, a plateau was reached; 28 day aged AgNP was not significantly different from 21 day aged suspension. The values for the pH 4.5 aged AgNPs were consistently lower than for pH 7.4. Significant differences compared to

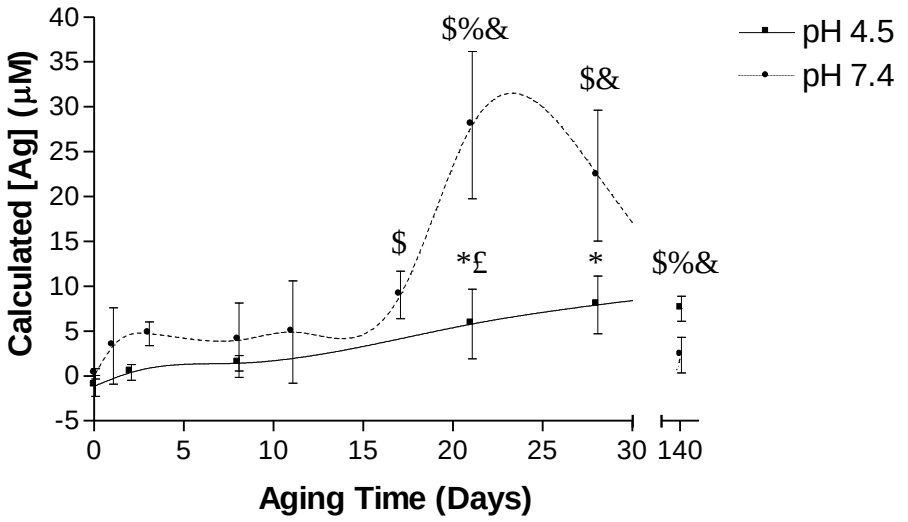

Figure 24. Silver concentrations recovered from AgNPs (100 µM) with time at pH 4.5 and pH 7.4. Data adjusted using the standard addition curve in Figure 23 intended to show total released Ag relative to day 0. Error bars represent +/- 95% CI. n = 3. A cubic spline fit curve has been superimposed upon data to show trend. Key to significance values: pH 4.5: * $p < 0.05$ relative to day 0, £ $p < 0.05$ relative to previous time point; pH 7.4: $ $p < 0.05$ relative to day 0, % $p < 0.05$ relative to previous time-point (all Tukey-Kramer test); & p 0.05 relative to pH 4.5 (*t*-test with Welch's correction for unequal variance where needed).

day 0 AgNPs were consistently lower than for pH 7.4. Significant differences compared to day 0 were seen at 21 days ($p < 0.05$) and above whereas at 8 days and below no significant differences were found (Tukey-Kramer test). At this pH, time-points between 8 and 21 days were not measured due to time constraints.

A subset of samples was left for extended aging for 5 months (140 days). After this time, at pH 7.4, significantly less ($p < 0.05$) Ag was found in the dialysate compared to any time point from 17 days onward. Other than this no significant differences between day 140 and any other time point, including day 0, were found. There was no significant difference between the concentrations of dialyzable Ag at day 140 relative to day 28 when aged at pH 4.5. At all time points where testing was possible (8, 21, 28, and 140 days), recovered Ag concentrations for pH 4.5 were significantly different to the same time point aged at pH 7.4 ($p < 0.05$).

To confirm that the process responsible for the formation of dialyzable Ag was oxidative dissolution, a set of samples was aged in the presence of the anti-oxidant, ascorbate. Silver nanoparticles aged for 28 days, at pH 7.4 in the presence of 50 mM ascorbate, yielded an

Ag (I) concentration that was below the detection limit of ICP-AES. This concentration was thus lower than fresh AgNP solution that had not been aged.

4.3.2. Effects of transition metal ions, copper and iron, on silver nanoparticles and the influence of chloride ions

Figure 25 and Figure 26 show the results of experiments designed to investigate the dissolution of AgNPs by the hypothesised oxidants, Cu (II) and Fe (III) ions, and how Cl^- ions affected this. Dissolution of AgNPs was followed as the loss of optical extinction of an AgNP suspension (100 μM) at λ = 409 nm (peak absorbance; see section 4.2.1). When added to AgNPs (100 μM), Cu (II) ions at 200 μM caused a slight reduction of the absorbance ($p < 0.05$) although there was no evidence of this changing with time (Figure 25A). To ensure that changes in absorbance were not due to alterations in ionic strength, Cl^- ions at 100 mM were also tested; these too, showed a slight reduction ($p < 0.05$) of absorbance that subsequently rose, within 60 s to a level just below the AgNP suspension without any additional ions, and remained unchanged for the remainder of the 240 s period (Figure 25A). Silver nitrate (100 μM) added to AgNPs (100 μM) showed no change relative to AgNPs alone either throughout the 5 minute experiment or after two weeks incubation. In the absence of AgNPs, the absorbance of $AgNO_3$ was almost zero. As the Cu and Fe ions reduced solution pH, a control was run at pH 3.5, just below the lowest pH value for any concentration of metal. No difference from the samples without additional ions was noted at any time point ($p < 0.05$) (Figure 25A).

The combination of 50 mM Cl^- ions with Cu (II) ions caused a rapid and complete disappearance of the absorbance caused by AgNPs, by 200 s. As the reaction was so rapid, lower concentrations of Cl^- ions were tested, and 20 mM Cl^- also resulted in a reduction in absorbance. At this concentration, the loss of absorbance with time was considerably reduced, but the reaction was still proceeding when the experiment was terminated at 480 s. Two millimolar Cl^- ions plus 200 μM Cu (II) ions added to AgNPs (100 μM) caused no change in absorbance over the course of the experiment (Figure 25B). The absorbance of AgNP suspensions following addition of Cu (II) ions (200 μM) at each chloride concentration (2, 20, 100 mM) was significantly different ($p < 0.05$) from the other two chloride concentrations at each time point where comparison was possible (some experiments were terminated early due to absorbance falling to zero). For comparison with

the additional Cl^- concentrations, further assays were performed using lower concentrations of Cu (II). Copper (II) ions (100 µM) reduced the absorbance of 100 µM AgNPs almost to zero in the presence of 50 mM Cl^- within 500 s, but reduction of the Cl^- ion concentration to 20 mM reduced the fall in absorbance ($p < 0.05$); the final absorbance at this concentration is difficult to estimate as despite the curve appearing linear, all other curves display an apparent exponential profile (Figure 25C). Lowering of Cu (II) ion concentration to 50 µM slowed the rate of absorbance loss even at Cl^- concentrations of 100 mM. However, 50 µM Cu (II) with 50 mM Cl^- still produced an absorbance drop that exceeded that seen for 100 µM Cu (II) plus 20 mM ($p < 0.05$).

Iron (III) ions reduced the absorbance of AgNPs very rapidly and the reaction was virtually complete within 15 s. The absorbance at which the curve became asymptotic was dependent upon the initial Fe (III) concentration. From the position and shape of the curve, 50 µM Fe (III) appeared to be the minimum concentration required to cause the loss of absorbance of AgNP suspensions (Figure 26A). Addition of Cl^- ions to the reaction mixture dramatically accelerated the loss of absorbance and lowered the point of asymptote (Figure 26B). With additional Cl^- (50 mM), 200 µM Fe (III) caused an almost total loss of absorbance within 15 s. Lowering the Cl^- concentration to 2 mM caused only a slight lag with the reaction complete at 90 s. Lower concentrations of Fe (III), in combination with Cl^-, caused an initially swift reduction in absorbance that plateaued before falling to 0.

To confirm that the loss of absorbance seen following the addition of oxidants was oxidation and not a different phenomenon such as agglomeration and precipitation, which may be seen in the presence of the reactants but the absence of an oxidative mechanism, the highest concentrations of each combination were tested in the presence of ascorbic acid (50 mM). Ascorbate virtually eliminated the loss of absorbance in both the Fe (III) only and Cu (II) plus Cl^- reactions although minor, but statistically significant, differences were seen at some time points ($p < 0.05$). Ascorbate almost prevented oxidation following the addition of Fe (III) and Cl^- (Figure 26C) producing a small ($p < 0.05$) reduction in absorbance at all times points.

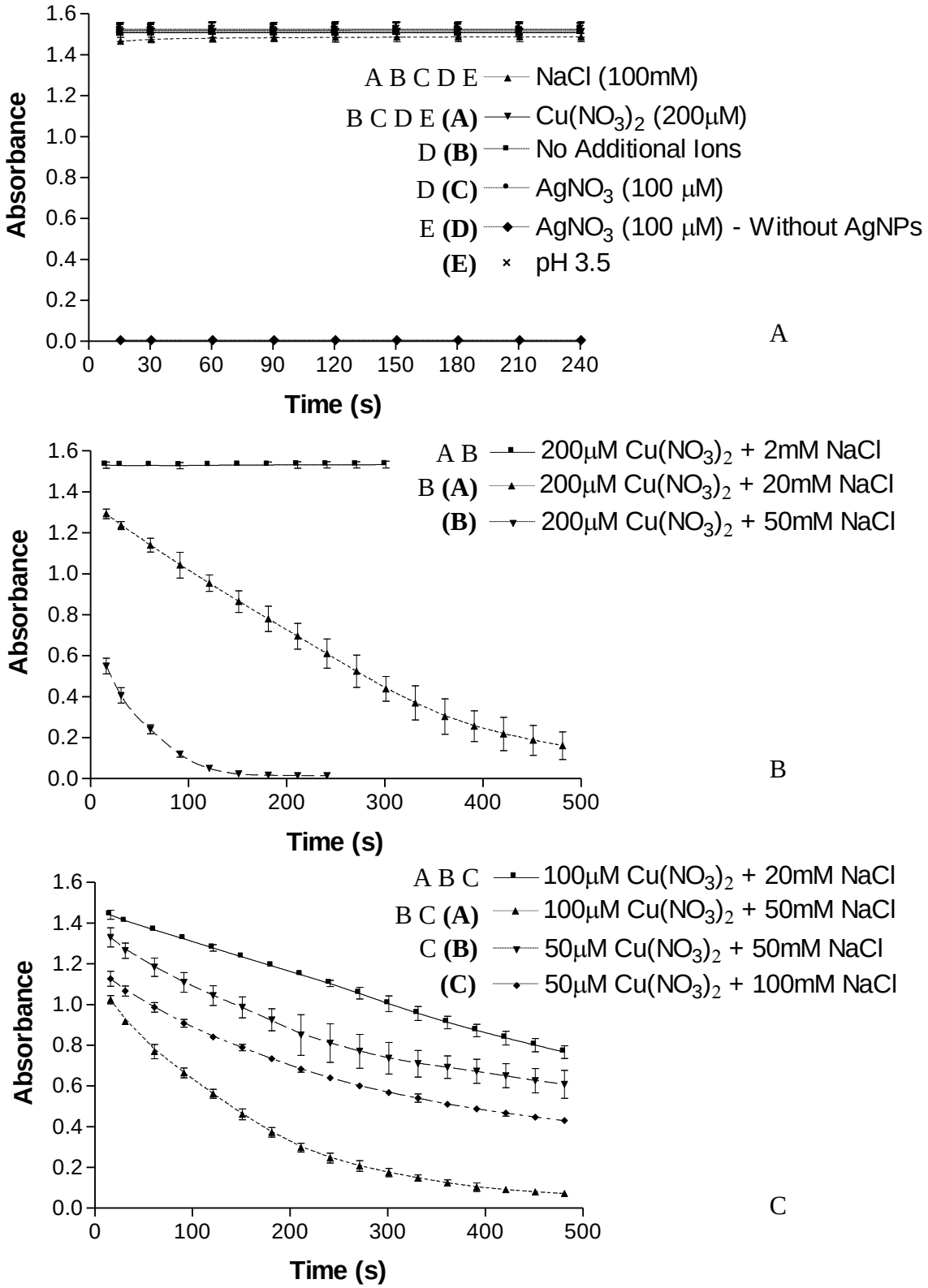

Figure 25. Copper (II) ion induced loss of AgNP absorbance. Absorbance of AgNPs (100 μM) at λ = 409 nm followed over time following addition of (A) Cl^- ions or Cu (II) ions individually, (B) Cu (II) ions (200 μM) plus various concentrations of Cl^- and (C) Cu (II) ions at 100 μM or 50 μM at various concentrations of Cl^-. Error bars represent +/- 95% CI interval. Each curve consists of 3 replicates followed over time. Key to significance values: A, B, C, D, and E $p < 0.05$ relative to the formulation with that letter displayed in brackets from that panel (Tukey-Kramer test); post-hoc tests for ANOVA factor: time are not shown for clarity.

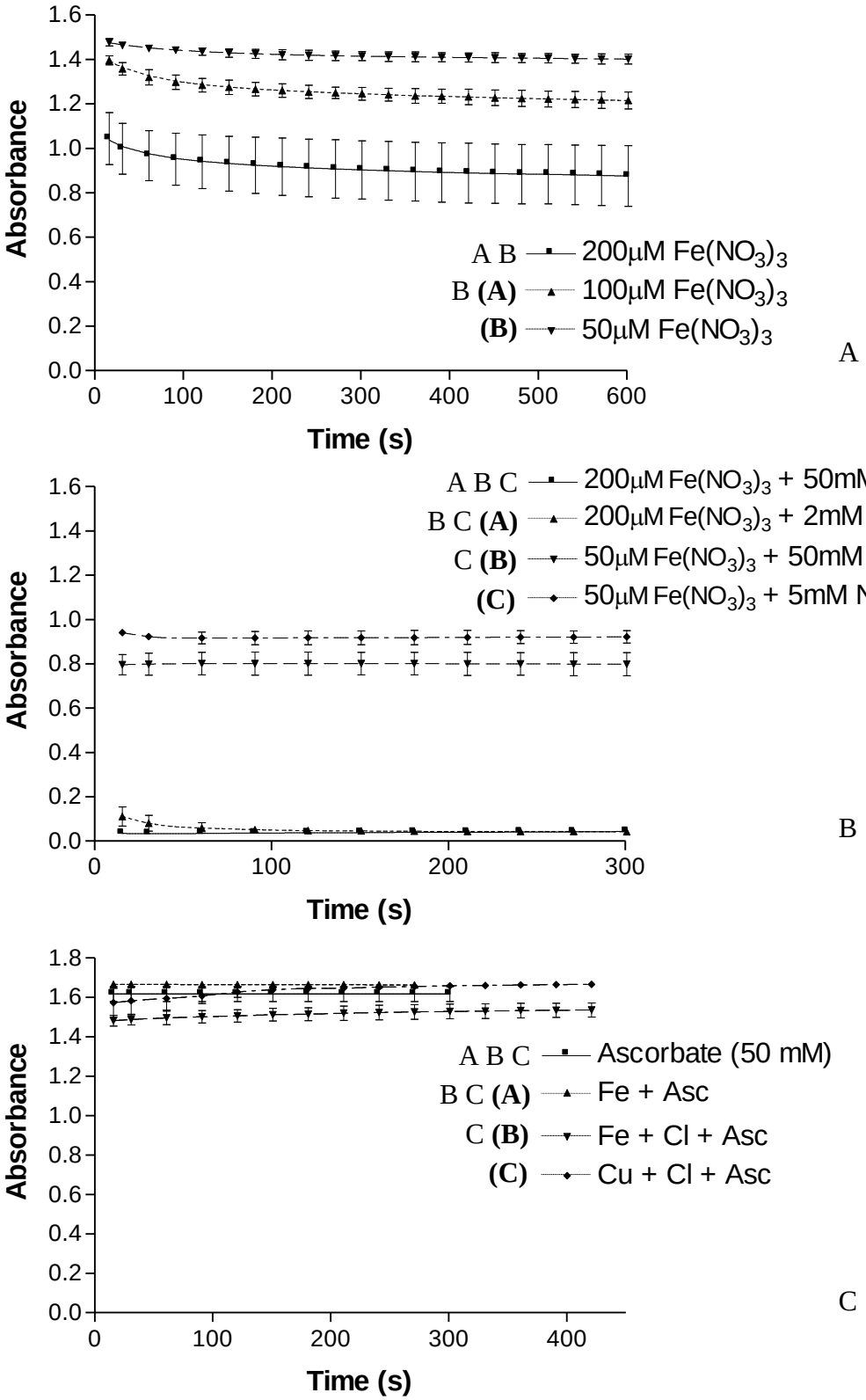

Figure 26 Ion mediated loss AgNP absorbance and inhibition by ascorbate. Absorbance of AgNPs (100 μM) followed over time following addition of (A) Fe (III) ions at various concentrations, (B) Fe (III) ions at 200 μM and 50 μM plus various concentrations of Cl^- and (C) Fe (III) and Cu (II) ions at 200 μM with Cl^- at 50 mM plus 50 mM ascorbate. Error bars represent +/- 95% CI interval. Each curve consists of 3 replicates followed over time. Key to significance values: A, B, and C $p < 0.05$ relative to the formulation with that letter displayed in brackets from that panel (Tukey-Kramer test); post-hoc tests for ANOVA factor: time are not shown for clarity.

4.3.3. Gustatory responses of *Drosophila* larvae to silver

Mass plate assays were performed to ascertain whether the taste of silver would elicit a behavioural response from *Drosophila* larvae. Figure 27A and B show that the aversive positive controls were significantly repellent relative to the no tastant control ($p < 0.05$). Sodium nitrate was not significantly different from control, but $AgNO_3$ was significantly repellent ($p < 0.05$) at both concentrations tested (Figure 27A). Silver chloride was significantly different from control ($p < 0.05$) at 100 µM but not 500 µM. AgNPs did not produce a response significantly different from control at any concentration (Tukey-Kramer test) (Figure 27B).

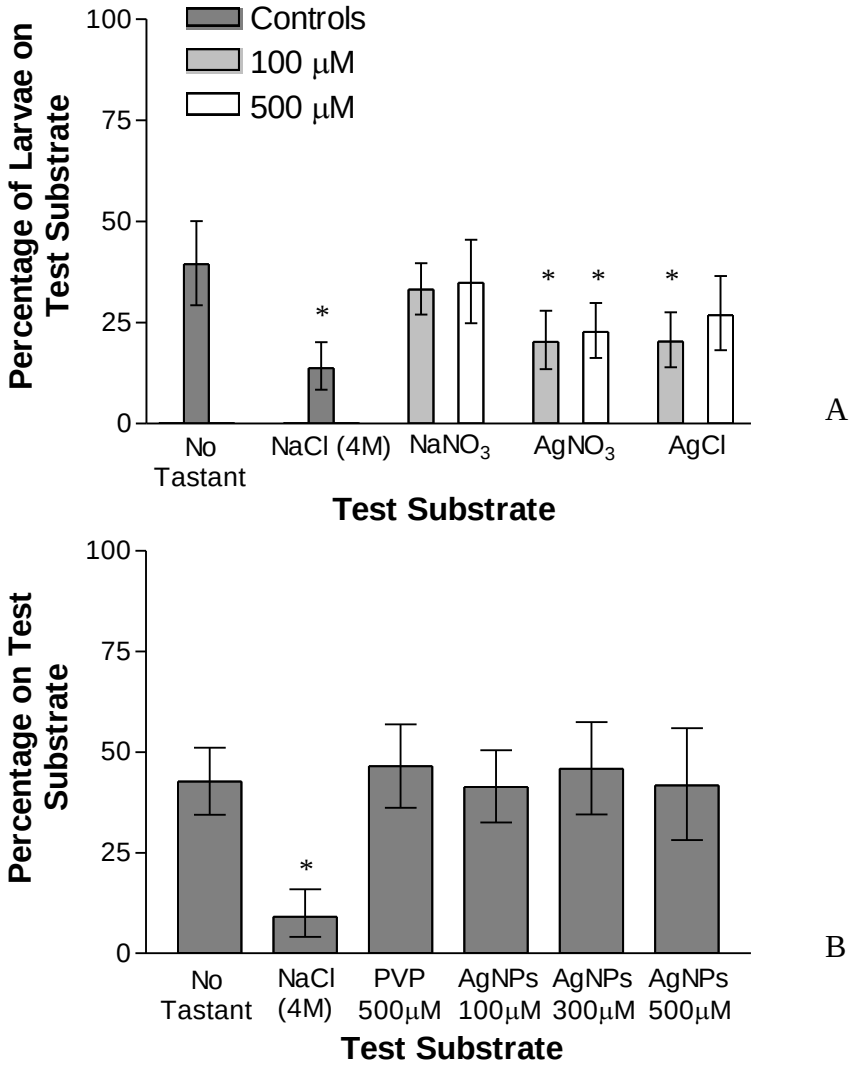

Figure 27. Gustatory responses of *Drosophila* to Ag. Mean percentage of larvae (+/- 95% confidence interval) on (A) ionic Ag test substrates and (B) AgNP test substrates after 5 minutes. n = 8 plates (~40 larvae/plate) all groups. Key to statistical significance: * $p < 0.05$ relative to no tastant control in that panel (Tukey-Kramer test)

4.3.4. TEM images of silver nanoparticles dispersed in fly food medium

To examine the behaviour of AgNPs in *Drosophila* medium, sections of medium were examined using TEM. The food medium appeared heterogeneous and husks of yeast cells were the only clearly identifiable components (Figure 28A, B and D). Control media (no additive and PVP only) sections contained some near spherical entities and small aggregates that were morphologically similar to NPs (Figure 28A - D). These unidentified bodies could make it difficult to identify an AgNP with certainty. However, they were rare; it is only at a concentration of 100 µM AgNPs that they may have occurred at a similar abundance to the genuine particles.

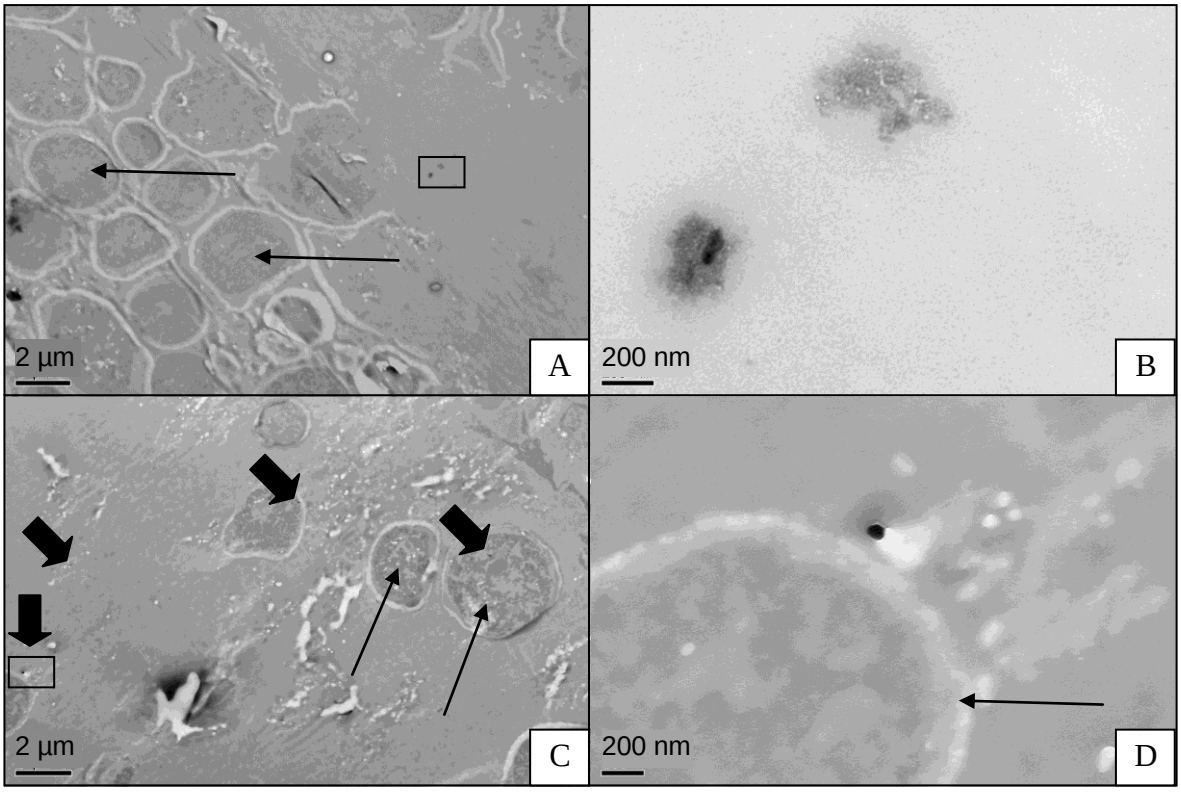

Figure 28. Transmission electron micrographs of control *Drosophila* medium. (A) A small electron dense region can be seen (boxed area) in control medium that has the appearance of a NP. (B) An enlargement of the boxed area in (A). At this magnification the particle bears less resemblance to a NP. (C) Small electron dense regions (thick arrows) resembling NPs can also be seen in medium supplemented with PVP. (D) An enlargement of the boxed area in (C). At this magnification this unidentified particle still has the appearance of a NP but is larger than would be expected for a non-agglomerated AgNP. Examples of yeast cells are marked with fine arrows.

Figure 29A and B shows food medium sections containing the highest concentration of AgNPs (10 mM) and reveals a rather different picture to that seen when the particles were dispersed in water (Section 4.2.1). As X-ray energy dispersive spectroscopy was not

available, it was impossible to be certain that all electron dense regions were AgNPs (or indeed particles at all) and all references to AgNPs and particles thus signify *apparent* AgNPs. There was no obvious sign of agglomeration, but the apparent particles appeared clustered into groups scattered throughout the medium (Figure 29A). Nonetheless, the apparent particles were definitely separate from one another (Figure 29B); a few, solitary, non-clustered particles were seen but these were in the minority.

The lower AgNP concentrations of 1 mM, 0.5 mM and 100 µM (Figure 29C - H) showed a distribution that was similar to neither the aqueous dispersion nor the 10 mM AgNPs in food medium. There was some evidence of agglomeration as particles were definitely larger that the diameter of the primary particles (Section 4.2.1). However, many of the presumed agglomerates had a circular shape in cross section yielding the appearance of a single, non-aggregated but larger nanoparticle. Other particles with irregular edges were clearly agglomerates. An additional characteristic exhibited by these lower concentrations (particularly 1 mM and 0.5 mM) was the tendency for the nanoparticles (and their agglomerates) to localise adjacent to the yeast cells, especially around the inner edge of the cell wall (Figure 29D and E).

4.3.5. Body loads of silver and copper following administration of silver nitrate or silver nanoparticles

To determine how the form of Ag influences uptake of Ag and tissue concentrations of Cu and whether aging of the medium affected these parameters, *Drosophila* were fed, for the entire larval period, on media containing either $AgNO_3$ and AgNPs that were either new (i.e. non-aged) or had been aged for 14 days; whole body tissue concentrations were determined in the adult. It was noted in this experiment that qualitatively similar changes to the colour of AgNP spiked medium supplemented with Cu (II) ions (See section 4.1) occurred without any additional ions over a longer period (three to four days) (Figure 30). Figure 31A shows that neither $NaNO_3$ nor PVP, when administered at a concentration equivalent to 100 µM of its respective Ag counterpart, showed a significant difference from absolute control with respect to Ag tissue concentration ($p = 0.9422$; ANOVA) or Cu tissue concentration ($p = 0.0889$; ANOVA). Upon exposure to Ag, concentration-dependent increases in whole body tissue concentrations of the metal were seen. Although shown on the graph in Figure 31B, AgNPs (200 µM) were not included in the ANOVA analysis that followed as the impossibility of including $AgNO_3$ at this concentration would

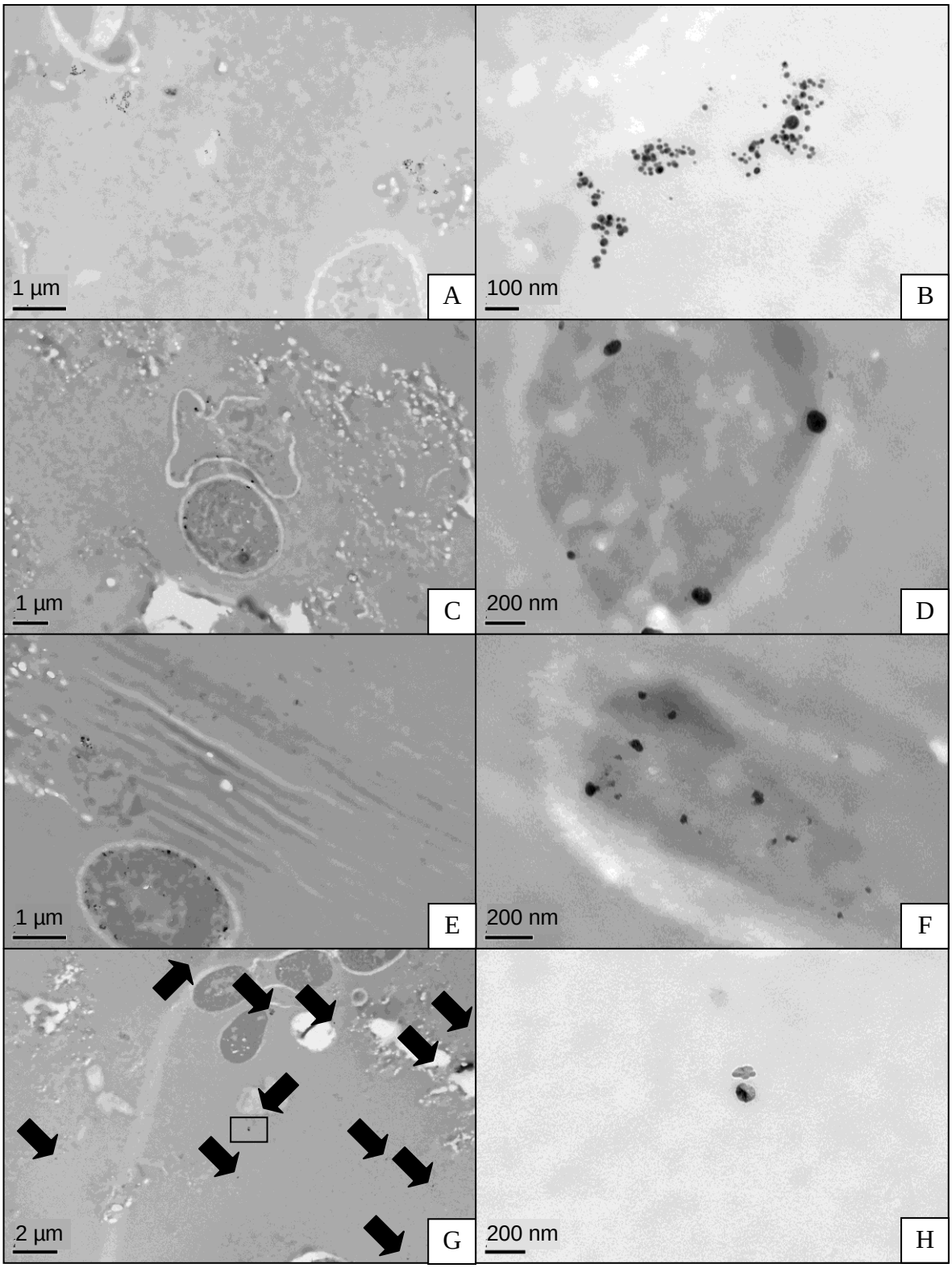

Figure 29. Transmission electron micrographs of *Drosophila* medium supplemented with AgNPs. Without X-ray energy dispersive spectroscopy, it is not possible to confirm particles are AgNPs and all references to such must be considered apparent AgNPs. (A) 10 mM AgNPs showed clustering but not agglomeration. (B) Cluster of 10 mM AgNPs showing individual particles. (C) 1 mM AgNPs were associated with a yeast cell and (D) when magnified were larger than expected. (E) 0.5 mM AgNPs also tended to associate with yeast cells and (F) when magnified seemed agglomerated. (G) 100 µM AgNPs (indicated with arrows) appeared dispersed but (H) Enlargement of the box in (G) showing individual particles were often actually agglomerates.

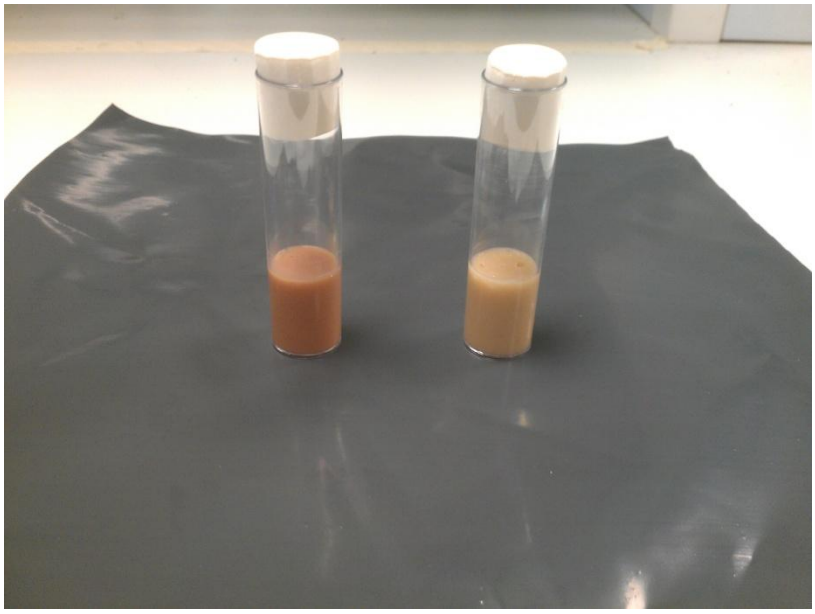

Figure 30. Colour of *Drosophila* medium supplemented with AgNPs. (Left) immediately after gelling of medium and (Right) after aging in the dark for four days

have led to missing cells and difficulty in determining interactions. Figure 31B shows that $AgNO_3$ and AgNP exposure resulted in similar tissue levels of Ag ($p = 0.1804$; factor: Ag type. 3-way ANOVA). However, both exposure concentration and medium age contributed to the tissue levels, each influencing the effect of the other ($p = 0.0031$; interaction term: Concentration, Age – 3-way ANOVA).

Although not significantly different, there was some evidence of raised Ag tissue concentration, relative to their respective controls, for 10 µM exposure groups, with the highest mean value belonging to non-aged AgNPs with $[Ag]_{DryMass} = 33.9$ µg/g (95% CI of 12.79 – 55.0 µg/g). When the exposure concentration was increased to 50 µM Ag (both forms are considered together due to the non-significant effect of Ag type) significant tissue concentrations, relative to control, were seen across both the aged and non-aged groups ($p < 0.05$). Likewise there was a significant increase in tissue concentration relative to control following exposure to 100 µM across both medium-age groups. However, the increase relative to control for the non-aged group was smaller than that of the aged group. Moreover, whilst there was no significant difference between the tissue concentrations of Ag in the 100 µM non-aged group versus the 50 µM non-aged group ($p > 0.05$; Tukey-Kramer test), a difference was found between the respective aged groups ($p < 0.05$). No significant difference in tissue Ag concentration ($p > 0.05$; student's *t*-test) was seen between the aged and non-aged AgNPs (200 µM) exposed groups.

Copper levels were measured in the same Ag exposed animals. No significant differences were found across any treatment group, regardless of the form of Ag, its age or the exposure concentration ($p > 0.05$; 3 – way ANOVA, all main effects (Ag type, Ag age and Ag concentrations [0 – 100µM]) and interactions) (Figure 31C). As with Ag tissue concentrations, the AgNP (200 µM) exposed groups were analysed separately; no significant difference in Cu levels was found between the aged and non-aged AgNP exposed *Drosophila* ($p > 0.05$; student's *t*-test).

4.4. Discussion

The mean primary diameter of the AgNPs was 19.4 nm, close to the nominal value. Likewise the Ag content was consistent with the stated proportions of metal to dispersant. No evidence of agglomeration of AgNPs was seen in water or fly food. However, oxidation by atmospheric O_2 was suggested in aged particles, which appeared more extensive at neutral pH. This was coupled with a clear pH dependent effect suggested to represent Ag binding to the PVP capping agent. A rapid loss of optical absorbance was seen when Cu and Fe were added to AgNP suspensions in combination with Cl^- which is interpreted as oxidative dissolution. Silver was found in the tissues of whole adult flies following $AgNO_3$ or AgNPs exposure in a concentration-dependent manner although no differences were seen between the forms of the metal despite some behavioural repellence seen in gustatory assay of $AgNO_3$.

4.4.1. Experimental limitations

The primary limitation with characterising the aqueous dispersions of AgNPs was that TEM can only give *direct* information about particle suspensions that have been dried (Section 4.2.1) and the resulting images therefore reflect the preparation process. Nonetheless, at no tested concentration was agglomeration seen, suggesting that even though agglomeration could occur during drying, it did not. It is difficult to envisage a plausible mechanism whereby an agglomerated aqueous suspension could dis-agglomerate upon drying. It was not considered necessary to measure metal concentrations in the medium and instead rely upon the nominal values (c.f. Al in medium (Section 3.4.2)). The second limitation in this chapter was related to aging of AgNPs at different pH values (Section 4.3.1). The standard addition method was valuable in determining the amount of

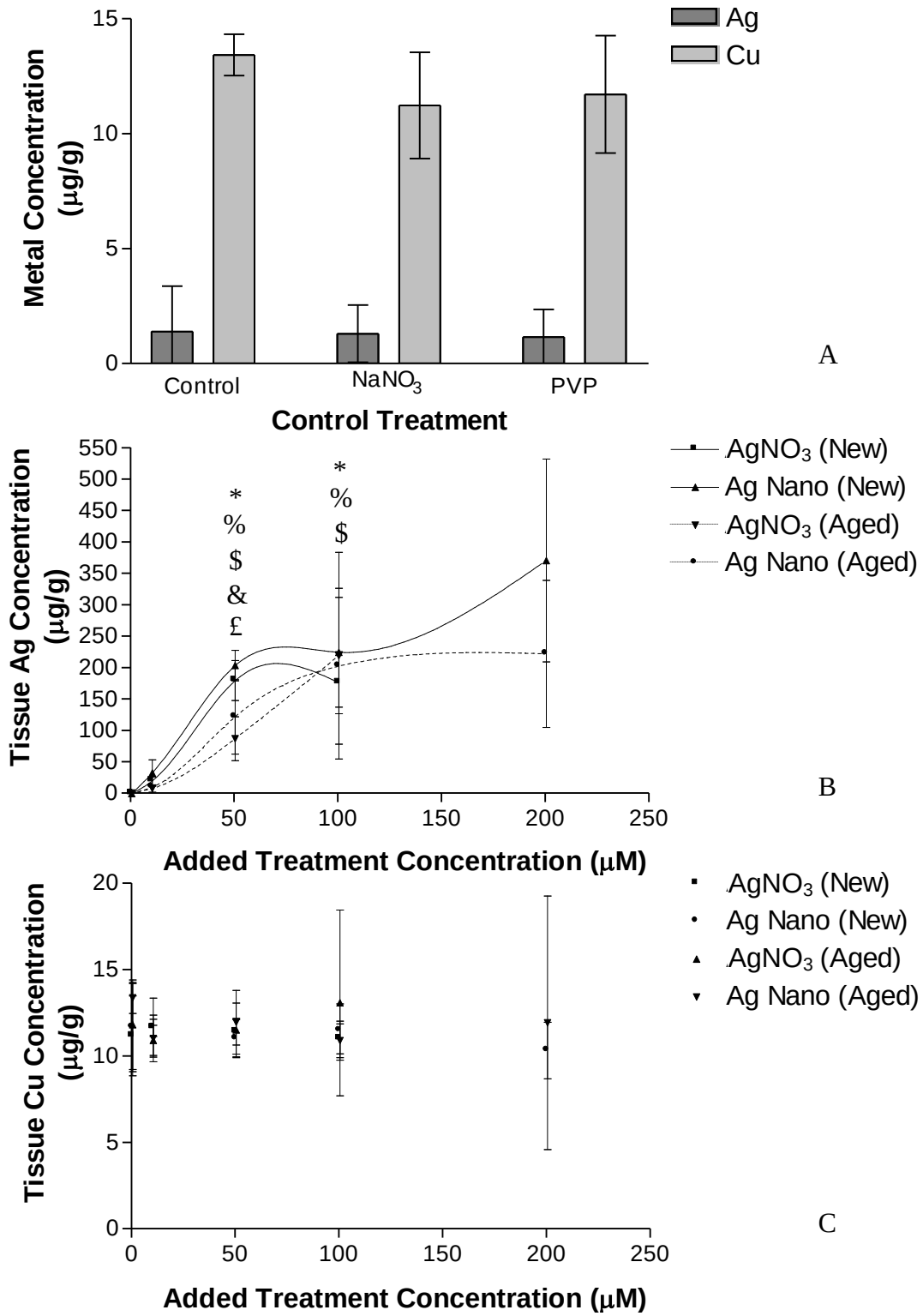

Figure 31. Tissue concentrations of Ag and Cu in adult *Drosophila* exposed to Ag for the entire larval period. (A) Tissue Ag and Cu concentration in *Drosophila* exposed to either $NaNO_3$ (100 µM), PVP (exposure concentration equivalent to that received from AgNPs 100 µM), or neither (absolute control). No significant differences were found (ANOVA – main effect). Tissue (B) Ag and (C) Cu levels in *Drosophila* exposed to 0, 10, 50 or 100 µM added $AgNO_3$ or AgNPs, or AgNPs (200 µM) in either freshly made or aged medium. Error bars represent +/- 95% CI interval. n = 4. Key to significance levels: * / % $p < 0.05$ relative to 0 µM (added) for new and aged respectively; $ / & $p < 0.05$ relative to lower dosage increment for new and aged respectively; £ $p < 0.05$ new relative to aged, equimolar counterpart. Only two-factor interactions were tested by Tukey-Kramer test as there was no significant difference between forms of Ag.

Ag released from AgNPs but assumes that equilibrium between Ag (I) ions and any binding sites was rapid. Furthermore, it is possible that the measured Ag content of the dialysate included some very small AgNPs that passed through the pores of the membrane.

4.4.2. The potential contribution of silver (I) ions to silver nanoparticle mediated toxicity

It is suggested here that Ag (I) ions released from AgNPs were a key mediator in the resulting biological effects as the data presented in this chapter indicate that dissolution of AgNPs occurs rapidly in the *Drosophila* medium. Later chapters compare the toxicity of both AgNPs and $AgNO_3$ to understand how much influence the Ag (I) ion has in AgNP induced toxicity. To the knowledge of the author, this is the first time that a standard addition curve has been used to correct for the adsorption of Ag (I) ions to the PVP capping agent, following AgNP oxidation by atmospheric oxygen. The use of this form of correction removes other potential sources of error, such as the dialysis not reaching equilibrium. The most compelling evidence for the existence of this adsorption phenomenon is the difference between the slopes of the standard addition plots at each pH (Figure 23). Even if the mismatch between measured and added Ag quantities were due to pre-equilibrium sampling or poor metal recovery from ICP-AES, the slopes would be equal. The difference would appear to arise from competition with the PVP binding sites and protons.

The dialysis data suggest that dissolution of AgNPs is fairly slow (< 10% over 28 days) at pH 4.5 but after a lag phase, at pH 7.4, dissolution rapidly reaches approximately 25% of the metal content of the particles by day 21 Figure 24. Silver nanoparticles would be expected to dissolve more rapidly at acid pH (Liu and Hurt 2010) but the reverse was observed here. It is possible that this is a spurious result, but an alternative hypothesis is proposed here. Any oxidative dissolution that occurs would release Ag (I) ions into solution, of which some would adsorb onto the PVP. This would disturb any equilibrium between AgNPs and Ag (I) ions and by Le Chatelier's principle would encourage further dissolution (Chang 2000). Such a phenomenon is likely to be greater here than in other studies due to the large amounts of PVP relative to metal (9:1). However, if the binding sites were occupied by H^+ ions, then such adsorption of the metal would be impaired and the equilibrium shifted less. If this effect exceeded the extra capacity of acidic solutions to promote dissolution, the net result would be less Ag (I) released from particles. As part of

the investigation, particles were aged in the presence of 50 mM ascorbate to demonstrate that an oxidative mechanism was at the root of the Ag release from the particles (Section 4.3.1). In this case the amount of Ag in the dialysate was below the limit of detection, and this not only supported the proposed mechanism but also demonstrated that Ag found in the dialysate was dissolved Ag (I) rather than very small particles that may pass through the dialysis membrane: small particles would be unaffected by ascorbate. The significant fall in Ag (I) seen at 140 days following aging at pH 7.4 raises questions regarding other processes that may occur during aging such as adsorption to the vessel wall and how the age of the PVP alters its affinity for Ag (I) ions. If the affinity of PVP for Ag (I) ions reduces with age or the adsorption equilibrium of Ag (I) on the vessel wall is achieved only slowly, the standard addition curve would produce underestimates of the true amount of Ag release. A similarly lowered reading would be seen if the Ag/PVP equilibrium was slow to establish.

Rapid dissolution of AgNPs was suggested in the presence of Cu (II) or Fe (III) plus Cl^- ions (Section 4.3.2). Standard electrode potentials suggest that in the absence of Cl^- these ions cannot oxidise Ag (s) (Vanýsek 2012) but these only offer a guide to the results described here, as the concentrations used were "non-standard" and the nanoscale properties of AgNPs alter their electrochemical behaviour (Plieth 1982, Redmond *et al*. 2005, Ivanova and Zamborini 2010). Consistent with expectations and the work of Ho *et al*. (2011), Cu (II) ions alone were unable to oxidise AgNPs, but Fe (III) ions did oxidise AgNPs suggesting that the deviations from standard conditions and/or the nanoscale properties shifted the E_{Cell} value. Addition of Cl^- ions markedly increased both the rate and extent of the dissolution observed. It is suggested that electrons are transferred from Ag atoms on the AgNP surface to Cu (II) or Fe (III) causing the release of an aqueous Ag (I) ion. This released Ag (I) ion is co-ordinated by one or more Cl^- ions, disturbing the equilibrium between the particle and the oxidising ion and encouraging further oxidation by Le Chatelier's principle. Chloride ions have since been observed to accelerate the oxidative dissolution of AgNPs when atmospheric oxygen is the presumed oxidant (Levard *et al*. 2013). Those Ag (I) ions that bound only a single Cl^- ion would likely precipitate, shifting the equilibrium still further; they are nonetheless still oxidised, even if now solid, and would not interfere with the measurement of AgNP induced absorbance. Indeed, the use of LSPR based absorbance assays have been suggested, specifically, for determining the concentration of AgNPs in the presence of Cl^- ions (Zook *et al*. 2011). The change in

ionic strength or pH was not responsible for agglomeration or dissolution as, in the absence of the suggested oxidising species, no change in absorbance was seen following incubation with 100 mM NaCl or at pH 3.5. One other possibility is that oxidation of the capping agent occurred. Silver (I) ions are able to oxidise PVP (over several days) (Hoppe *et al.* 2006) but logically cannot result in net oxidation of Ag (s) as the oxidation of an Ag atom would reduce an Ag (I) ion. Addition of Ag (I) ions to AgNP suspension resulted in no change in absorbance over two weeks relative to a no added ions suspension, suggesting that the PVP was not oxidised to a degree sufficient to cause agglomeration of the particles.

4.4.3. Body loads of silver following silver nitrate and silver nanoparticle administration

The similarity of whole body Ag concentrations in adult flies following either $AgNO_3$ or AgNP administration (Figure 31) suggests that similar processes are involved in the absorption of the metal, namely uptake of Ag (I) ions; this is supported by considering the potential for dissolution of AgNPs in the medium. The absorption mechanism is not known but is suggested that Ag (I) ions are absorbed into the cells of the gut by the Cu transporter Ctr1A. However, as no differences in Cu tissue concentrations were found, an alternative pathway for Cu uptake must be operational to compensate for the resulting inhibition of Ctr1A by Ag. Armstrong *et al*. (2013), similarly, found no difference in *Drosophila* tissue concentrations of Ag following treatment with either $AgNO_3$ or AgNPs (50 µg/ml). The absorption of ions, rather than particles is supported by the gustation data that show that Ag salts are more repellent than AgNPs. As body burdens were similar following either form of Ag exposure it is possible that there was no difference in repellence between the forms of Ag when presented in the medium (rather than in plain agar as in the gustatory assay) as the AgNPs had dissolved. However, the possibility that the absorption of AgNPs was lower than that of $AgNO_3$ to a degree that was exactly offset by any decreased consumption of $AgNO_3$ cannot be excluded.

The effect of allowing the AgNP medium to age before administration was examined to investigate how dissolution may affect uptake. There was, however, little difference between aged and non-aged samples for either $AgNO_3$ or AgNPs except at 50 µM where aged preparations led to reduced uptake. It is suggested that the reason for a lack of difference between the non-aged and aged AgNPs in most cases was that the dissolution

process was far more rapid than the aging period allowed. This is supported by the observation that AgNP spiked medium gradually faded (over three to four days) (Section 4.3.5) even in the absence of supplementary Cu (II) or Fe (III) ions, suggesting oxidative dissolution occurred throughout the course of the experiments. Why reduced tissue Ag concentrations were found following administration of aged $AgNO_3$ and AgNPs is not known.

4.4.4. Conclusions

The AgNPs used in this study are suggested to be oxidised and dissolved via exposure to atmospheric O_2, and the capping agent was able to bind a proportion of the released Ag (I) ions. These released ions were likely in competition with protons for binding sites and it is suggested that this drove a higher rate of dissolution at higher pH. The transition ions Cu (II) and Fe (III) were able to rapidly oxidise AgNPs in the presence of Cl^-; these ions are all likely to be present in the medium even without further additions, and coupled with the slow loss of colour in AgNP spiked medium it is highly likely that AgNPs were dissolving within the time-frame of the experiment. This suggests that the similarity between body tissue levels from AgNP exposed and $AgNO_3$ exposed *Drosophila* is a consequence of the absorbed Ag species being the same.

Chapter 5. Developmental toxicity and evidence for oxidative stress following administration of silver

5.1. Introduction

Future anthropogenic emissions of Ag are likely to be dominated by engineered AgNPs (Tolaymat *et al*. 2010) with lesser, but significant, contributions from releases of ionic Ag (Purcell and Peters 1998, Johnson *et al*. 2005). Although AgNPs comprise zero-valent Ag, the unique properties that nanoscale dimensions bestow upon materials mean that they are toxicologically distinct from the bulk material despite superficial similarity (Tolaymat *et al*. 2010, Yu *et al*. 2013). However, nanomaterials are subject to no additional regulation over their bulk counterparts, but may display toxic properties unique to the nanoscale such as induction of oxidative stress: an effect common to many nanoparticles (Kovacic and Somanathan 2010, Mocan *et al*. 2010). Furthermore, the enormous surface area that AgNPs present allows the release of Ag (I) ions (as described in Chapter 4) which are already established as highly toxic.

The majority of aquatic Ag toxicity studies (both vertebrate and invertebrate) have focused upon adult animals and the respiratory surfaces that are the primary toxic target of Ag ions. In aquatic organisms the Na^+/K^+ ATPase and carbonic anhydrase enzymes are inhibited by Ag (Morgan *et al*. 1997, Bianchini and Wood 2003). Mammals, including humans, develop argyria following exposure to Ag ions. This syndrome is not entirely understood but results in a blue-grey discolouration of the skin and cornea as a result of deposition of insoluble Ag salt (Drake and Hazelwood 2005, Lansdown 2010). A considerable quantity of elemental Ag (>2 g in total) must be ingested over months or years to produce argyria (Jonas *et al*. 2007). Although argyria is usually benign there are isolated cases of neurological toxicity existing concurrently (Lansdown 2007).

With environmental concentrations of AgNP derived silver expected to rise exponentially (Fabrega *et al*. 2011), the likelihood of exposure to organisms, including those that are still developing, is increased. Furthermore, the application of AgNPs, especially for their antibacterial properties, entails deliberate human contact, which as a recent lawsuit demonstrates may involve disproportionate exposure of infants (Seltenrich 2013). It is therefore vital that any developmental toxicity of AgNPs be promptly identified and

studied. Given the variety of AgNPs available, with diverse characteristics, this is a large undertaking. Furthermore, AgNPs must be compared with ionic Ag to gain an appreciation of the contribution that dissolution makes to toxicity; there is however a relative dearth of studies into the developmental effects of ionic Ag. The Japanese medaka *Oryzias latipes* (Wu *et al*. 2010) and *D. rerio* (Lee *et al*. 2007) embryos sustain developmental delay, morphological malformations and histological changes when exposed to AgNPs. Mouse blastocysts exposed *in vitro* to AgNPs are prone to implantation failure or post-implantation re-sorption when returned to the uterus, but nanoparticulate Ag was not as potent as the equivalent concentration of $AgNO_3$ (Li *et al*. 2010). Silver nitrate more effectively inhibits the differentiation of cardiomyocytes *in vitro* than AgNPs although smaller particles (20 nm versus 80 and 113 nm) are almost as potent as $AgNO_3$ (Park *et al*. 2011b). However, when citrate-capped AgNPs were administered orally to rats, no evidence for reproductive or embryonic toxicity was found (Hong *et al*. 2013). The particulate nature of AgNPs could perhaps give rise to developmental toxicity, independently of the metallic/elemental composition, through mechanical means such as chromosomal breaks or interference with the mitotic spindle.

Recently, four studies into the effects of AgNPs on *Drosophila* development have been published (Gorth *et al*. 2011, Key *et al*. 2011, Panacek *et al*. 2011, Posgai *et al*. 2011) Before this, however, the developmental toxicity of $AgNO_3$ to *Drosophila* had been studied only once but had been shown to be concentration-dependent (Kroman and Parsons 1960). Of the studies of AgNPs using *Drosophila*, the most intriguing was an apparent association between *increasing* particle size and toxicity (Gorth *et al*. 2011). This association is difficult to explain given that it runs counter to much current understanding of AgNP toxicity. All (Key *et al*. 2011, Panacek *et al*. 2011, Posgai *et al*. 2011) but one (Gorth *et al*. 2011) of these studies noted a concentration-dependent toxicity although the specific concentrations, and the size and coating of the particles were variable. Despite this interest in AgNP toxicity in *Drosophila*, to date, only one study (Armstrong *et al*. 2013) directly compared the developmental toxicity of AgNPs and $AgNO_3$ to *Drosophila*, and it found no difference in the number of pupae successfully eclosing between control, $AgNO_3$ or AgNPs, when Ag was administered in food at 50 mg/l (~500 μM).

Oxidative stress is a mechanism common to the toxicity of many nanomaterials and may contribute to the developmental defects that have been observed. However, both ionic (Wagner *et al*. 1975, Cortese-Krott *et al*. 2009) and nanoparticulate (Kim and Ryu 2013) Ag are able to induce oxidative stress by a variety of mechanisms. Therefore, understanding the ability of AgNPs to upset the oxidative balance of an organism is complicated by the fact that AgNPs liberate free Ag (I) ions. Various strategies, including the use of anoxic environments (Xiu *et al*. 2012), the application of antioxidants and metal ion chelators (Yang *et al*. 2012) have been used to help elucidate the contributions for each form of Ag with varying degrees of success. Posgai *et al*. (2011) found that ascorbic acid was able to ameliorate some of the toxicity associated with AgNPs in *Drosophila*, but without comparison to Ag (I) ions, the form of Ag responsible for causing the implied oxidative stress cannot be known.

Many methods that purport to measure oxidative stress do so only by inference and rely upon certain assumptions. The most direct method would be to visualise ROS using fluorescent probes (Soh 2006) but such a method is difficult to employ when studying *in vivo* biology and without a suite of probes, over-selective. Administration of antioxidants provides an alternative, but, as seen later (in Chapter 6), it can be difficult disentangle the direct interactions of the antioxidant and the metal from those with any ROS produced. Changes in expression of antioxidant genes can also be difficult to interpret as ultimately such changes could derive from many factors, causing trouble in attributing cause and effect. If the primary parameter of interest for a particular toxicant, relating to oxidative stress, is its effect upon the health and viability of the whole organism then examining the susceptibility of an exposed organism to an externally applied oxidative stressor such as paraquat (Bus and Gibson 1984, Bonilla *et al*. 2006, Magwere *et al*. 2006, Hosamani and Muralidhara 2013) or hydrogen peroxide (Slack *et al*. 2010, Vrailas-Mortimer *et al*. 2012) can yield valuable information.

The aims of this chapter were to compare the developmental toxicity of $AgNO_3$ and AgNPs, both in terms of maturation and physical growth, through assays of pupation and eclosion success, and larval length, respectively. The contributions of Ag ions and NPs are assessed using Cu ions as an antagonist to Ag (I) ions. Alongside the AgNP studies are parallel investigations using inert, diamond nanoparticles to ascertain if ingestion of nano-sized particulates alone (i.e. without specific bioactive properties) may affect development.

The chapter also aims to perform a preliminary examination of how Ag may induce, or affect susceptibility, to oxidative stress.

5.2. Methods

5.2.1. Developmental toxicity of silver and diamond nanoparticles to *Drosophila*

5.2.1.1. Pupation success, eclosion success, and time to pupation following silver exposure

Pupation success, eclosion success and time to pupation (Section 2.7.1) were performed in four experiments: one experiment for each form of silver ($AgNO_3$ and AgNPs), each paired with its usual control ($NaNO_3$ and PVP) to gauge toxicity ranges; a third to directly compare equimolar concentrations of $AgNO_3$ and AgNP with a finer resolution between concentrations; and a fourth using diamond nanoparticles to ascertain if any possible effects of AgNPs could simply be attributed to the presence of nanoparticulate matter without specific bioactive properties. In the first experiment, 10 replicate vials, each containing 50 eggs, were prepared containing $AgNO_3$ spiked medium (Section 4.2.4) at added concentrations of 0 μM, 10 μM, 50 μM, 100 μM, 500 μM, 1mM and 50 mM, and their development followed as described in Section 2.7.1. Equimolar equivalent vials were prepared using $NaNO_3$ as a control for the nitrate counter ion. In the second experiment 10 replicate vials were prepared containing AgNP spiked medium at added concentrations of 0 μM, 100 μM, 500 μM, 1 mM and 10 mM. Vials containing the equivalent concentration of PVP (Section 4.2.4) were prepared in parallel. In the third experiment, the added concentrations of $AgNO_3$ used were 0 μM, 10 μM, 50 μM, 100 μM and 200 μM, whilst the AgNP experiments had additional vials at 300 μM, 400 μM and 500 μM; each Ag experimental treatment had an equivalent $NaNO_3$ or PVP control. In the fourth experiment, diamond nanoparticles were tested in the same manner using concentrations of 50 μM, 100 μM, 200 μM and 500 μM.

5.2.1.2. The effects of copper administration upon silver mediated developmental toxicity

To test the influence of supplementary Cu on any Ag induced developmental changes, larvae were co-exposed to Cu and Ag at the same time. Inverted 1.5 ml micro-centrifuge tubes (Star-Labs, UK) were made into individual fly cages by removing the lid and the tip at the bottom of the tube with a scalpel and plugging the latter with a small piece of cotton

wool to allow gas exchange. Standard *Drosophila* medium spiked with 100 µM $AgNO_3$, 100 µM or 200 µM Cu $(NO_3)_2$, a combination of Cu with Ag, or control was placed in the hollow of the separated lid. Further groups of medium prepared with AgNPs and Cu(NO_3) in various combinations were made, but it was here that their interaction in the medium was first seen (described in Section 4.1 and studied in detail in section 4.2.3), making it clear that it was not possible to administer the two together. An individual *Drosophila* egg was placed on the food substrate contained within the lid and the tube body placed onto it. One hundred replicate tubes per Ag treatment group and 60 per Cu only and control groups were prepared.

5.2.1.3. Larval length as a measure of silver toxicity and its modulation by copper

To assess the effects of Ag on the physical growth of *Drosophila* larvae and whether these could be modulated by Cu, larval length at 90 hours after hatching was determined. The *in vitro* interaction between AgNPs and Cu (Section 4.2.3) made it impossible to co-administer copper with AgNPs, and to circumvent any chemical interaction between the metals in the media, a pre/post-load experimental design was used.

For Cu pre-loading experiments, three vials of 50 *Drosophila* eggs per vial were placed on Cu $(NO_3)_2$ (200 µM) containing, or control medium (Section 4.2.4) and incubated at 25°C in the dark. Sixty-six hours AEL (45 hours post-hatching) larvae were removed from the medium (Section 2.5) and transferred to either control medium or that containing $AgNO_3$ or AgNPs at 100 µM. At 111 hours AEL (90 hours post-hatching) larvae were harvested, killed and measured as described in Section 2.7.2). For Cu post-loading experiments the procedure was identical except that the Ag and Cu treatments were temporally reversed. In both cases equal number of control larvae were prepared, handled and transferred between vials in an identical manner.

For comparison to the pre/post loading studies, an additional study was performed examining continuous Ag and Cu exposure throughout larval life. Three vials of fifty *Drosophila* eggs per vial were placed on media containing $AgNO_3$ (100 µM), Cu $(NO_3)_2$ (200 µM) or $AgNO_3$(100 µM) + Cu $(NO_3)_2$ (200 µM) and incubated at 25 °C in darkness until 111 hours AEL (90 hours post-hatching). Larvae were then removed from the medium and measured as described in Section 2.7.2)

5.2.2. Oxidative stress resistance and the effect of antioxidants in *Drosophila melanogaster*

5.2.2.1. The effects of ascorbic acid administration upon silver mediated developmental toxicity

To determine the effect of an exogenously administered antioxidant upon Ag mediated developmental toxicity, ascorbic acid was administered simultaneously with either $AgNO_3$ or AgNPs. Five vials of fifty *Drosophila* eggs per vial were placed upon medium spiked with either no additive, $AgNO_3$ (100 μM), AgNPs (100 μM) or each of the former plus ascorbic acid (50 mM) as used by Posgai *et al*. (2011) for the amelioration of AgNP mediated toxicity. Vials were incubated at 25°C in the dark and the time to pupation was determined as described in Section 2.7.1.

5.2.2.2. Paraquat resistance in silver exposed *Drosophila*

To examine the resistance of Ag exposed *Drosophila* to externally applied oxidative stress, larvae were exposed to Ag, and the subsequently eclosed adults exposed to paraquat. Individual fly cages were made from upturned micro-centrifuge tubes (Section 5.2.1.2). Control, standard *Drosophila* medium or that spiked with either $AgNO_3$ (50 μM) or AgNPs (50 μM) was placed in the hollow of the separated tube lid; unspiked medium was used for controls. Forty individual *Drosophila* eggs were placed on the food substrate contained within the lid and the tube body placed onto it. The eggs were incubated under standard conditions (Section 2.2) until emergence of the adult flies. The emerged adults were placed on fresh food, spiked with the same form of Ag at the same concentration, by replacing the cage base (tube lid), and allowed to mature for 5 days.

Paraquat stock solution (100 mM) was prepared freshly by adding paraquat (125 mg) to 4861 μl de-ionised H_2O. Thirty mature flies from each treatment group then had their food substrate replaced with 1% plain agar supplemented with 5% sucrose as a food source plus 20 mM paraquat (adapted from Ali *et al*. (2011)) Ten controls from each treatment group received 5% sucrose agar without paraquat. Death counts were then made periodically (~3 hourly) until all animals had died.

5.3. Results

5.3.1. Developmental toxicity of silver nitrate and silver nanoparticles

5.3.1.1. Pupation success following administration of silver nitrate and silver nanoparticles

Silver nitrate displayed marked toxicity (Figure 32A). A steep concentration – response relationship was seen; 100 μM exposed animals pupated with the same degree of success as the controls ($p > 0.05$; Tukey-Kramer test), whereas 500 μM exposed animals exhibited complete lethality ($p < 0.05$). Additional concentrations of 1 mM, 10 mM and 50 mM also resulted in no pupae forming (data not shown). $NaNO_3$ had no effect at any of the concentrations tested, even at the high concentrations (data not shown).

To test the effects of AgNPs on the development of *Drosophila*, the protocol used for $AgNO_3$ was used with adjusted concentrations. In this instance the medium-concentrations used were guided by the $AgNO_3$ results but constrained by the practical limitations of suspending AgNPs. Figure 32B shows that AgNPs exhibited a different toxicity profile from $AgNO_3$. Up to 100 μM there was no effect of AgNPs on pupation success, as was seen with $AgNO_3$. At 500 μM, rather than the complete mortality seen with the $AgNO_3$, a large drop in the number of pupae formed occurred. Above this concentration a trend reversal occurred such that by 10 mM, 68.25% (95% CI of 48.19 – 85.27%) of eggs pupated versus control pupation success of 87.47% (95% CI of 81.17 – 92.69%) ($p > 0.05$). The capping agent, PVP, had no effect at any concentration.

5.3.1.2. Eclosion success following administration of silver nitrate and silver nanoparticles

The eclosion success of the larvae from Section 5.3.1.1 was assessed to see how Ag affected further progression towards adulthood. A small but significant reduction was seen in the fraction of emerging adults following $AgNO_3$ (100 μM) treatment compared to both control and $NaNO_3$ of the same concentration. This was true regardless of whether the fraction of emerging adults was calculated from the initial egg count (Figure 33A) or the number of animals that pupated (Figure 34A). This confirms that there were additional losses throughout metamorphosis and the reduced number of adults was not simply due to fewer pupae to start with. In common with $AgNO_3$ exposed *Drosophila*, those that had

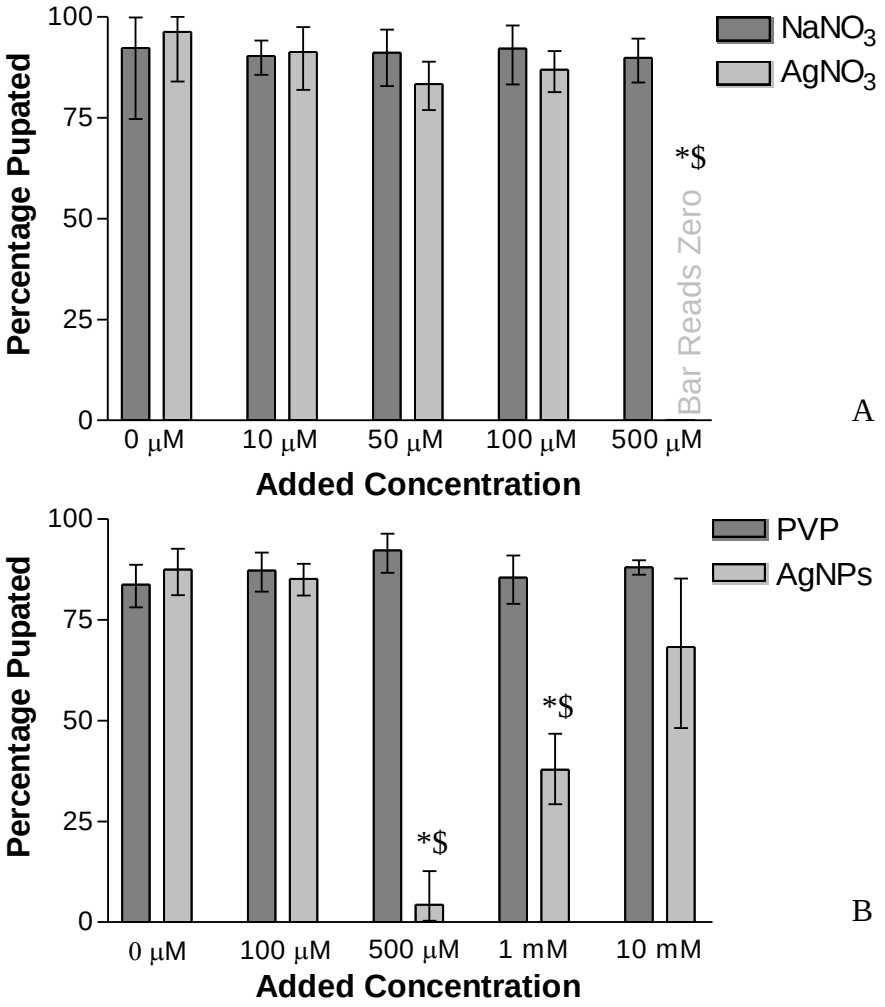

Figure 32. Pupation success following Ag administration. Mean percentage of eggs forming pupae (+/- 95% confidence interval) following exposure to $AgNO_3$ (A) and AgNPs (B). Responses to (A) $NaNO_3$ and (B) PVP (in amounts equivalent to that supplied by stated concentration of AgNPs rather than the concentration of PVP *per se*) are included as a counter-ion or capping agent control respectively. n = 10. Key to p values: * $p < 0.05$ (Tukey-Kramer test) relative to respective 0 μM (added) group in each panel; $ $p < 0.05$ (Tukey-Kramer test) Ag relative to (A) $NaNO_3$ and (B) PVP of the same concentration.

received AgNPs experienced greater losses at eclosion compared to pupation. A small but significant decrease in the numbers of emerging adults was seen at 100 μM exposure ($p < 0.05$). The U-shaped concentration-response profile observed for pupation was still evident when studying eclosions, but the reduced toxicity was confined to the highest concentration of AgNPs (10 mM) regardless of the denominator used for fractional calculation (Figure 33B and Figure 34B).

Following eclosion a marked reduction in pigmentation in $AgNO_3$ and AgNP exposed animals was noted along with a soft and seemingly more pliable cuticle. Indeed,

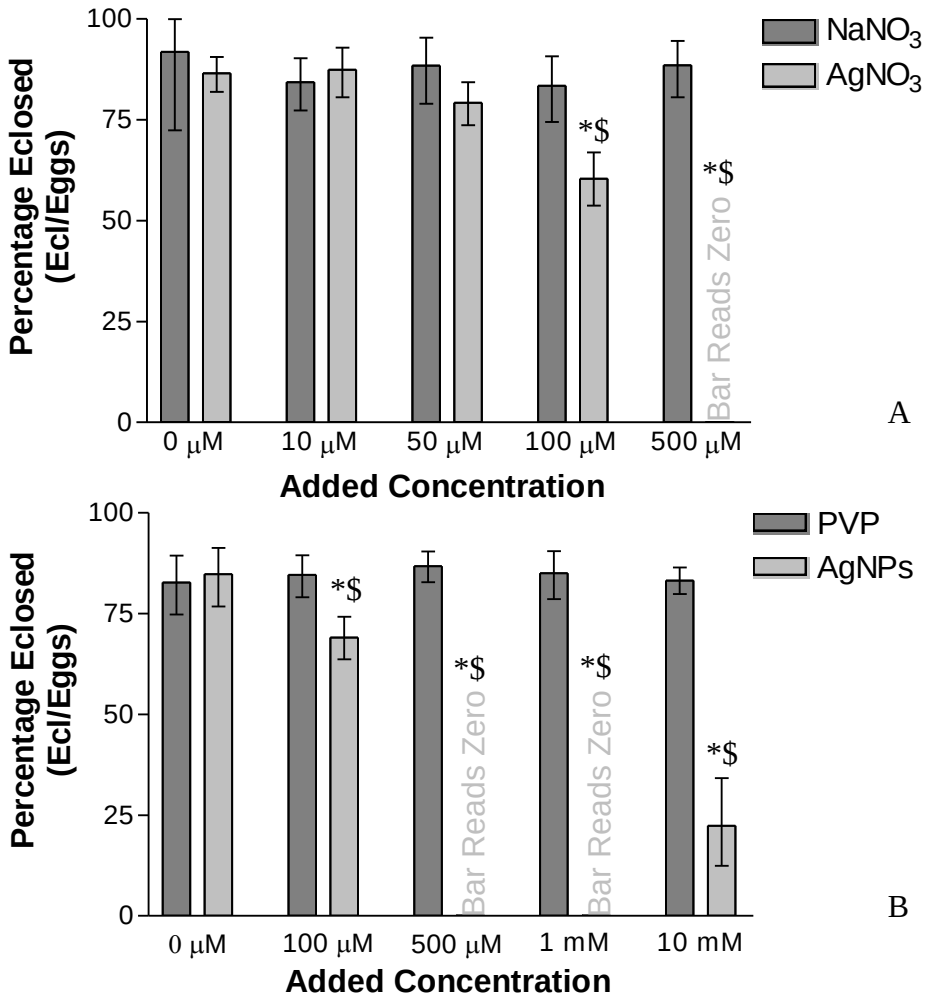

Figure 33. Eclosion success following Ag administration. Mean percentage of eggs successfully eclosing (+/- 95% confidence interval) following exposure to $AgNO_3$ (A) and AgNPs (B). Responses to (A) $NaNO_3$ and (B) PVP (in amounts equivalent to that supplied by stated concentration of AgNPs rather than the concentration of PVP *per se*) are included as a counter-ion or capping agent control respectively. n = 10. Key to p values: * $p < 0.05$ (Tukey-Kramer test) relative to respective 0 μM (added) group in each panel; $ $p < 0.05$ (Tukey-Kramer test) Ag relative to (A) $NaNO_3$ and (B) PVP of the same concentration.

occasional flies (~ 3%) tried to emerge from their pupal case but were unable to extricate themselves and died.

5.3.1.3. Pupation and eclosion success directly compared between silver nitrate and silver nanoparticles

Having ascertained the effective and lethal ranges of both forms of Ag, a direct comparison between $AgNO_3$ and AgNP toxicity was made, under identical conditions, with finer resolution between 100 μM and 500 μM concentrations. Silver nitrate resulted in 100% mortality at millimolar concentrations, so no comparison could be made with AgNPs at such concentrations. Sodium nitrate and PVP controls were not used as they had been

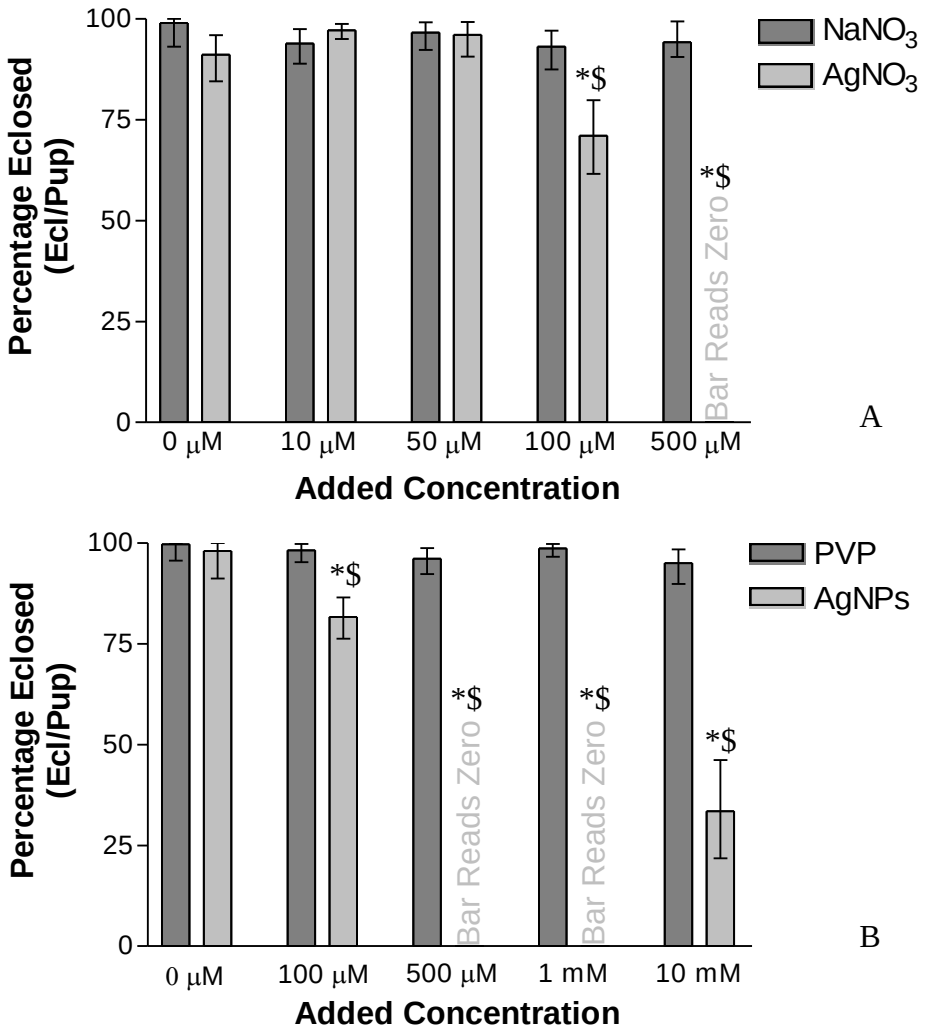

Figure 34. Eclosion success following Ag administration. Mean percentage of pupae successfully eclosing(+/- 95% confidence interval) following exposure to $AgNO_3$ (A) and AgNPs (B). Responses to (A) $NaNO_3$ and (B) PVP (in amounts equivalent to that supplied by stated concentration of AgNPs rather than the concentration of PVP *per se*) are included as a counter-ion or capping agent control respectively. n = 10. Key to p values: * $p < 0.05$ (Tukey-Kramer test) relative to respective 0 μM (added) group in each panel; $ $p < 0.05$ (Tukey-Kramer test) Ag relative to (A) $NaNO_3$ and (B) PVP of the same concentration.

consistently found to be non-toxic in earlier experiments.

There was no significant difference between the number of pupae formed at concentrations 100 μM and below (Figure 35A). Above this clear differences emerged; $AgNO_3$ exposed *Drosophila* exhibited marked toxicity at 200 μM reducing the pupation fraction from 73.2% (95% CI of 52.1 – 89.9%) in controls to 7.2% (95% CI of 5.8 – 7.7%) ($p < 0.05$). Above 300 μM no animals survived to pupation when exposed to $AgNO_3$ ($p < 0.05$). In contrast, the pupation fraction after exposure to AgNPs at concentrations of 200 μM and above was significantly ($p < 0.05$) reduced relative to control but a substantial proportion

nevertheless managed to pupate. The pupation success at 500 µM was 28.8% (95% CI of 5.4 – 61.3) compared to a control pupation fraction of 87.3% (95% CI of 52.3 – 100%) ($p < 0.05$). Despite the lack of significance between some adjacent concentrations there was an obvious monotonic concentration-response relationship with a decline in pupation corresponding to increased concentrations. Although not tested, the reversal of the concentration-response relationship lies between 0.5 mM and 1 mM.

Figure 35B and C show that in this experiment the eclosion fraction (measured as fraction of eggs and pupae respectively) at 100 µM of either form of Ag remained unchanged compared to control. The higher concentrations of $AgNO_3$ yielded no emerging adults as no pupae were formed (Figure 35B and C). A single adult eclosed following 300 µM $AgNO_3$ and at 200 µM the percentage of eclosed adults as a proportion of eggs was 6.0% (95% CI of 4.3 – 7.9%) ($p < 0.05$ compared to control). A large proportion of losses at the eclosion stage were accounted for in losses before pupation as the fraction of pupae successfully eclosing was 78.0% (95% CI of 47.8 – 97.2%) (Figure 35C). Following 200 µM AgNP administration, considerable losses were evident by the time of eclosion; 34.2% (95% CI of 25.3 – 43.8%) of eggs reached adulthood compared with 82.7% (95% CI of 35.2 to 100%) in controls ($p < 0.05$; Tukey-Kramer test). Further losses were seen at increased concentrations with only minimal survival above 300 µM AgNPs. When measured as a fraction of pupae, the number of eclosions was not significantly affected by the form of silver (two-way ANOVA; Factor: form of silver; $p = 0.76$). Therefore, although a sizable proportion of eggs went on to eclose following AgNP exposure (200 µM), when measured as proportion of pupae it was no different to $AgNO_3$ (200 µM).

5.3.1.4. Time to pupation following administration of silver nitrate and silver nanoparticles

To achieve greater differentiation of toxicity, time to pupation was measured. $AgNO_3$ and AgNPs experiments were performed separately such that they could be compared to $NaNO_3$ and PVP respectively. A total of 15 hazard ratio (HR) comparisons were made in the $AgNO_3$ experiment with a family-wise error rate (α) of 0.05 yielding a significance threshold of 0.0033 following a Bonferroni correction. The 24 comparisons made for AgNPs gave a threshold of 0.0021.

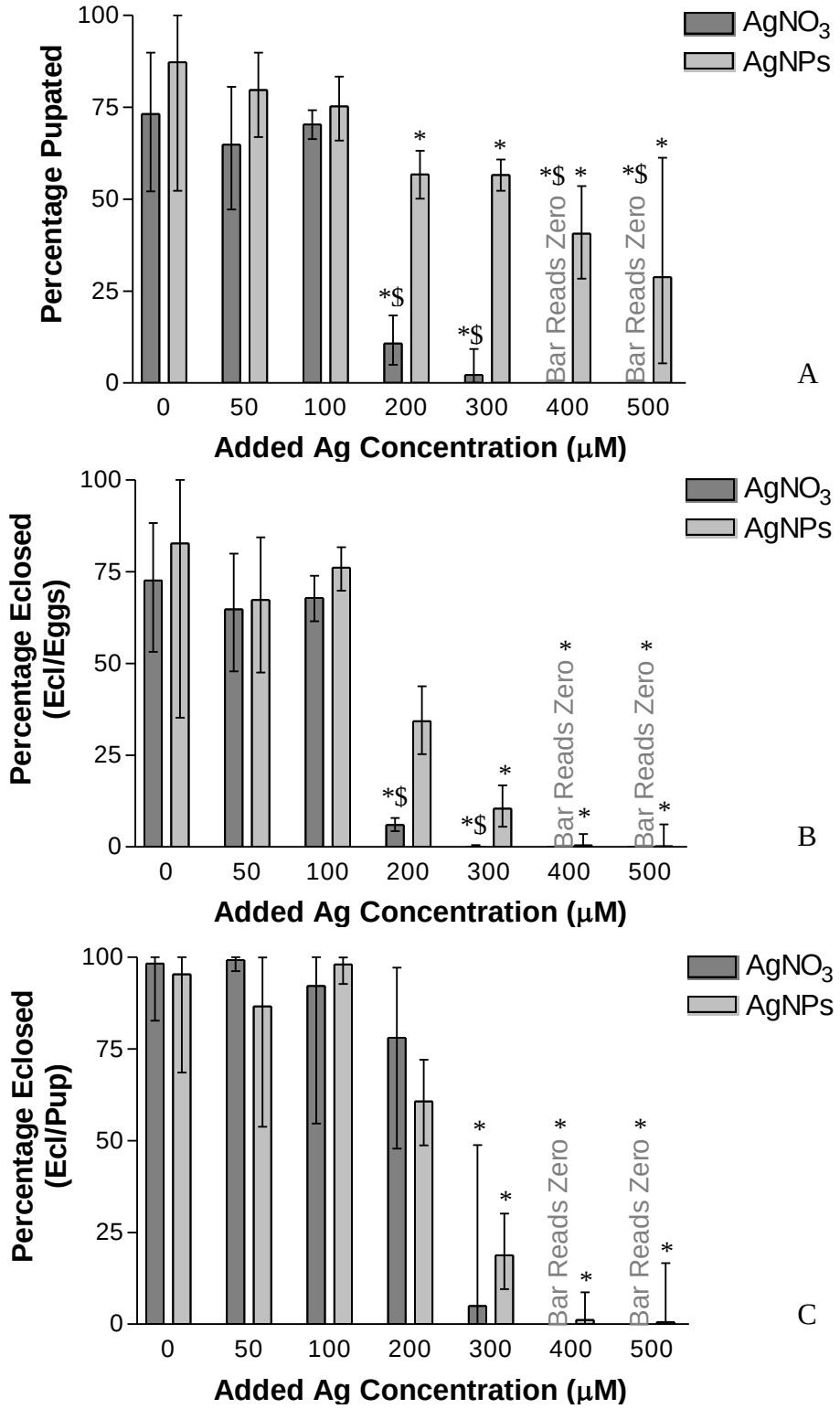

Figure 35. Pupation and eclosion success following $AgNO_3$ or AgNP administration under direct comparison. Added concentrations used ranged from 50 µM to 500 µM. (A) Mean percentage of eggs successfully pupating and (B) eclosing (+/- 95% confidence interval) following exposure to $AgNO_3$ or AgNPs. (C) Percentage of pupae eclosing (+/- 95% confidence interval) following exposure to $AgNO_3$ or AgNPs. n = 10. Key to p values: * $p < 0.05$ (Tukey-Kramer test) relative to respective 0 µM (added) concentration in that panel; $ $p < 0.05$ (Tukey-Kramer test) $AgNO_3$ relative to AgNPs of same concentration in that panel.

Following administration of the $NaNO_3$, no significant differences were seen between any concentration and control or between the individual concentrations, suggesting that the NO_3^- ion at these concentrations does not affect the development time of *Drosophila* larvae (Figure 36A). Following exposure to $AgNO_3$ a significant delay in development was seen for both 50 µM and 100 µM with hazard ratios, relative to control, of 0.62 (95% CI of 0.54 – 0.71; $p < 0.0033$; log-rank test) and 0.40 (95% CI of 0.34 – 0.46; $p < 0.0033$; log-rank test). There was no significant difference between 10 µM $AgNO_3$ and control ($p > 0.0033$; log rank test) (Figure 36A).

All concentrations of AgNPs tested caused a significant increase in the time to pupation (all $p < 0.0021$; log-rank test) (Figure 36B). In a manner similar to that seen with the pupation success, a U-shaped concentration-response curve trend was observed, although none of the concentrations above 0.1 mM were significantly different from one another. The animals that took longest to develop were also those that had the poorest survival to the pupation stage (c.f. Section 5.3.1.1).

Some of the PVP controls exhibited differences in the rate of pupation, relative to control. At 0.1 mM there was no significant difference but at 0.5 mM a significant *increase* in pupation rate was seen, a disparity that disappeared at 1 mM only to re-appear at 10 mM. However, the actual differences between PVP exposed larvae and controls, although significant, were small; for example the largest difference was seen between control and 10 mM PVP but the actual effect size is represented by $HR_{Control:PVP} = 0.73$ (95% CI of 0.61 – 0.86; $p = 0.00012$) and a median ratio of 1. All concentrations of AgNPs retarded progression to pupation relative to their respective PVP only controls ($p < 0.0021$).

5.3.1.5. Time to pupation compared directly between silver nitrate and silver nanoparticles

Having established the pupation rates within the viable concentration range for both forms of Ag and compared them with their usual controls (NO_3^- and PVP), the pupation rates between the two forms of Ag were compared directly in the same experiment at added concentration ranges of 0 – 300 µM for $AgNO_3$ and 0 – 500 µM for AgNPs. The threshold for statistical significance was designated as 0.00091 following Bonferroni correction for 55 comparisons.

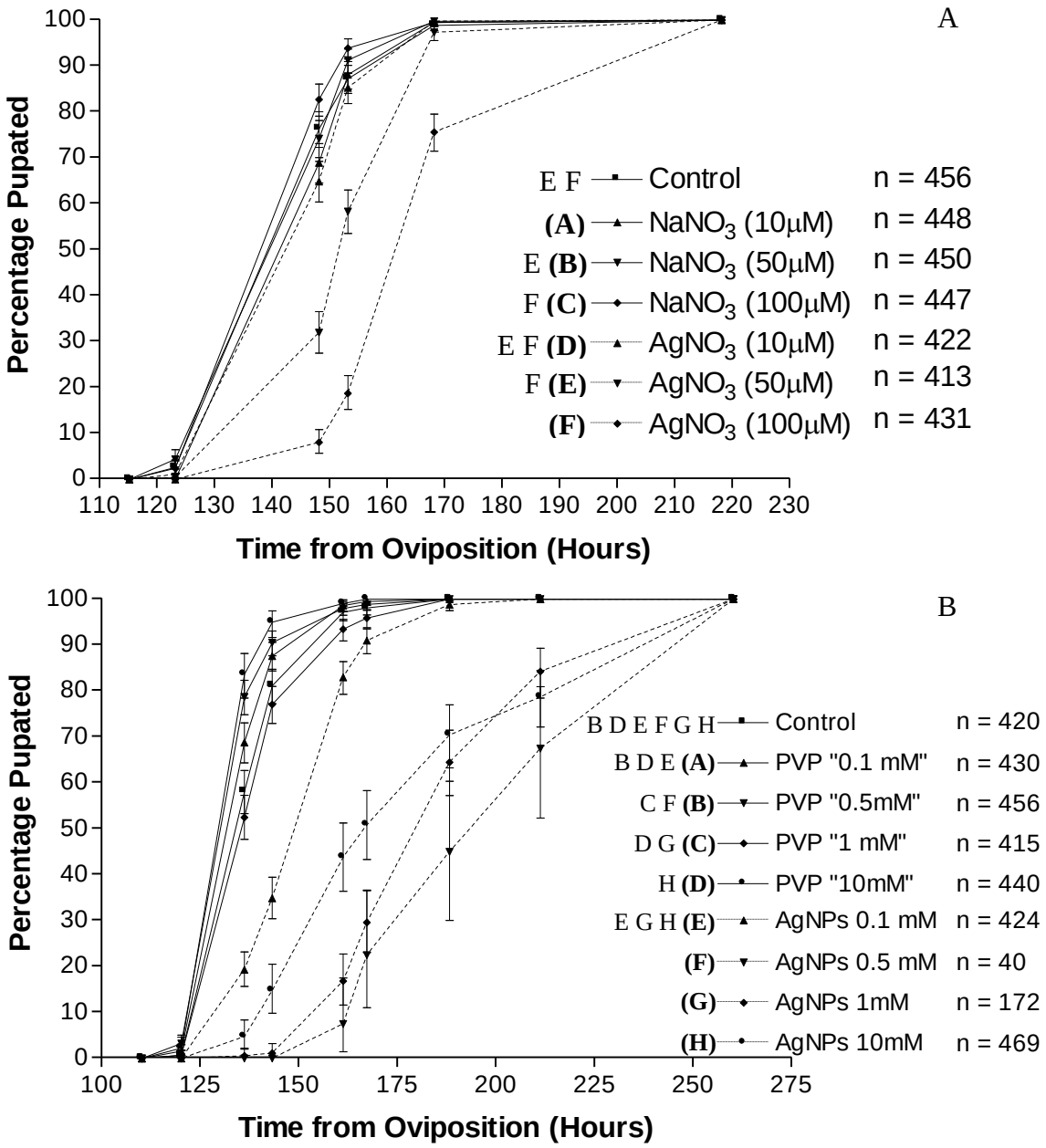

Figure 36 Time to pupation following administration of Ag from hatching. (A) Added concentrations of $AgNO_3$ used were 10, 50 and 100 µM. Also shown are responses to equimolar concentrations of $NaNO_3$. (B) Added concentrations of AgNPs used were 0.1, 0.5, 1 and 10 mM. Also shown are responses to PVP at concentrations equivalent to those found in AgNPs of the specified concentrations. Error bars represent +/- 95% CI of fraction pupated. Replicates for each group are shown in the treatment group key. Key to significance values: (Panel A) A, B, C, D, E, and F $p < 0.0033$ relative to the group with that letter displayed in brackets (Log-rank test); (Panel B) A, B, C, D, E, F, G, H $p < 0.0021$ relative to the group with that letter displayed in brackets (Log-rank test).

Pupation rates at all concentrations for both forms of Ag (except for $AgNO_3$ at 50 µM) were significantly different from control (Figure 37). The non-significant concentration of $AgNO_3$ showed a trend towards significance with $HR_{Control:AgNO_3(50\mu M)} = 1.24$ (95% CI of 1.04 - 1.46; $p = 0.0150$; log-rank test) and was also not significantly different

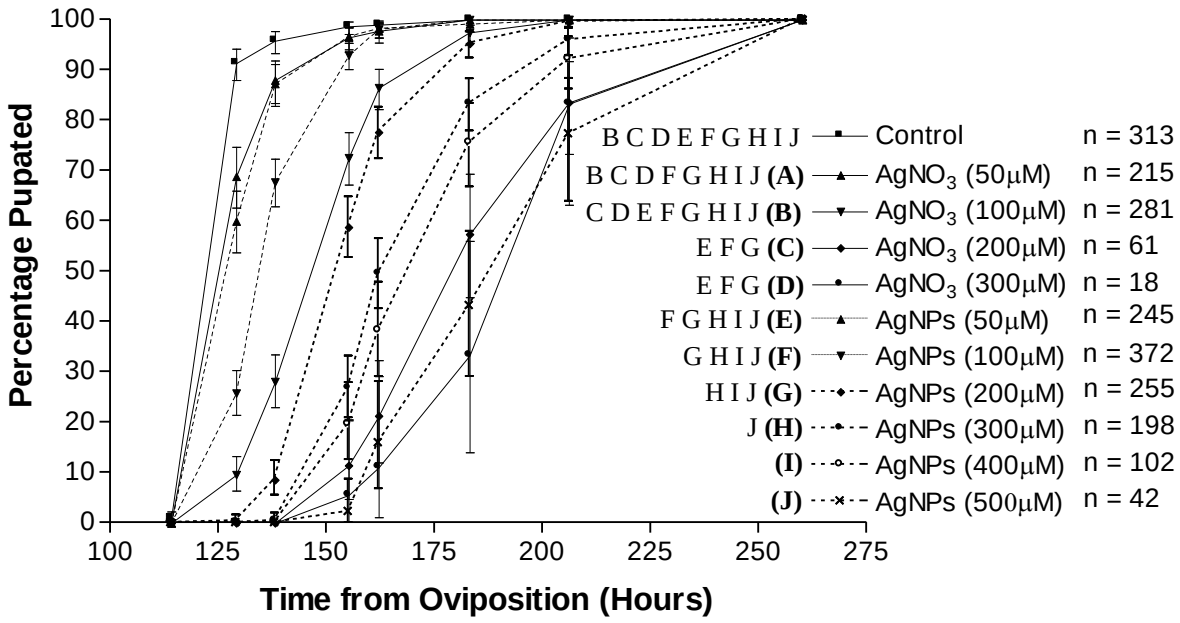

Figure 37. Direct comparison of time to pupation following administration of $AgNO_3$ or AgNPs. Added concentrations range from 50 μM to 300 μM for $AgNO_3$ and 500 μM for AgNPs and were administered throughout development from hatching. Error bars represent +/- 95% CI of fraction pupated. Replicates for each group are shown in the treatment group key. Key to significance values: A, B, C, D, E, F, G, H, I and J $p < 0.00091$ relative to the group with that letter displayed in brackets (Log-rank test).

from 50 μM AgNPs ($p = 0.4790$), the latter of which was significantly different from control with $HR_{Control:AgNPs(50\mu M)} = 1.34$ (95% CI of 1.1 - 1.58; $p = 0.0006$). Above 50 μM the toxicity profiles of the two forms of Ag diverged with 100 μM $AgNO_3$ showing a significantly greater effect on pupation rate than AgNPs with $HR_{AgNO3(100\mu M):AgNPs(100\mu M)} = 0.57$ (95% CI of 0.49 to 0.66; $p < 5 \times 10^{-11}$). This difference was also present at 200 μM exposure with a $HR_{AgNO3(200\mu M):AgNPs(200\mu M)} = 0.45$ (95% CI of 0.36 - 0.57); $p = 8.6 \times 10^{-9}$). An additional observation was that, although 200 μM AgNPs delayed development to a greater degree than 100 μM $AgNO_3$, the magnitude of the difference was less than that caused by 100 μM $AgNO_3$ relative to 100 μM AgNPs; that is, the difference between 100 μM $AgNO_3$ and 100 μM AgNPs was larger than the difference between 200 μM AgNPs and 100 μM $AgNO_3$. As the concentrations increased, most differences between consecutive concentrations failed to reach statistical significance despite an apparent trend; above 200 μM, the only significant difference seen within each type of Ag was 500 μM AgNP exposed animals were slower to pupate than 300 μM exposed. The only difference to reach significance above 200 μM between the two forms of Ag was that 300 μM $AgNO_3$ exposed animals took longer to pupate than 200 μM AgNPs with

$HR_{AgNO3(300\mu M):AgNPs(200\mu M)}$ = 0.40 (95% CI of 0.28 – 0.55; p = 8.4 x 10^{-5}). Overall, $AgNO_3$ was more potent at delaying pupation than an equimolar concentration of AgNPs.

5.3.2. Developmental toxicity of diamond nanoparticles

To ascertain if any of the effects seen with the AgNPs could be replicated by the presence of nanoparticulates with no specific bioactive properties, a pupation and eclosion based testing schedule was used during the administration of diamond nanoparticles. Figure 38A shows there were no significant differences (ANOVA- main effect) in pupation success at any of the concentrations tested up to and including 500 µM, indicating that biologically inert particles of a similar size cannot mimic the larval mortality observed with AgNPs. Likewise, no significant differences from control were seen in the number of eclosing adults at any concentration of diamond nanoparticles whether the measurement was made as a fraction of total eggs (Figure 38B) or number pupated (Figure 38C) (p = 0.70 and 0.22 respectively; ANOVA – main effect).

To assess the developmental toxicity of diamond nanoparticles with greater sensitivity, time to pupation was measured. The threshold for statistical significance between treatment groups was designated as 0.005 after Bonferroni correction for 10 comparisons. When exposed to diamond nanoparticles *Drosophila* larvae showed no significant difference (p > 0.005; log rank test) in the time to pupation for any concentration (Figure 39). Overall diamond nanoparticles have no observable impact on the gross developmental cycle of *Drosophila*.

5.3.3. Amelioration of silver-induced developmental toxicity by copper

The developmental impact of Ag was tested for its interaction with $Cu(NO_3)_2$ by examining how the latter affected the delay in pupation induced by Ag. AgNPs could not be tested due to interaction with Cu (II) ions (See section 4.3.2). Bonferroni correction for 13 comparisons yielded a statistical significance threshold of 0.0038 (α = 0.05). Administration of 100 µM $AgNO_3$ delayed the pupation relative to control with $HR_{Control:AgNO3(100\mu M)}$ = 3.67 (95% CI of 2.29 – 5.87; p < 0.0038; log-rank test) (Figure 40). Administration of the Cu salt alone at either 100 or 200 µM had no significant effect on rate of pupation (p = 0.7688 and p = 0.4229 respectively; log-rank test). When co-administered with $AgNO_3$, Cu $(NO_3)_2$ at both concentrations facilitated a marked recovery

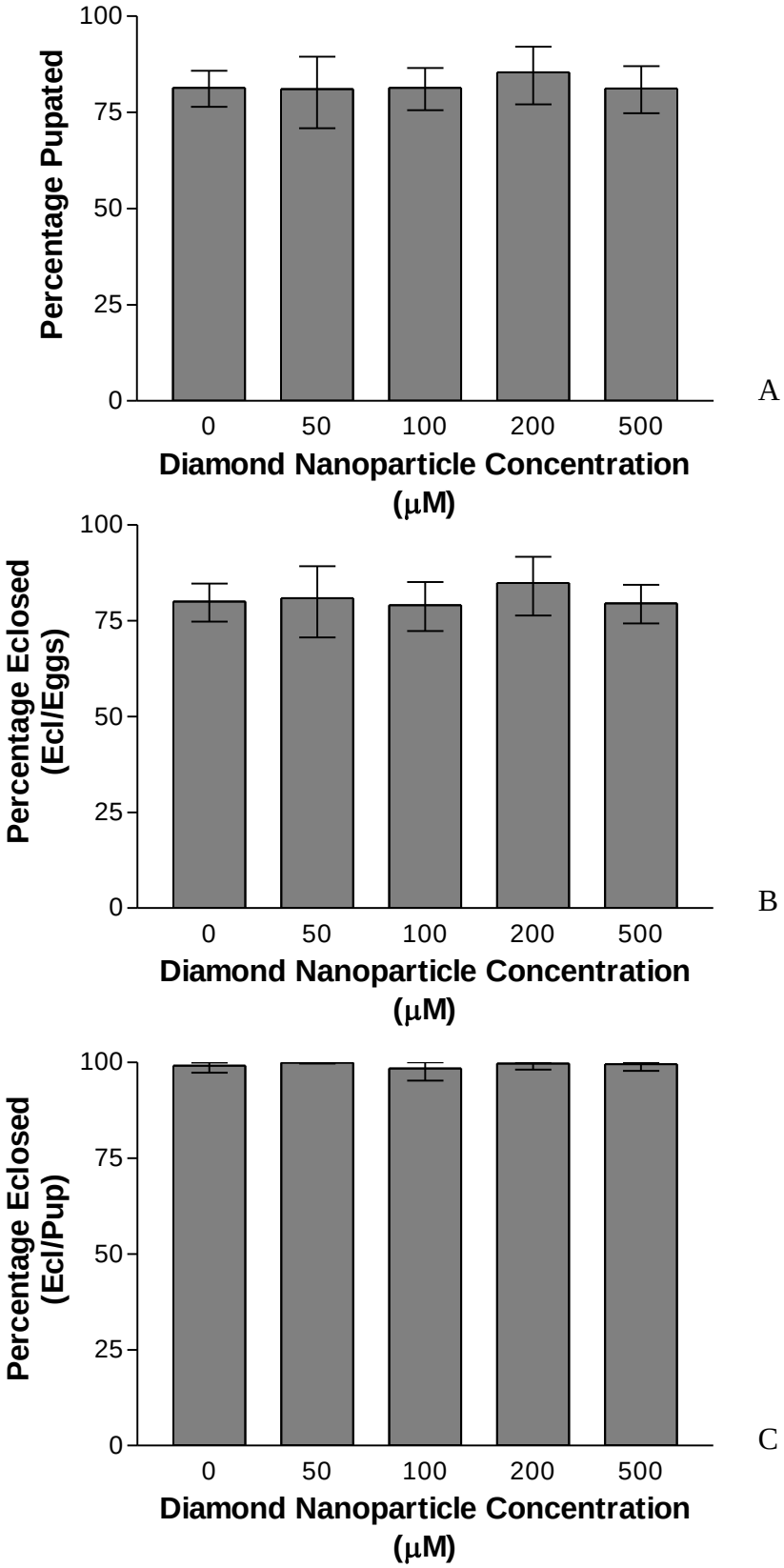

Figure 38. Pupation and eclosion success following diamond nanoparticles. Mean percentage (A) pupation and (B and C) eclosion (+/- 95% CI) following administration of diamond nanoparticles throughout development from hatching at concentrations of 0, 50, 100, 200 and 500 µM AgNPs. Eclosions are presented as fractions of both eggs laid (B) and number pupated. n = 10. No significant differences were seen between any groups in any panel; (ANOVA – main effect).

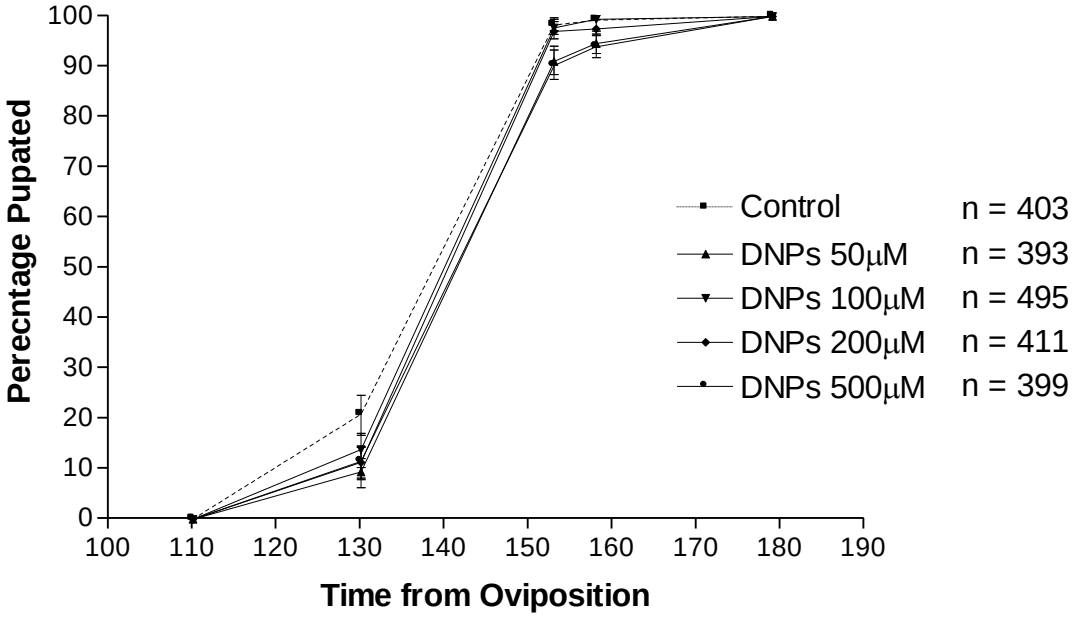

Figure 39. Time to pupation following administration of diamond nanoparticles throughout development from hatching. Concentrations used were 0, 50, 100, 200 and 500 μM. Error bars represent +/- 95% CI of fraction pupated. Replicates for each group are shown in the treatment group key. No significant differences were seen between any groups ($p > 0.005$; log-rank test).

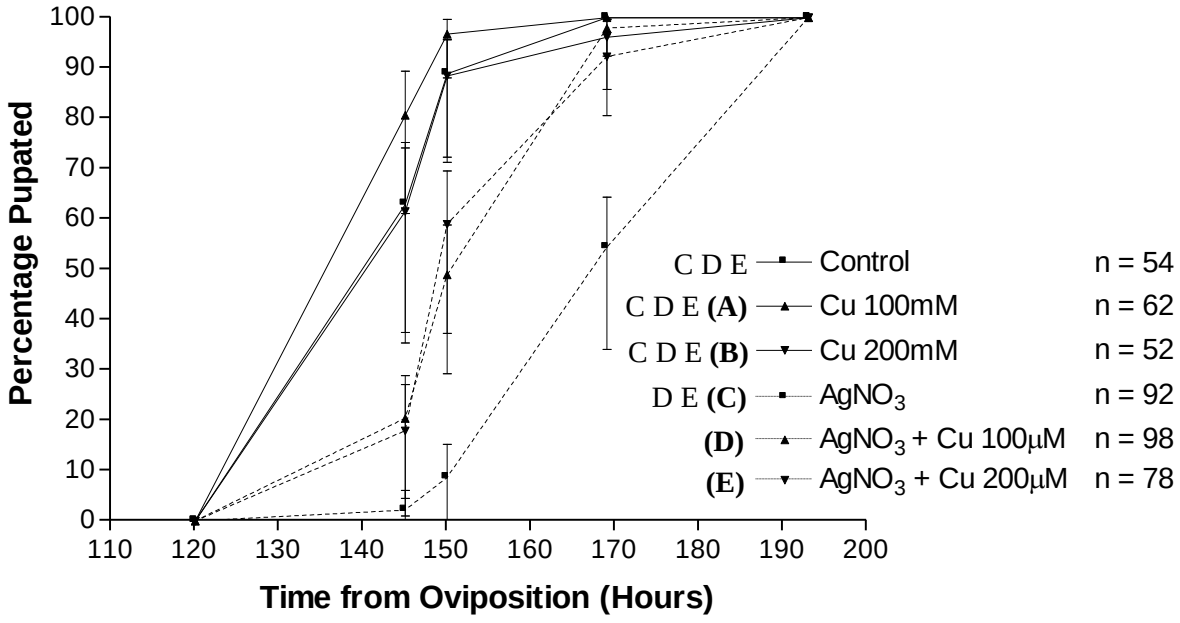

Figure 40. Time to pupation following co-administration of silver nitrate and copper nitrate. $AgNO_3$ was administered at 100 μM (added), Cu at 100 or 200 μM (added) and AgNPs at 100 μM (added) throughout larval life from hatching. Error bars represent +/- 95% CI of fraction pupated. Replicates for each group are shown in the treatment group key. Key to significance values: A, B, C, D, and E $p < 0.0038$ relative to the group with that letter displayed in brackets (Log-rank test).

in the rate of pupation relative to $AgNO_3$ alone with $HR_{AgNO_3 + Cu(NO_3)_2[100\mu M]: AgNO_3} = 2.16$ (95% CI of 1.60- 2.93; $p = 5.2 \times 10^{-8}$; log-rank test) and $HR_{AgNO + Cu(NO3)2[200\mu M]: AgNO3} = 2.16$ (95% CI of 1.55 – 3.02; $p = 2.85 \times 10^{-7}$). Despite this reduction in toxicity, mitigation was incomplete as neither concentration of $Cu(NO_3)_2$ restored pupation rates to control levels, with significant differences ($p < 0.0038$) still apparent between these groups and those exposed to no added Ag. There was no significant difference between the $AgNO_3$ exposed animals that received either 100 or 200 µM Cu ($p = 0.8778$; log-rank test).

5.3.4. Effect of silver on larval length and its interaction with copper administration

To determine how Ag affected physical growth of larvae and if Cu was able to alter any effect, larval length was measured after exposure to Ag with larvae either pre- or post-loaded with Cu. Larval length was also determined after administration of $AgNO_3$ for the entire larval period from hatching, with and without simultaneous administration of Cu.

Significant differences in larval length were found in neither the $AgNO_3$ group nor the AgNP group ($p > 0.05$; two-way ANOVA – main effects) following Ag administration during the first 45 hours of larval life (Figure 41A). As such, Cu exposure during the second 45 hours of larval life had no physical growth defect to ameliorate. Similarly, when Ag was administered to larvae from 45 - 90 hours after hatching, no significant effect ($p > 0.05$; two-way ANOVA – main effects) on larval length was seen (Figure 41B) with or without Cu administration for the first 45 hours of larval life. Following continuous exposure throughout larval life (90 hours), once again, no significant effect was observed for the Ag treated animals versus the control, nor was any difference seen between the Cu treated animals and the controls. The only significant difference ($p < 0.05$) was between Ag only treated animals and Cu treated only animals (Figure 41C). Overall, Ag in either form has little, if any, effects on the physical growth of *Drosophila* larvae.

5.3.5. Amelioration of silver induced developmental toxicity by ascorbic acid

To test if the reported amelioration of developmental toxicity to *Drosophila* caused by AgNPs (Posgai *et al*. 2011) extended to $AgNO_3$, time to pupation was determined for AgNP and $AgNO_3$ in the presence and absence of ascorbate. The threshold for significance after Bonferroni correction for 11 comparisons was 0.0045. Figure 42 shows that administration of either form of Ag resulted in a significant delay in pupation relative to

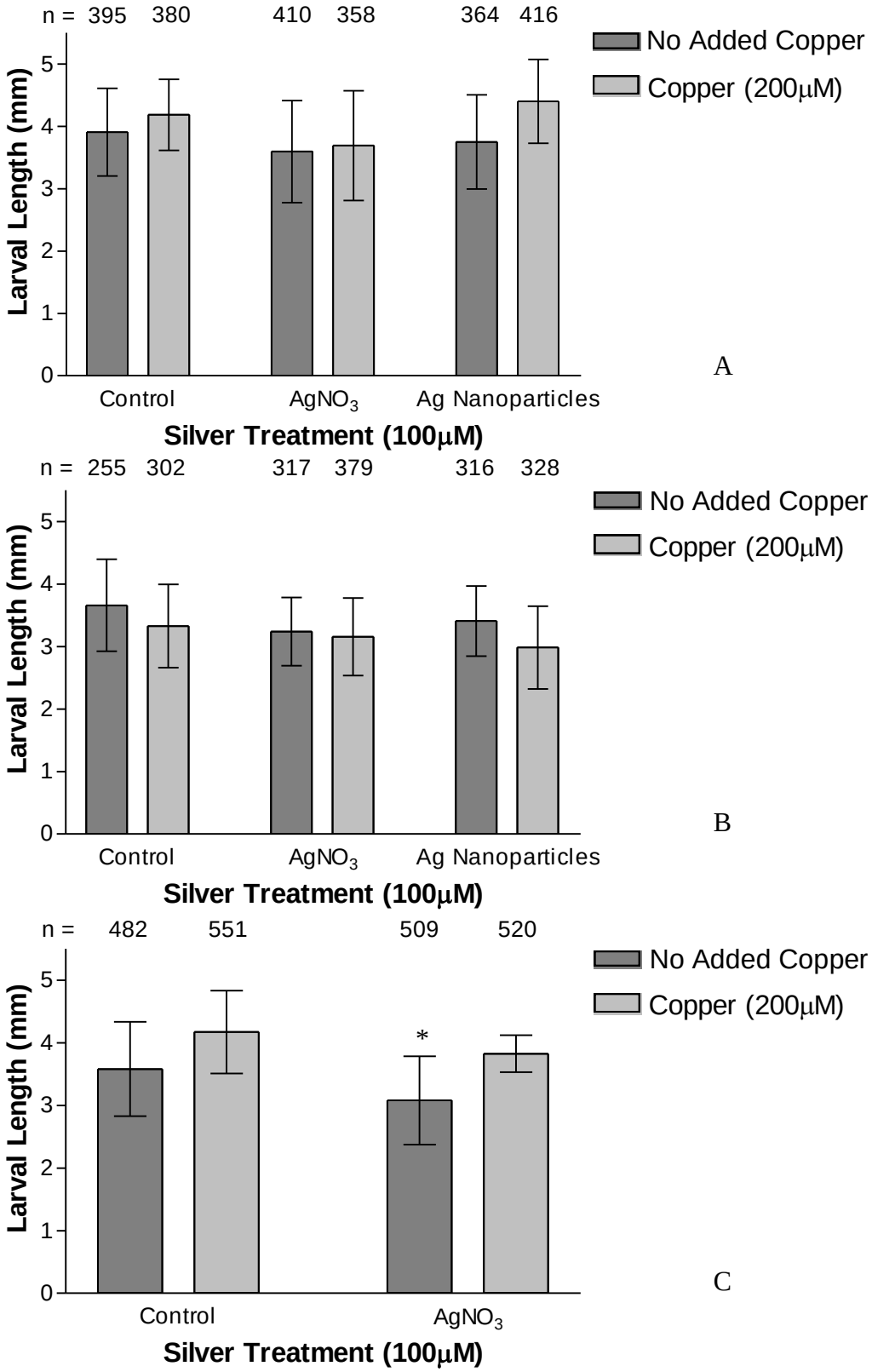

Figure 41. Larval length following 100 μM $AgNO_3$ or AgNP exposure and treatment with 200 μM Cu. Larvae were either (A) preloaded or (B) post-loaded with Cu, receiving Ag after or before Cu administration respectively. (C) Other larvae were exposed to $AgNO_3$ and Cu simultaneously. Error bars represent +/- 95% CI. Replicates for each group are shown above the bar. Key to statistical significance: (panel C) * $p < 0.05$ relative to Cu exposed control. No other significant differences were found between any groups in any panel (two-way ANOVA – main effects).

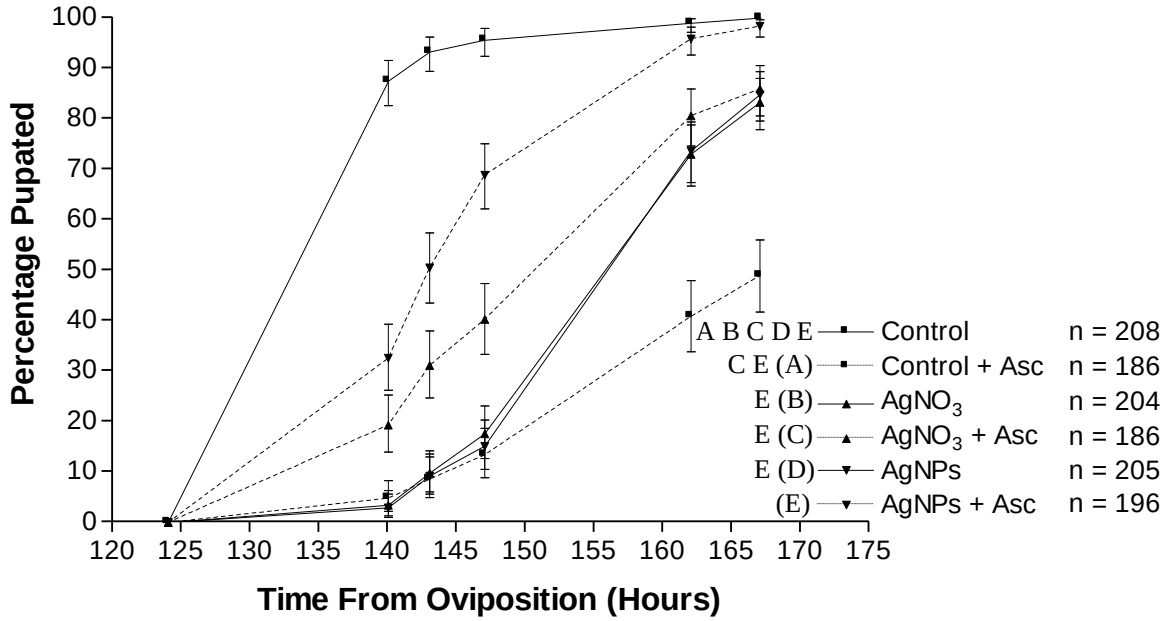

Figure 42. Time to pupation following co-administration of $AgNO_3$ or AgNPs with ascorbic acid (50 mM). Ag was administered at 100 µM (added) for the entire larval lifespan from hatching. Error bars represent +/- 95% CI of fraction pupated. Replicates for each group are shown in the treatment group key. Key to significance values: A, B, C, D, and E $p < 0.0045$ relative to the group with that letter displayed in brackets (Log-rank test).

control ($p < 0.0045$), but they were not significantly different from one another. The group with the most developmental retardation was the ascorbate only group. This group was significantly slower to develop than any other including both Ag treated groups ($p < 0.0045$). However, when ascorbate was co-administered with AgNPs a marked recovery in pupation rate was seen relative to AgNPs or ascorbate alone ($p < 0.0045$). Despite the ascorbate treated $AgNO_3$ group showing a trend towards a restored pupation rate this group failed to reach significance relative to $AgNO_3$ group only ($p > 0.0045$; log rank test). Nonetheless, the ascorbate treated $AgNO_3$ group did show significant recovery relative to the ascorbate only group ($p < 0.0045$). Both ascorbate treated AgNP and $AgNO_3$ exposed animals were significantly slower to pupate ($p < 0.0045$) than controls not exposed to ascorbate.

5.3.6. Effects of silver nitrate and silver nanoparticles on paraquat resistance of adult *Drosophila*

To explore how Ag exposed flies responded to an externally applied oxidative stress, a paraquat resistance assay was performed on adult flies (5 days post-eclosion) that had been exposed to Ag throughout their lifespan from hatching. Bonferroni correction for 3 HR ratio comparisons yielded a significance threshold of 0.017 ($\alpha = 0.05$). Paraquat

administration to control flies caused rapid death of exposed flies with a median lifespan following paraquat exposure of 48 hours. None of the animals exposed to Ag in the absence of paraquat died within the timescale of the experimental animals (data not shown) indicating that the induction of premature death was attributable to the paraquat and not simply an artefact of the nutritionally limited diet. Figure 43 shows that the animals that received AgNPs died more rapidly in response to treatment with paraquat than the paraquat exposed control with $HR_{AgNPs:Control} = 2.13$ (95% CI of 1.18 – 3.86; p = 0.0061). The $AgNO_3$ group, however, showed no increase in their susceptibility to the toxicity of paraquat compared to control (p = 0.1217; log-rank test). The $AgNO_3$ plus paraquat group compared to AgNP plus paraquat group showed no significant difference in pupation rate (p = 0.1196; log-rank test).

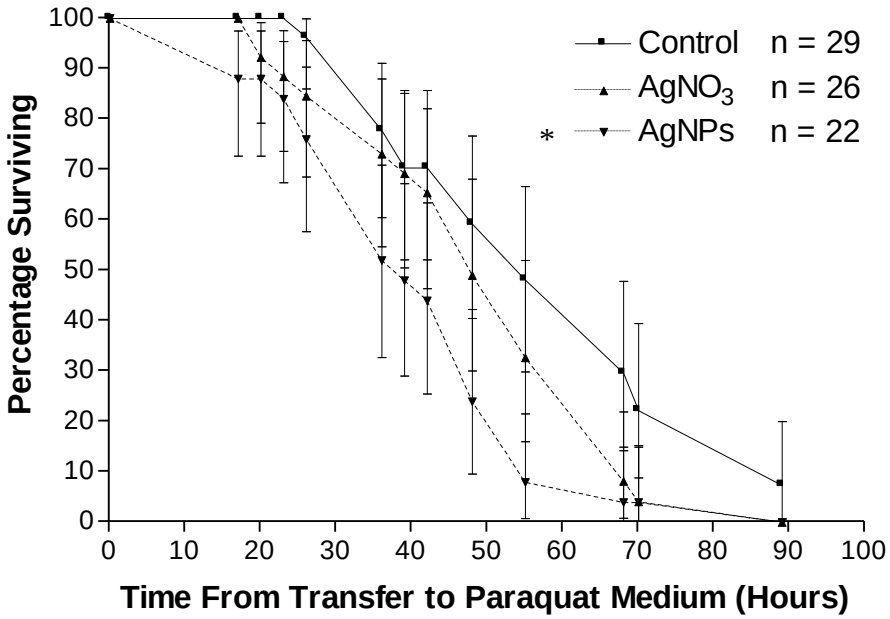

Figure 43. Survival plot of *Drosophila* exposed to 20 mM paraquat subsequent to receiving 100 μM $AgNO_3$ or AgNPs until five days post eclosion. Error bars represent +/- 95% CI of mortality fraction. Replicates for each group are shown in treatment group key. Key to significance values: * p < 0.17 relative to the paraquat exposed control (log-rank test).

5.4. Discussion

When exposed to $AgNO_3$ or AgNPs, *Drosophila* larvae exhibited a concentration-dependent developmental toxicity up to 500 μM. Larvae were considerably more sensitive to $AgNO_3$ and increasing the concentration of AgNPs above 500 μM resulted in a reversal of the toxicity trend. Larval length was unaffected by either form of Ag, even when development was delayed. The developmental delay caused by $AgNO_3$ was mitigated by

additional Cu (II) ions whereas both $AgNO_3$ and AgNP toxicity were reduced in the presence of ascorbic acid although this effect was more pronounced with the latter. Although $AgNO_3$ and AgNP exposed animals were both more sensitive to paraquat than controls, the effect was greatest after AgNP exposure. That is, AgNP exposed animals were significantly more susceptible than controls whereas $AgNO_3$ exposed animals were significantly different from neither the control or AgNP groups, suggesting an intermediate effect but a statistically underpowered experiment.

5.4.1. Experimental limitations

The first limitation of this study is that AgNPs clearly interact with the medium. The brown colouration of the medium caused by AgNPs fades over the course of a few days even without additional ions Figure 30. This, combined with the prevention of this fading by ascorbic acid, suggests that oxidative dissolution is occurring even in otherwise unadulterated medium. However, as discussed below this has allowed some insight into particle versus ion mediated toxicity. The second limitation is that viability estimates at later stages of development may be an underestimate of the toxicity due to the unavoidable selection of Ag tolerant larvae.

5.4.2. Comparison of the developmental toxicity of silver nitrate and silver nanoparticles

Up to concentrations of 500 µM both $AgNO_3$ and AgNPs caused a concentration-dependent reduction in pupation and eclosion success (Section 5.3.1.3) along with delayed pupation (Section 5.3.1.5). The toxicity of $AgNO_3$ appeared greater than AgNPs in these assays. This differential in developmental toxicity is not due to reduced body burdens as no significant differences in tissue levels were found between the forms of Ag (Figure 31) although it is possible tissue distribution patterns differed. As no difference in physical growth was found (Section 5.3.4), it appears that the toxicity is mediated through delay of maturation rather than reduced food intake or a metabolic effect that delayed the larvae's ability to attain critical mass. As interference with Cu mediated processes could mediate some of the toxic effects of Ag, (Yaffe 1955, Bertinato *et al*. 2010, Ilyechova *et al*. 2011) developmental toxicity of $AgNO_3$ was assessed in the presence of added Cu ions. Co-administration of Cu and Ag, despite ameliorating the developmental delay could not mitigate it completely, suggesting that Cu independent mechanisms also operate. It was

not possible to test the effects of Cu on AgNP induced developmental toxicity due to their interaction within the medium (see Section 4.3.2 for examination of this interaction). Three studies have reported that AgNP exposure causes developmental toxicity in *Drosophila* in an exposure concentration-dependent manner (Key *et al*. 2011, Panacek *et al*. 2011, Posgai *et al*. 2011). Panacek *et al*. (2011) found a threshold for reduced pupation success at ~400 µM versus 200 µM seen in this work. In this study, a U-shaped concentration-response curve was found for AgNPs that was not found for $AgNO_3$ (See Sections 5.3.1.1, 5.3.1.2, and 5.3.1.4). Although this has not been explicitly described in the literature, there are suggestions that it may have been present in some studies. For instance, when Key *et al*. (2011) increased AgNP exposure concentrations from 830 µM to 1.6 mM a recovery in the number of successful eclosions was seen. The reasons for this U-shaped concentration-response are not clear but certain explanations may be excluded. Increased particle agglomeration at higher concentrations is not supported as a mechanism as the TEM images of high concentrations of AgNPs in fly food medium show no evidence of agglomeration. It is possible that less Ag was taken up by the larvae at these concentrations but this hypothesis cannot be tested with the data available here. The literature suggests that dissolution rates increase with AgNP concentration so increased free Ag (I) is an unlikely cause (Lee *et al*. 2012). However, Ho *et al*. (2011) have found that Cl^- ions retarded dissolution of AgNPs due to the formation of a passivation layer of AgCl on the AgNP surface. That this effect was not evident in the dissolution studies described in Section 4.3.2 is probably attributable to the stochiometric ratios of Ag to Cl^- ions, allowing the formation of multi-chloro Ag complexes. Perhaps, at high AgNP concentrations in the food medium Cl^- became limiting and instead of the generation of soluble multi-chloro Ag complexes, an AgCl layer forms preventing further dissolution.

Whatever similarities may exist between the toxic effects of $AgNO_3$ and AgNPs, one fundamental difference is the particulate presentation of AgNPs. These particles, being of nanometre dimensions are potentially able to evade the normal barriers to absorption (Park *et al*. 2010b, Lin *et al*. 2012) and it is therefore possible that a component of toxicity from nanoparticles may arise simply from the presence of foreign particulates within the cells and tissues, regardless of the surface chemistry of the particle. However, there was no evidence for such toxicity suggesting that the effects of AgNPs are specific to either the elemental composition of particles themselves or the ions that they release.

Posgai *et al*. (2011) found a slight amelioration of developmental delay in *Drosophila*, following simultaneous exposure to AgNPs and ascorbic acid and suggest that oxidative stress is critical for toxicity. A similar effect was observed here and a similar, but lesser, effect was also noted for $AgNO_3$ (Section 5.3.5). Similarly, the paraquat assay showed that both forms of Ag are able to exacerbate externally applied oxidative stress, with AgNPs being more potent (Section 5.3.6). This suggests that the oxidative stress derived from AgNPs must have a particle specific component as even total *ex vivo* dissolution would be unable to exceed the toxicity of an equal concentration of Ag (I) ions supplied as such. However, neither of these observations provides unequivocal evidence for oxidative stress mediating the developmental toxicity. One possible reason for reduced toxicity of AgNPs when administered with ascorbic acid is reduced dissolution. The data of Section 6.3.2 suggest that this is the mechanism behind ascorbate based amelioration of AgNP induced de-pigmentation and it is reasonable to hypothesise that it contributed to the reduction in developmental toxicity also. The moderating effect of ascorbic acid upon $AgNO_3$ toxicity suggests that an *in vivo* antioxidant effect is also possible following exposure to either form of Ag.

5.4.3. *Drosophila* as a model for Ag induced developmental toxicity

Considerable differences in the tolerance of *Drosophila* to Ag are seen across the literature compared to the work here (Rapoport 1939, Di Stefano 1943, Kroman and Parsons 1960) and the reasons are likely to include strain differences and medium composition. The greatest impediment to the implementation of *Drosophila* as a model for AgNP toxicity is the interaction of AgNPs with the medium. That fading of AgNP induced colouration of the medium is prevented by addition of ascorbate suggests that oxidative dissolution is occurring without the addition of any oxidising species and, that *Drosophila* may not be exposed to the form of Ag that is expected from the original formulation. This need not be an impassable obstacle but does require some thought as to how it may be overcome. Daily changing of the medium is a possible solution. Ascorbate cannot be used *specifically* for this purpose owing to its inherent antioxidant effects. Regardless of the developmental toxicity of liberated Ag (I) ions, it would seem that non-dissolved AgNPs exert developmental toxicity to *Drosophila* and that this should be pursued further.

5.4.4. Conclusions

Drosophila exhibited developmental toxicity in response to both $AgNO_3$ and AgNPs. Like Al, Ag seems to affect *Drosophila* during the potentially vulnerable periods of growth and tissue remodelling that occur during larval life and especially during metamorphosis. Under the conditions used here the toxicity of AgNPs appears to derive from both particle specific and dissolution mediated effects. To gain a better understanding of the relative contributions of these effects, it is vital that a better understanding of the interactions of the particles with the food medium is gained.

Chapter 6. Cuticular abnormalities and preliminary screens for other silver induced toxicity

6.1. Introduction

The Ag (I) ion shares many chemical similarities with its isoelectronic counterpart Cu (I) (Wiberg *et al*. 2001) although only Cu has a physiological role (Cowan 1997). Therefore Ag (I) ions have the potential to interfere with processes involving Cu (I), especially as Ag (I) ions often have a stronger affinity than Cu for the ligating groups of cuproproteins (Lee *et al*. 1998). For example, the mammalian copper transporter, Ctrl1 is competitively inhibited by Ag (I) ions whilst facilitating the entry of Ag (I) into cells (Bertinato *et al*. 2010). Silver is incorporated into ceruloplasmin in both rats (Whanger and Weswig 1970, Ilyechova *et al*. 2011) and mice (Ilyechova *et al*. 2011), resulting in a loss of enzyme activity. The copper dependent enzyme, tyrosinase, which is responsible for oxidising various phenolics and precursors in the melanization cascade (Sugumaran 2002), is also potently inhibited by Ag (I) ions; such inhibition has been demonstrated with enzyme isolated from mammals (Lerner 1952), cephalopods (Lacoue-Labarthe *et al*. 2009), and *Drosophila* (Yaffe 1955).

Rapoport (1939) was the first to note de-pigmentation in *Drosophila* following administration $AgNO_3$. Finding an apparent mimicry of the pigmentation mutant, "yellow", he classified it as a phenocopy, a then recently coined term signifying a phenotypic change, usually ascribable to a mutation, caused by an environmental agent (Mamun *et al*. 2011). A more detailed analysis by Di Stefano (1943) described how the effect extended to the bristles, microchaetae and malpighian tubules but rightly disputed the assertion that the animal resembled a yellow mutant. That work also noted a softening of the "chitin". Yaffe (1955), using haemolymph derived from pre-pupae and tyrosine as a substrate, suggested that the mechanism involved inhibition of either the enzyme tyrosinase or an enzyme further downstream in the melanin synthesis cascade. Tyrosinase is closely related to laccase, another multifunctional enzyme with similar functions but differing substrate specificities (Nappi *et al*. 1991). Together, these enzymes are classed as polyphenoloxidases (PPOs) and their presence and activity in insect cuticle is often simply referred to as PPO.

The PPO system is not confined to melanization reactions; it is also integral to sclerotization of the cuticle (Andersen 2010). These exoskeletons are toughened by a complex set of sclerotization reactions that cross link proteins forming a hard, waterproof matrix that protects the soft tissues of the animal beneath. These reactions involve many highly reactive intermediates, but many have tyrosine as their ultimate building block. Through initial reactions, substrates for PPO are formed that then go on to be oxidised resulting in the process of sclerotisation (Hopkins and Kramer 1992). $AgNO_3$ exposed animals are not only de-pigmented but also appear softer than their unexposed counterparts, although this apparent difference has not been tested experimentally (Di Stefano 1943). With PPO forming a key component of the sclerotization process, inhibition of this enzyme by Ag (I) ions is a likely mechanism.

Five studies have noted de-pigmentation in *Drosophila* exposed to AgNPs of varying size and surface chemistry (Gorth *et al*. 2011, Key *et al*. 2011, Panacek *et al*. 2011, Posgai *et al*. 2011, Armstrong *et al.* 2013). However, four of these made no suggestion for the role of Ag (I) ions (Gorth *et al*. 2011, Key *et al*. 2011, Panacek *et al*. 2011, Posgai *et al*. 2011) and the study conducted by Armstrong *et al.* (2013) concluded that Ag (I) ions were definitely *not* responsible for de-pigmentation. The conclusion of Armstrong *et al*. (2013) was reached after noting minimal Ag (I) ion release from AgNPs in water and comparing the resulting Ag (I) ion concentrations with those used by Di Stefano (1943). The author hypothesises that Ag (I) ions, not intact AgNPs, are the driver behind the de-pigmenting effect of both $AgNO_3$ and AgNPs. This hypothesis is based on the knowledge that Ag (I) ions are definitely capable of causing de-pigmentation, that no alternative ion-free, particle-specific mechanism was proposed, and that extrapolating the concentrations of Ag (I) ions released in water to *Drosophila* medium is unwise. The last point is made clear when considering that additional ions, e.g. Cu (II), Fe (III) and Cl^- (all likely to be present in the medium) substantially alter the rate of dissolution (Section 4.2.3). Simultaneous administration of ascorbic acid prevents the de-pigmentation seen in AgNP exposed flies (Posgai *et al*. 2011), possibly as a consequence of reduced dissolution in the medium (Section 5.4.2). Furthermore, a wide range of sensitivities of *Drosophila* to many metals is reported across the literature, and the tolerance of *Drosophila* in this study to $AgNO_3$ is many fold lower than those reported by Di Stefano (1943) and other authors (Rapoport 1939, Kroman and Parsons 1960) meaning such comparisons should be made with caution.

Panacek *et al*. (2011) suggest that the importance of dopamine as an intermediary in the melanization cascade and its role in the general stress response might mean that a disturbance in its synthesis is responsible for the effects of AgNPs in *Drosophila*. In work that has only so far been published as a conference abstract, an increase in transcription (from a total body mRNA extraction) at the pale genetic locus (*ple*) has been reported (Reaves *et al*. 2012). The gene product of this locus is tyrosine hydroxylase (TH) (Neckameyer and Quinn 1989, Neckameyer and White 1993) and is important for the initial oxidation of tyrosine to L-DOPA during melanin and dopamine synthesis (True *et al*. 1999). Increased transcription of *ple* seems an unlikely cause of de-pigmentation but rather the consequence of disruption of a complex feedback loop involving PPO. However, if increased *ple* transcription ultimately increases TH activity, then altered levels of L-DOPA and dopamine are likely to ensue (Daubner *et al*. 2011). Dopamine is a potent biogenic amine with diverse biochemical effects and Ag could therefore potentially affect behaviour, neurological function and the heart.

The aims of this chapter were to clarify the role that Ag (I) ions play in the de-pigmenting effects of AgNPs and if the interference with tyrosine metabolism that drives Ag (I) ion induced de-pigmentation may extend to other processes requiring tyrosine metabolites. To address these aims, firstly, the de-pigmenting effects of $AgNO_3$ and AgNPs were compared. The Cu concentration of the media was manipulated to antagonise any Ag (I) ion mediated effects, such that the relative contributions of Ag (I) ions and AgNPs to AgNP induced de-pigmentation may be teased apart. Next, the effects of ascorbic acid on Ag induced de-pigmentation were examined in an attempt to understand how it is able to mitigate the de-pigmenting effect of Ag in the form of NPs (Posgai *et al*. 2011). Finally, preliminary screens, examining other possible physiological disturbances mediated by alterations to Cu metabolism, were made by assessing the effect of Ag on a simple behavioural paradigm and on heart-rate.

6.2. Methods

6.2.1. Production of a pigmentation scale for grading purposes

In order to quantify and compare the cuticular pigmentation effects of both forms of Ag, and to simultaneously determine whether Cu administration was able to alter an AgNP induced toxic phenomenon, a method was devised to measure colour of the cuticle. To

produce a scale for assessing fly colouration, a graded array of de-pigmented flies was produced by placing 50 eggs into vials containing a range of $AgNO_3$ concentrations, in standard medium, from 10 μM – 120 μM (in 10 μM increments). These were incubated in the dark at 25°C until eclosion. Once eclosed these flies were placed in fresh vials containing the same concentration of $AgNO_3$ in standard medium for 5 days to mature. The flies were then collected and killed by freezing at -18°C for 24 hours. As male and female *Drosophila* have different pigmentation patterns (Kopp *et al*. 2000), two scales of pigmentation were produced: one for females and one for males. Flies were selected visually from any of the vials (regardless of Ag concentration and solely upon the basis of colour) to create a graduated scale in which each level was distinctly lighter or darker than the others. With females, four pigmentation levels were produced but males could not be separated into more than three unambiguously different pigmentation levels. With the scales established (Figure 44) (images used for assessment were full colour), flies were placed on black filter paper and photographed using a Leica DFC 420 C camera attached to a Leica MZ10F dissection microscope.

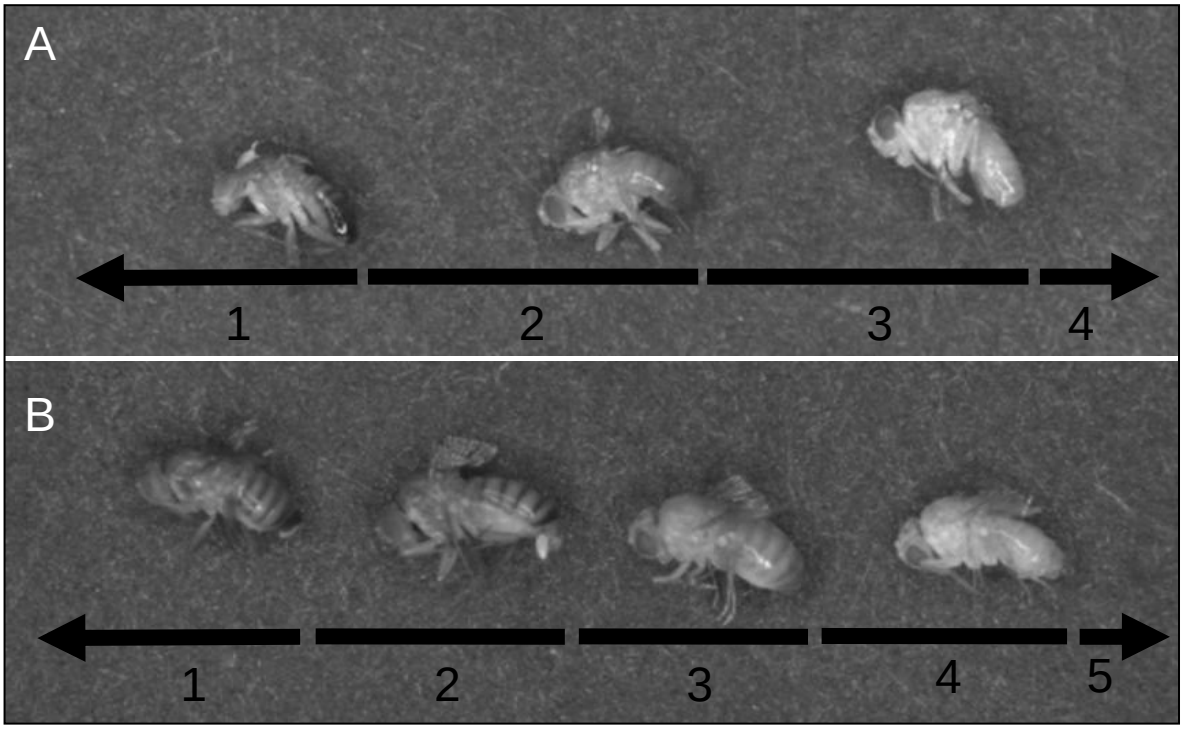

Figure 44. Pigmentation Scales for *Drosophila*. (A) Male and (B) female. Arrows illustrate the range of each pigmentation level.

The scale was used later (Section 6.2.2) to score a fly based upon overall pigmentation level. A blinded assessor was instructed to score a fly with the level that was **at least as**

dark as equivalent number on the scale but **darker** than the number below it. At the pale end of each scale was an additional level that was classified as **not as dark as** the highest level associated with a fly image (3 for males and 4 for females) making a total range of scores of 1 - 4 and 1 - 5 for males and females respectively.

6.2.2. Determining the effects of silver and copper on adult cuticle pigmentation

To test the effects of $AgNO_3$ and AgNPs, administered throughout development, on pigmentation as well as the effects of simultaneous Cu exposure upon $AgNO_3$ induced effects, 50 eggs were added to each of five vials containing either control, $AgNO_3$ (100 μM), AgNPs (100 μM), $Cu(NO_3)_2$ (200 μM) or a combination of $AgNO_3$ (100 μM) and $Cu(NO_3)_2$ (200 μM). The eggs were incubated in the dark at 25 °C until eclosion, at which point the adult flies were transferred to fresh vials containing media of the same composition to that which they had developed on. The flies were left to mature for a further 5 days, killed and individually photographed as described above.

As with the larval length studies (Section 5.2.1.3), temporal separation of AgNP and Cu administration was needed and a preload based design chosen as a pilot suggested that this was the best arrangement for maximum de-pigmentation to be seen. For this experiment, 50 *Drosophila* eggs per vial (five vials in total) were placed on Cu $(NO_3)_2$ (200 μM) containing medium or control medium and incubated at 25°C in the dark. Sixty-nine hours AEL (48 hours post-hatching) larvae were removed from the medium (Section 2.5) and transferred to either control medium or that containing $AgNO_3$ or AgNPs (100 μM). These larvae were then incubated until eclosion. Following a five day maturation period flies were killed and photographed as described above. Individual fly images were separated on the basis of sex, and each sex randomised and assessed blind against the relevant scale described above by an independent assessor. Once scoring was complete, the blinding was removed and data analysed.

6.2.3. Testing oxidative stress as a mechanism underlying de-pigmentation

6.2.3.1. Paraquat administration throughout larval life

To test the hypothesis that systemic oxidative stress was sufficient to induce de-pigmentation, larvae were fed paraquat throughout development. Paraquat was added directly to standard *Drosophila* medium as it has been shown to not interact adversely

Drosophila medium (Rzezniczak *et al*. 2011). Fifty *Drosophila* eggs per vial were placed upon medium spiked with a sub-lethal concentration of paraquat (10 mM); a pilot using 20 mM was lethal to all larvae. Paraquat spiked medium (10 mM) was produced by adding 10 ml, freshly prepared paraquat stock solution (100 mM; prepared as in Section 5.2.2.2 but at x 3 volume) to 84.9 g (90 ml) molten *Drosophila* medium. Five paraquat and 5 control vials were then incubated in the dark (to be comparable with Ag exposure) at 25 °C until eclosion. Animals were then scored for pigmentation as in Section 6.2.2.

6.2.3.2. Effect of ascorbic acid supplementation upon silver induced de-pigmentation and developmental delay

To test the interaction of antioxidants with the de-pigmentation effects of $AgNO_3$ and AgNPs, larvae were exposed to each form of Ag either alone or in combination with ascorbic acid. Fifty eggs, for each of five vials, were placed on either control media or that spiked with $AgNO_3$ (100 μM), AgNPs (100 μM), ascorbic acid (50 mM) alone, or ascorbic acid in combination with either form of Ag. These vials were treated as those in Section 6.2.2 with eclosed adults allowed to mature for five days on medium identical to that on which they developed. Mature flies were then killed and scored for pigmentation.

To examine the hypothesis that the interaction between Ag and ascorbic acid occurred *ex vivo*, sequential additions, in differing orders, of Ag and ascorbic acid were performed. Vials were prepared as described above with the following modifications. Three methods of preparation were used for media containing both $AgNO_3$ and ascorbic acid. The first of these involved adding ascorbic acid (50 mM) to the molten medium, stirring continuously for three minutes before adding $AgNO_3$ (100 μM) and mixing thoroughly; the second had the reagents added in reverse order with the same timing. The third method involved direct mixing of $AgNO_3$ and ascorbic acid in the volume of water to be added to the medium creating concentrations of 0.77 mM and 385 mM, respectively. This solution was left to stand for three minutes before being added in its entirety to the medium and mixed, resulting in a final dilution of reagents identical to that in the other two methods.

Fifty eggs per vial were collected and placed upon each type of medium prepared above, plus three further treatments containing, respectively, no additive, $AgNO_3$ (100 mM) or

ascorbic acid (50 mM). Five vials per treatment were incubated and adult pigmentation was scored as described in Section 6.2.2.

6.2.4. Testing cuticle integrity through desiccation and starvation resistance

To determine whether any similarities, regarding pigmentation, that may exist between $AgNO_3$ or AgNPs extend to other cuticular processes, sclerotization was examined. Rather than attempting to measure any property of the cuticle directly, its ability to retain water was assessed by investigating desiccation resistance in Ag exposed flies. Males and females were analysed separately as they have different tolerances to both desiccation (Matzkin *et al*. 2007) and starvation (Matzkin *et al*. 2009). Five vials of 20 eggs per vial were placed upon medium spiked with either $AgNO_3$ (50 µM) or AgNPs (50 µM). These eggs were incubated under standard conditions until all animals had eclosed. Flies were then transferred to fresh, spiked medium and allowed to mature for 5 days before desiccation. Desiccation chambers were made from a *Drosophila* vial with a 5 g silica gel sachet placed at the bottom. To prevent direct contact between flies and the desiccant a Droso-Plug™ (Dutcher Scientific UK Ltd, Romford, UK) was pushed down into the tube. This would form the platform upon which flies would stand. Flies were transferred under CO_2 anaesthesia from their feeding vials into the desiccation vials which were then covered with Parafilm pierced in three places with a needle to permit gas exchange. To ensure that any effects seen from this assay were due to desiccation and not starvation, identical assays were performed but the desiccant was replaced with a 3% non-nutritive plain agar creating a starvation chamber. Fly deaths were recorded at approximately 2 hourly intervals for desiccation resistance and every 8-12 hours for the starvation assay. Flies were not removed as they died, due to the short time course of the experiment; removal of corpses has been shown to be unnecessary as cannibalism does not occur (Huey *et al*. 2004).

6.2.5. Effects of silver exposure upon pre-pupal heart rate in *Drosophila*

To ascertain if Ag administration had any effect upon metabolic rate, the pre-pupal heart rate was measured as a marker. Furthermore, any effects on this organ could represent interference with the synthesis of the biogenic amine, dopamine (Johnson *et al*. 1997) and thus prompt further study. Pre-pupal heart rate was measured manually as has been reported (Robbins *et al*. 1999). Five vials of 50 eggs per vial were collected and placed on

control medium or medium spiked with either $AgNO_3$ (100 µM) or AgNPs (100 µM). The vials were then incubated under the conditions specified in Section 2.2 until 3rd instar wandering (post-feeding) larvae were seen. These larvae were watched closely for the next few hours to determine when they began to pupate. Larvae first slow down and ultimately stop crawling, while at the same time everting their anterior spiracles. As the spiracles become maximally everted the larval body shortens and becomes a white pre-pupa (Demerec 1994). At this stage body wall movements have all but ceased and the dorsal tube (heart) can be observed beating unimpeded by extraneous muscle contractions. The pre-pupa was carefully removed from the vial wall with a moist paintbrush and transferred to a glass microscope slide.

The slide was placed under a dissection microscope and the pre-pupa observed at x 50 magnification. The pre-pupa was allowed to rest for 5 minutes to prevent any disturbance to heart-rate resulting from handling. Heart rate was determined by counting the number of contractions per minute, counted over two minutes. Forty-five pupae were examined for each treatment group.

6.2.6. Effects of silver upon negative geotactic behaviour in *Drosophila*

To test for a possible behavioural deficit that may emerge in *Drosophila* following exposure to Ag at concentrations lower than those that produced developmental delays, the negative geotaxis assay was performed upon adult flies that had received Ag throughout development. In addition to control, the experimental groups tested were $AgNO_3$ and AgNPs at concentrations of 10 µM, 50 µM and 100 µM. The assay was performed as described in Section 3.2.6.

6.3. Results

6.3.1. Effects of silver nitrate and silver nanoparticles on cuticle pigmentation and its reversal by copper nitrate administration

To determine how Ag affected pigmentation and if Cu interacted with any such effect, flies were blind scored against a scale formed from sets of male or female flies (separate scales were used as males and females have different pigmentation patterns, meaning they are incomparable) of gradually diminishing colour (four levels for male (Figure 44A) and five

for female (Figure 44B). Firstly, de-pigmentation was measured following $AgNO_3$ or AgNP (100 µM) exposure in food during the entire larval period, with the former also tested in combination with Cu. The effects of Cu on AgNP induced de-pigmentation could not be assessed in this way owing to the suggested oxidative dissolution of AgNPs (examined in Section 4.2.3). Consistent with previous non-quantitative observations made earlier in this study, a marked de-pigmentation occurred in *Drosophila* that had received $AgNO_3$, whether male (Figure 45A) or female (Figure 45B) ($p < 0.001$). In contrast, *Drosophila* that had received Cu only showed no difference from control ($p > 0.05$; Dunn's test) demonstrating that Cu does not alter pigmentation. However, animals that had received both $AgNO_3$ and $Cu(NO_3)_2$ (200 µM) were significantly darker than those exposed to $AgNO_3$ alone ($p < 0.001$) and not significantly different (Dunn's test) from either control (no Ag or Cu) or Cu only exposed.

As it was not possible to determine if the mitigating effect of supplementary Cu extended to the loss of colouration caused by AgNPs, owing to chemical incompatibility, a pre-loading schedule was tested. A pilot suggested that Ag administration in the first 48 hours of larval life hours did not alter colouration whereas exposure to Ag from 48 hours after hatching until pupation was sufficient to cause this effect. For this reason only Cu was administered for 48 hours prior to administration of Ag, so that the two treatments could not interact. Both forms of Ag administered in this fashion, in the absence of Cu pre-loading, caused a significant change in colouration in both sexes compared to control ($p < 0.001$ in all cases) signifying that Ag exposure in the last half of larval development was able to robustly cause this effect (Figure 46A and B). No change in colouration was seen following $Cu(NO_3)_2$ administration alone during the first 48 hours of larval life ($p > 0.05$; Dunn's test). However, when preloaded into larvae that subsequently received either form of Ag, the Cu abolished the de-pigmentation caused by Ag.

Significant differences were seen in both sexes between the Ag exposed animals that had and had not received Cu preloading ($p < 0.001$ in all cases; Dunn's test). Furthermore, no significant difference was seen between any copper treated group and its respective control ($p > 0.05$; Dunn's test), indicating apparently complete recovery. Examples of flies from different groups are presented in Figure 47.

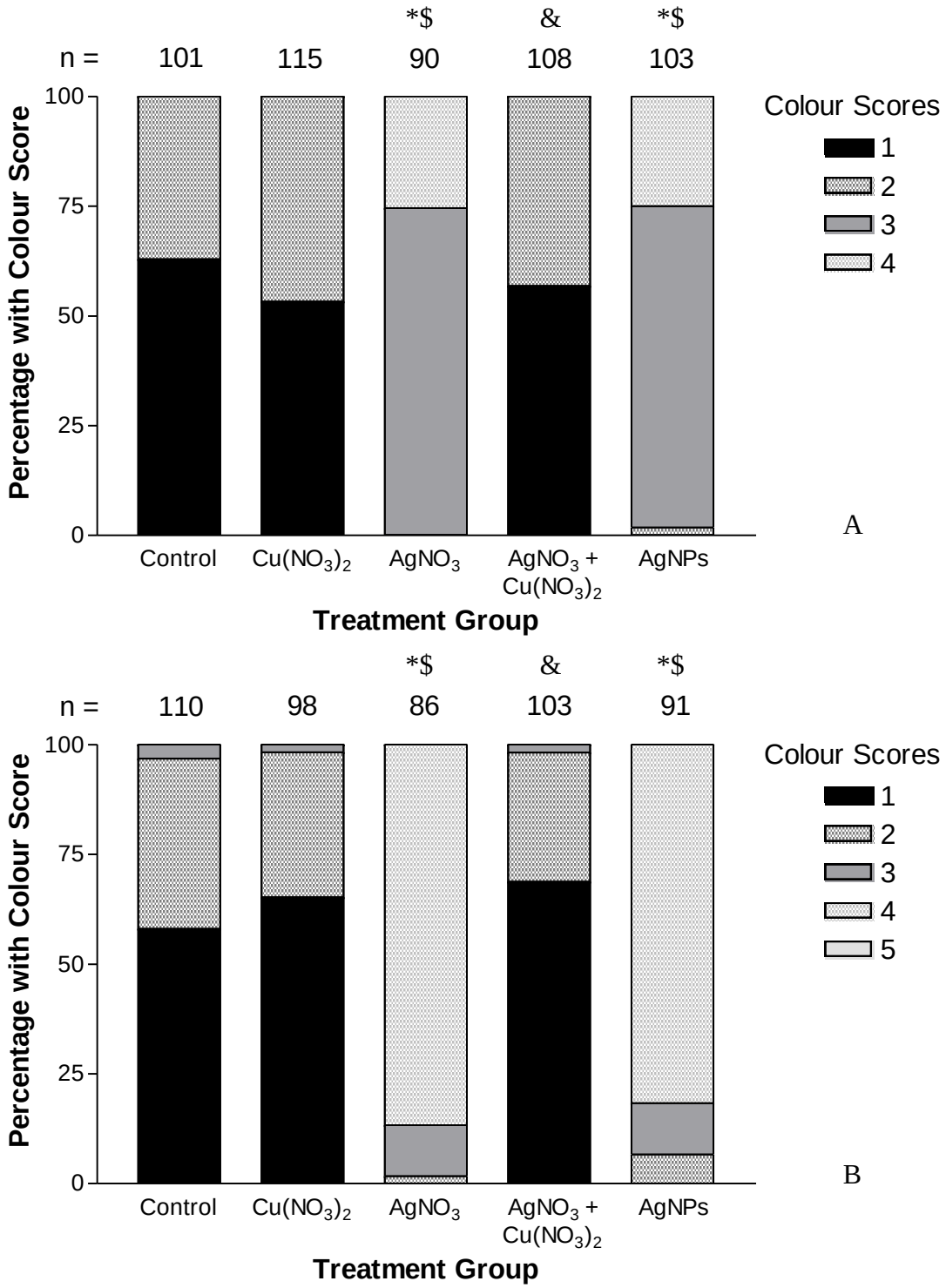

Figure 45. Pigmentation score following Ag and Cu administration. (A) Males and (B) females received (via food) either Cu $(NO_3)_2$ (200 µM), $AgNO_3$ (100 µM), both simultaneously or AgNPs (100 µM) throughout their larval period from hatching and five days post eclosion. Segments represent percentage of flies scoring at each pigmentation level where 1 is fully pigmented and 5 is de-pigmented. Replicates for each group are shown above the respective group. Key to p values (Dunn's test following Kruskal-Wallis test): * $p < 0.001$ relative to control (no added Ag); \$ $p < 0.001$ relative to Cu; & $p < 0.001$ relative to $AgNO_3$.

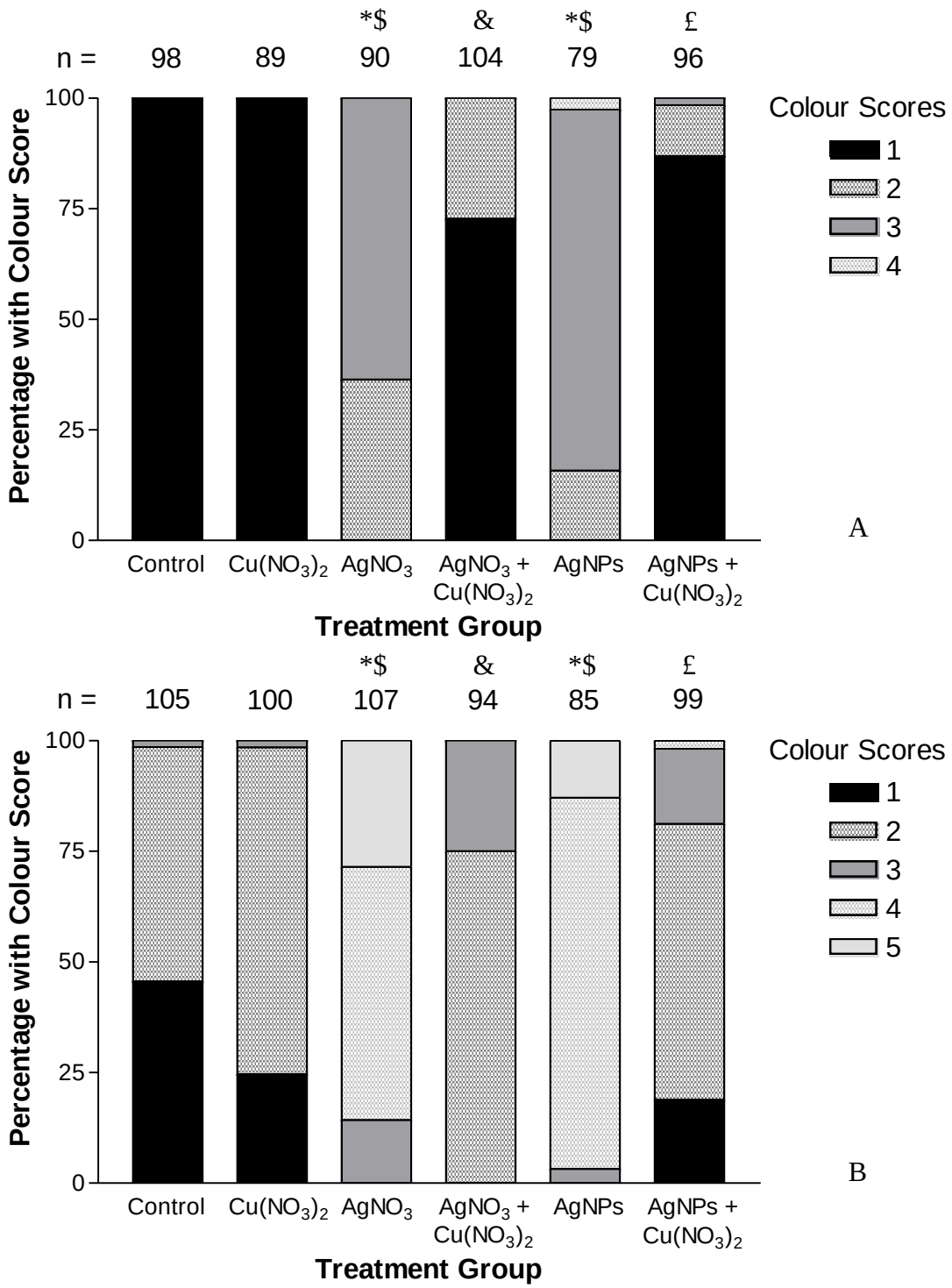

Figure 46. Pigmentation score following Ag and Cu administration. (A) Males and (B) females received (via food) $AgNO_3$ or AgNPs (100 µM) at 45 hours after hatching until 5 days post eclosion having been preloaded with $Cu(NO_3)_2$ (200 µM) for 45 hours from hatching. Segments represent percentage of flies scoring at each pigmentation level. Replicates for each group are shown above the respective group. Key to p values (Dunn's test following Kruskal-Wallis test): * $p < 0.001$ relative to control (No added Ag); $ $p < 0.001$ relative to Cu; & $p < 0.001$ relative to $AgNO_3$; £ $p < 0.001$ relative to AgNPs.

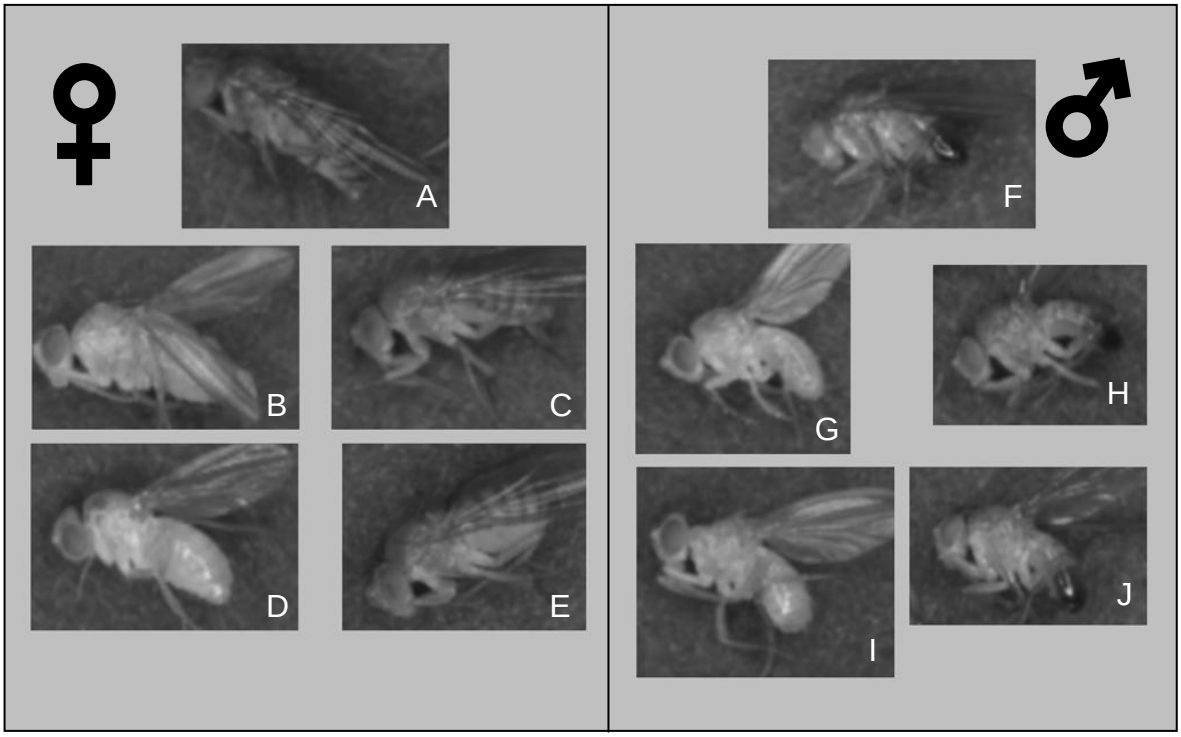

Figure 47. Effects on adult cuticle pigmentation of $AgNO_3$ (100 μM) and AgNPs (100 μM) from 48 hours after hatching until pupation, alone and after 48 hours of $Cu(NO_3)_2$ (200 μM) preloading. $AgNO_3$ caused loss of pigmentation of the cuticle in both (B) female and (G) male as did AgNPs ((D) female and (J) male) when compared to no added metal controls ((A) female and (F) male). The pigmentation was restored when the animals were preloaded with Cu: $AgNO_3$, (C) female and (H) male; AgNPs, (E) female and (J) male.

6.3.2. Effects of ascorbic acid on Ag induced de-pigmentation

The effect of ascorbate on de-pigmentation induced by both AgNPs and $AgNO_3$ was compared. Figure 48 shows that co-administration, in the food, of AgNPs and ascorbic acid (50 mM) resulted in no significant difference (Dunn's test) in pigmentation from control in either sex (Figure 48A and B for males and females respectively; AgNPs alone produced de-pigmentation in both sexes ($p < 0.001$). Silver nitrate also caused de-pigmentation but there was no reversal of this effect following ascorbate administration. To gain insight into how the order of treatment addition to the medium altered the efficacy of ascorbate the treatments were added to the medium sequentially (with a three minute gap to ensure no mixing of the treatments before contact with the medium) or together. As can be seen in Figure 49, ascorbate did not ameliorate the de-pigmentation ($p > 0.05$; Dunn's test) caused by $AgNO_3$ when added to the medium first followed by a three minute gap with a final addition of $AgNO_3$. Likewise, the reverse sequence with $AgNO_3$ first and ascorbate last

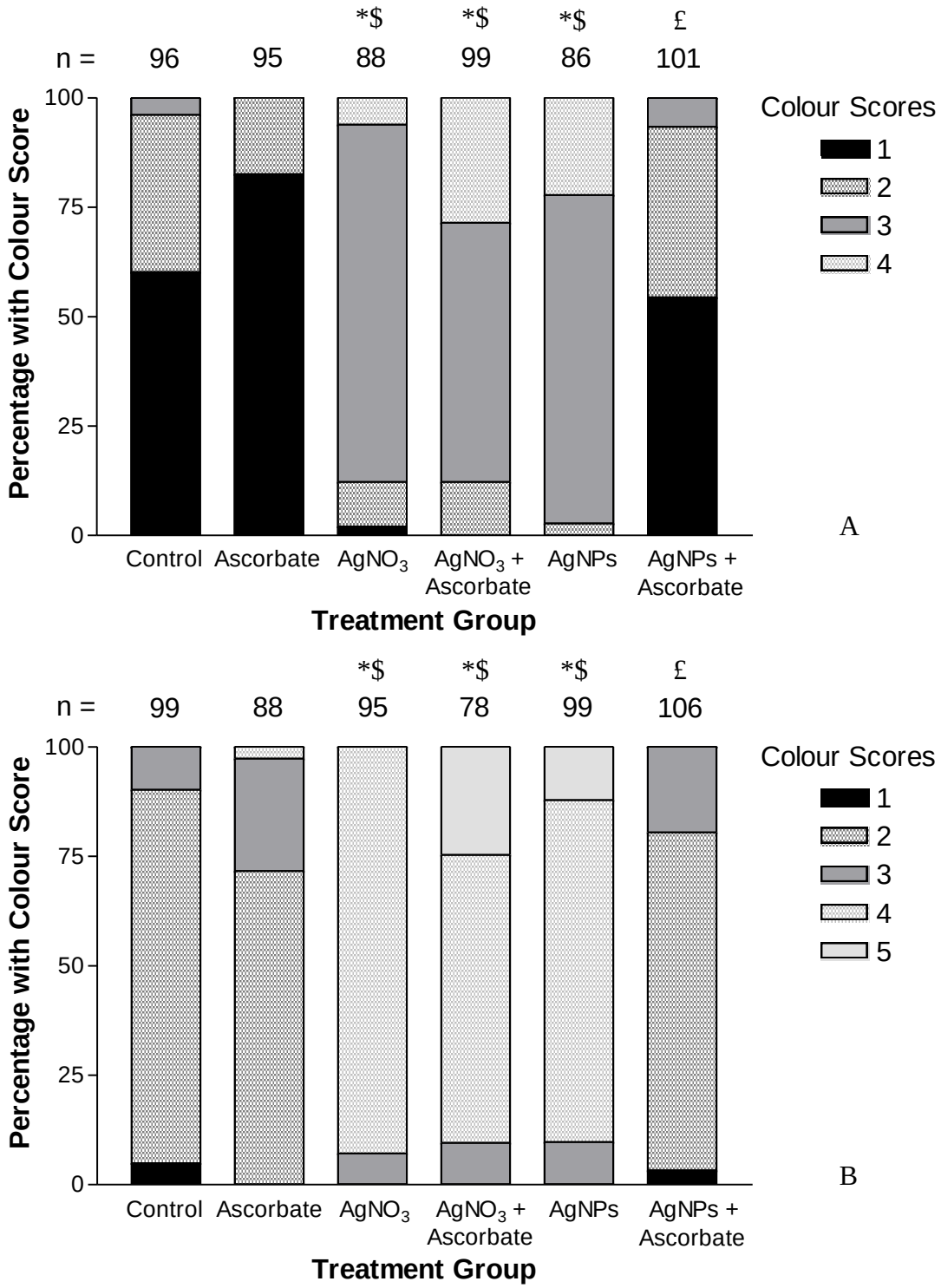

Figure 48. Pigmentation score following Ag and ascorbic acid administration. (A) Males and (B) females received $AgNO_3$ or AgNPs (100 µM) plus ascorbic acid (50 mM) throughout larval life and five days post eclosion. Segments represent percentage of flies scoring at each pigmentation level. Replicates for each group are shown above the respective group. Key to p values (Dunn's test following Kruskal-Wallis test): * $p < 0.001$ relative to control (no added metal); $ $p < 0.001$ relative to ascorbate; £ $p < 0.001$ relative to AgNPs (all symbols are relative within the panel).

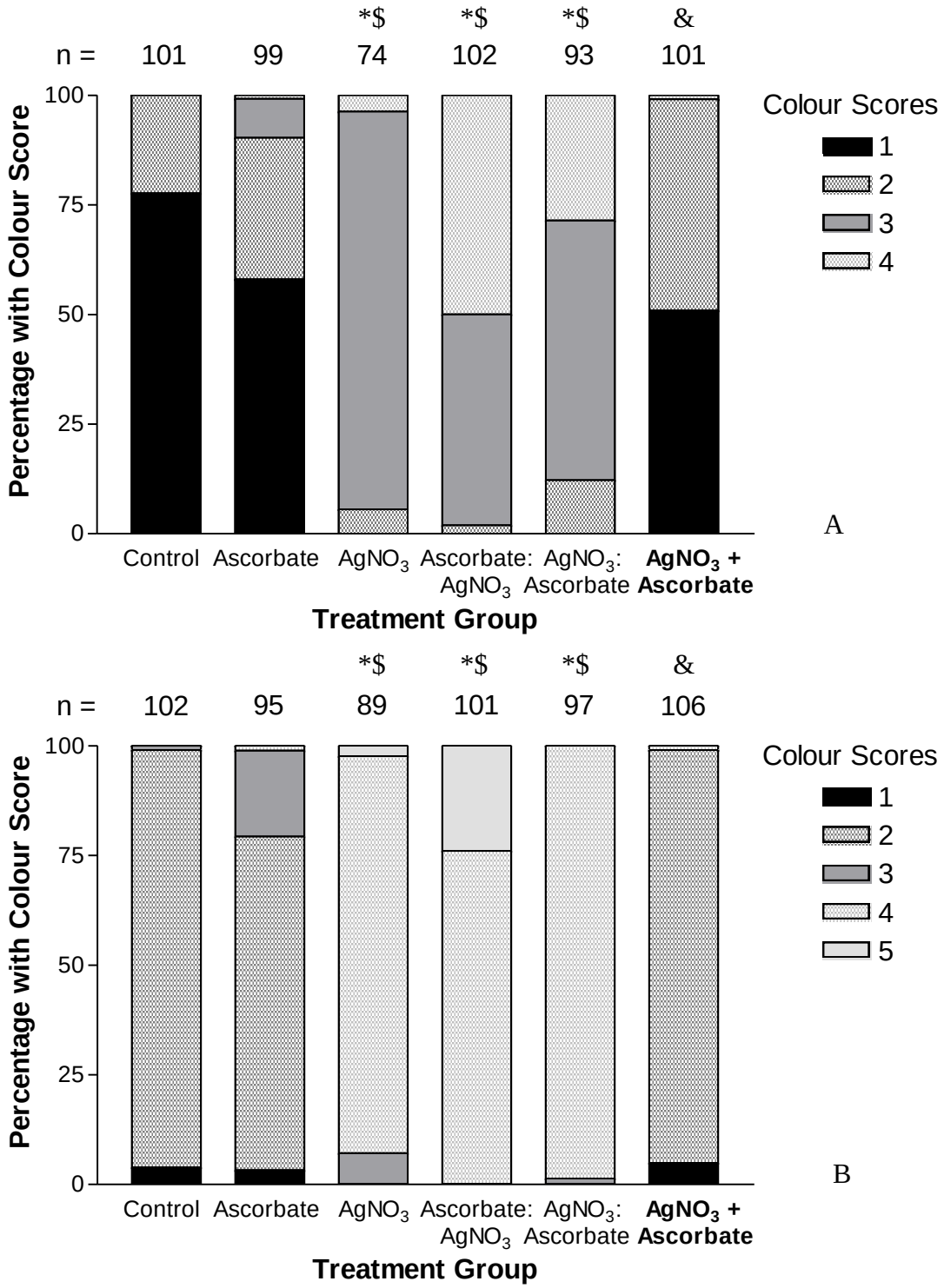

Figure 49. Pigmentation score following sequentially mixed Ag and ascorbic acid administration. (A) Males and (B) females received $AgNO_3$ (100 µM) plus ascorbic acid (50 mM) throughout larval life and for 5 days post eclosion. Boxes represent inter-quartile range with dark line as median. Segments represent percentage of flies scoring at each pigmentation level. Replicates for each group are shown above the respective group. Key to p values (Dunn's test following Kruskal-Wallis test): * $p < 0.001$ relative to control (no added metal); $ $p < 0.001$ relative to ascorbate; & $p < 0.001$ relative to $AgNO_3$ (all symbols are relative within the panel).

produced no reversal of de-pigmentation ($p > 0.05$; Dunn's test. However, a significantly ($p < 0.001$) darker pigmentation relative to $AgNO_3$ exposed animals was seen in those exposed to $AgNO_3$ and ascorbate that had been pre-incubated in a micro-centrifuge tube before addition to the medium. This group was not significantly different from control ($p > 0.05$; Dunn's test). Larvae that had been subjected to oxidative stress throughout their entire life, via administration of the strong pro-oxidant, paraquat, from hatching showed no evidence of a change in pigmentation in either males or females ($p > 0.05$; Dunn's test) (data not shown).

6.3.3. Effects of silver nitrate and silver nanoparticle administration on desiccation and starvation resistance of adult *Drosophila*

To test the effect of Ag on sclerotization, desiccation assays were performed alongside starvation assays as controls (to ensure it was water and not food that was limiting). Males and females were analysed separately as they have different tolerances to both desiccation and starvation. The threshold for significance was set at 0.017 after Bonferroni correction for three comparisons ($\alpha = 0.05$). Both male (Figure 50A) and female (Figure 50B) *Drosophila* demonstrated a shorter lifespan upon desiccation after being reared on 50 µM $AgNO_3$ or 50 µM AgNPs compared to control. Hazard ratios for the males were 1.90 ($p < 0.017$) and 2.39 ($p < 0.017$) for $AgNO_3$ and AgNPs respectively whilst for the females HRs were 3.25 ($p < 0.017$) for $AgNO_3$ and 3.61 ($p < 0.017$) for AgNPs. There was no significant difference ($p > 0.017$; log-rank test) in lifespan reduction following desiccation whether flies had been exposed to $AgNO_3$ and AgNP (males or females).

To ensure that the desiccated flies were dying from desiccation and not starvation, additional experiments were performed in which the flies were starved but in the presence of moisture. In the starvation assay males succumbed far more quickly than the females exactly as they did in the desiccation assay. However, there were no significant differences between control, $AgNO_3$ or AgNPs in either sex ($p > 0.017$; log-rank test) (Figure 51). Moreover, virtually no flies had died in the starvation assay by the time mortality had reached 100% in the desiccation test. Overall, $AgNO_3$ and AgNP exposed flies are have increased sensitivity to desiccation, but not starvation.

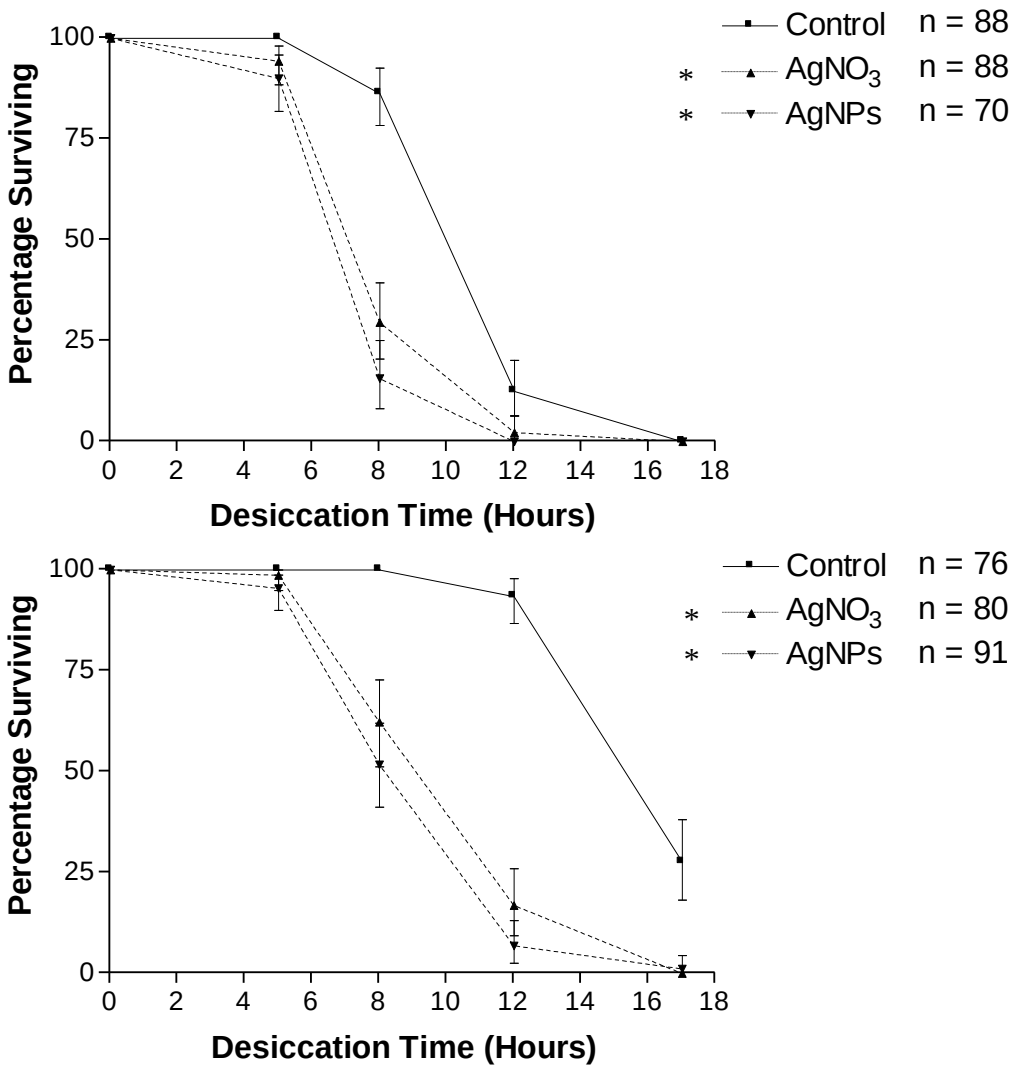

Figure 50. Desiccation resistance of Ag exposed *Drosophila*. Survival plots of (A) male and (B) female *Drosophila* exposed to desiccating conditions subsequent to receiving $AgNO_3$ or AgNPs (50 μM) for their entire larval period and five days post eclosion. Error bars represent +/- 95% CI of mortality fraction. Replicates for each group are shown in treatment group key. Key to significance values: * $p < 0.017$ relative to control (no added Ag) in that panel.

6.3.4. Additional broad toxicity screens

To ascertain if any gross physiological defects occurred following Ag exposure, heart-rate and negative geotaxis assays were performed. Following exposure to $AgNO_3$ or AgNPs (both 100 μm) throughout the larval period no significant differences ($p = 0.1627$; ANOVA – main effect) in pre-pupal heart rate were seen between either form of Ag or the control (data not shown). The negative geotaxis behaviour of *Drosophila* adults that had received $AgNO_3$ or AgNPs (at concentrations of 10, 50 and 100 μm) until five days

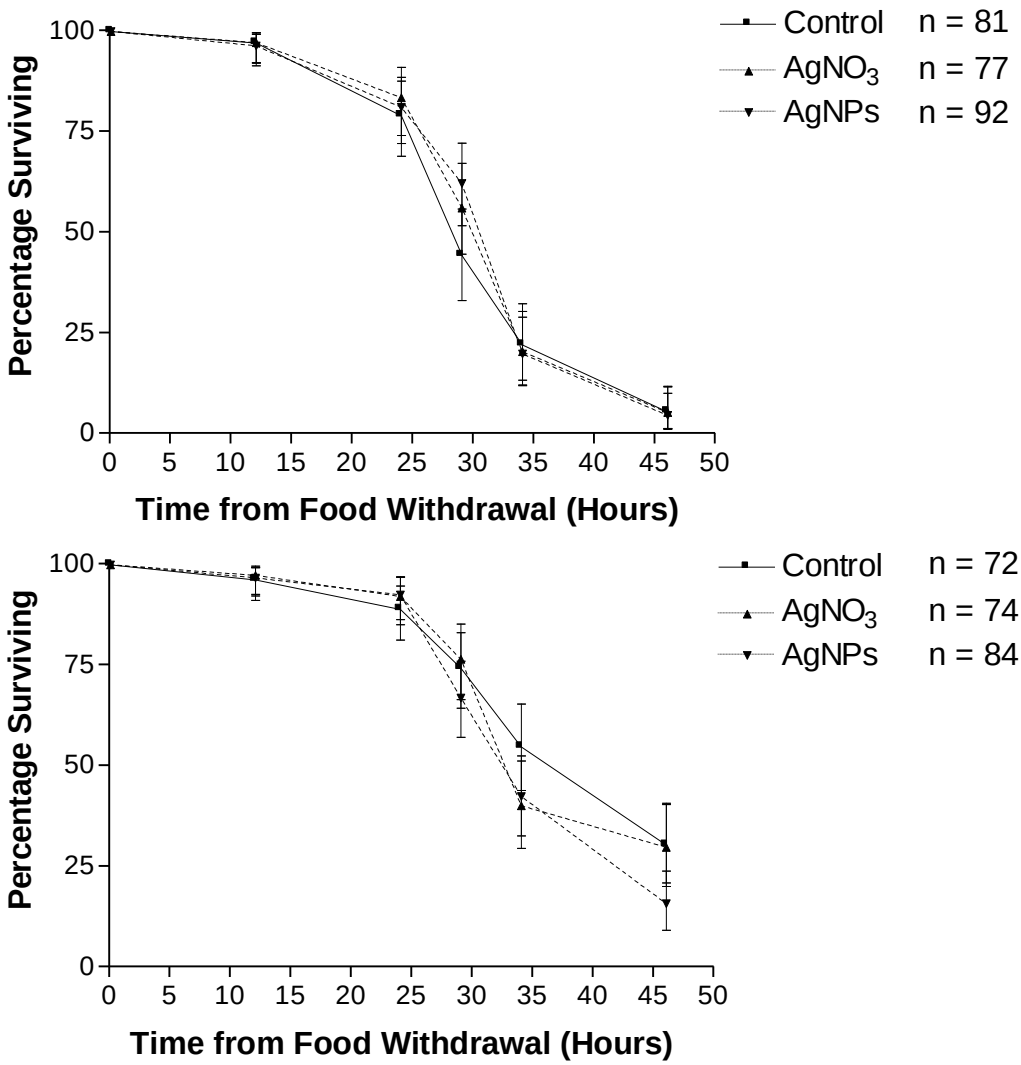

Figure 51. Starvation resistance of Ag exposed *Drosophila*. Survival plots of (A) male and (B) female *Drosophila* that were starved subsequent to receiving $AgNO_3$ or AgNPs (50 μM) for their entire larval period and five days post eclosion. Error bars represent +/- 95% CI of mortality fraction. Replicates for each group are shown in treatment group key. No significant differences were found between any groups within each panel (log-rank test).

post-eclosion was not significantly affected ($p = 0.37$ (Factor: Ag Form), $p = 0.14$ (Factor: Concentration); two-way ANOVA – main effects) (data not shown).

6.4. Discussion

One of the final stages of *Drosophila* development is the post-eclosion pigmentation and sclerotization of the cuticle. *Drosophila* larvae exposed to $AgNO_3$ or AgNPs exhibited de-pigmentation and a loss of desiccation tolerance. De-pigmentation could be prevented by supplementing the larvae with additional Cu, suggesting an antagonistic relationship

between Cu and Ag; desiccation tolerance was not tested in the presence of supplemental Cu because of time constraints. Ascorbic acid mitigated the de-pigmentation induced by AgNPs but not $AgNO_3$ and it is probable that this is a direct result of reduced dissolution of the AgNPs. Despite the possible perturbations, by AgNPs, to systemic dopamine synthesis suggested by the work of by Reaves *et al*. (2012), no effect of Ag was seen on negative geotaxis or heart rate.

6.4.1. Experimental limitations

The main limitation with the pigmentation studies (Sections 6.3.1and 6.3.2) is simply that the resolution of the assay was restricted by the ability of an observer to distinguish between pigmentations levels and that measurements made in this way are, to a degree, subjective. However, quantifying melanin levels in a completely objective fashion is time consuming, technically demanding (Oikawa and Nakayasu 1973, Borovansky 1978), and sometimes inaccurate (Latocha *et al*. 2000) and therefore premature without more preliminary evidence to support the effort. Five previous studies (Gorth *et al*. 2011, Key *et al*. 2011, Panacek *et al*. 2011, Posgai *et al*. 2011, Armstrong *et al*. 2013) have observed de-pigmentation following AgNP exposure but none have quantified this, making only subjective visual comparisons and giving descriptions such as being "totally bleached", being "devoid of pigmentation" or having "reduced pigmentation". The method used in this work therefore represents an advance over these studies by creating a predefined scale that was used by a disinterested observer to blindly score pigmentation.

6.4.2. Exploring the mechanism of silver nitrate induced de-pigmentation

Yaffe (1955) established that *Drosophila* tyrosinase/polyphenoloxidase (PPO) was inhibited by Ag (I) ions. The mechanism behind the inhibition of mammalian tyrosinase involves the displacement of Cu (I) ions from the active site (Lerner 1952). It is therefore likely that *Drosophila* PPO is inhibited by Ag (I) ions through displacement of Cu (I) but it is plausible given the different evolutionary lineages of the two enzymes (van Holde *et al*. 2001, Sugumaran 2002) that the inhibitory mechanism is different. To investigate whether Ag ions cause de-pigmentation through antagonism with copper *Drosophila* were co-exposed to Ag (I) ions and Cu (II) ions (which are more bioavailable than Cu (I) ions (Vancampe and Gross 1968) but are reduced to the latter upon absorption (Lee *et al*. 2001)) (Section 6.3.1). Consistent with the hypothesis that Ag (I) displaces Cu (I) in

tyrosinase, Cu administration completely prevented Ag induced de-pigmentation (Figure 45). To complement the de-pigmentation experiments a quantitative measure was devised for sclerotization (Section 6.3.3). Consistent with the role of PPO in sclerotization, Di Stefano (1943) noted that the adult cuticle was softened after larval exposure to $AgNO_3$. The effect of Ag upon sclerotization was analysed quantitatively here by using desiccation resistance as a proxy for normal cuticle structure as it forms a barrier to moisture loss (Noble-Nesbitt 1991). Desiccation resistance confirmed that the cuticle was likely to be compromised but alternatives are considered here. Reduced moisture intake during the larval period leading to a more limited water reserve is possible but unlikely given that larval size was no different (Section 5.3.4). Silver exposed flies may have been able to survive only with a higher bodily percentage of water than controls but it is difficult to conceive a mechanism for this. The observed softening and stickiness (See Section 5.3.1.2) of the cuticle strongly supports the notion that increased water loss was the cause of premature death. Although not tested explicitly by desiccation, Cu administration appeared to prevent this softening of Ag exposed flies.

6.4.3. Role of silver ions in the cuticular effects of AgNPs

When pigmentation and desiccation resistance of AgNP exposed animals were compared to those administered $AgNO_3$ no significant differences were seen (Sections 6.3.1 and 6.3.3), suggesting that the same pathways might be involved and that Ag (I) ions are the driving force behind the effect. Cu supplementation reversed the de-pigmentation effect of AgNPs exactly as seen with $AgNO_3$, adding further weight to the theory that these effects are mediated by Ag (I) ions competing for Cu binding sites in PPO. It is surprising that, despite noting the de-pigmentation in AgNP exposed animals, none of the studies examining the effects of AgNPs in *Drosophila* have suggested a role for Ag (I) ions in the process. Alternative mechanisms involving oxidative stress have been suggested (Key *et al*. 2011, Panacek *et al*. 2011, Posgai *et al*. 2011) and, in one study, apparently confirmed by the remedial action of ascorbic acid (Posgai *et al*. 2011). Armstrong *et al*. (2013) suggest that the mechanism is directly related to the particulate nature of the AgNP via an unspecified interaction with Cu metabolism.

The amelioration of AgNP induced de-pigmentation by ascorbate (a powerful antioxidant (Du *et al*. 2012)) led Posgai *et al*. (2011) to propose that oxidative stress is the mediator of

de-pigmentation. Within the same study, however, they could not cause de-pigmentation with titanium dioxide NPs although these are known to produce ROS (Posgai *et al*. 2011). It was suggested that an additional mechanism may also be operating (Posgai *et al*. 2011). Furthermore, this work found no de-pigmenting effect of paraquat administration (Section 6.3.2) (known to produce profound oxidative stress in *Drosophila*) (Bus and Gibson 1984). Nonetheless, ascorbic acid could clearly prevent AgNP induced de-pigmentation and this was investigated further.

The above discussion strongly implicates Ag (I) ions in AgNP induced de-pigmentation. Given this fact and that simultaneous administration of ascorbate can attenuate AgNP induced de-pigmentation, it was possible that ascorbate would have affected $AgNO_3$ induced de-pigmentation also; ascorbate was, however, ineffective under these circumstances. Two possible interpretations are proposed for this. The first is that despite both $AgNO_3$ and AgNPs causing de-pigmentation, the mechanism behind this effect is different. The second is that ascorbate prevents the release of Ag (I) ions from the particle surface; when ions are supplied as such no amelioration occurs. This latter hypothesis is supported by the data from Sections 4.3.1 and 4.3.2 that show ascorbate attenuates the dissolution of AgNPs even under strongly oxidizing conditions and that the colour of the medium spiked with AgNPs does not fade when supplemented with ascorbate. However, as a strong reducing agent, ascorbate might be expected to reduce Ag (I) ions when supplied as $AgNO_3$ as well as those liberated from AgNPs, raising the possibly of a unique effect of AgNPs. However, this appears not to be the case as when $AgNO_3$ is pre-incubated with ascorbate, in water before addition to the medium, rather than the two being added to the food medium sequentially the ameliorating effect is seen (Figure 49), suggesting that reduction to metallic Ag is central to the mechanism. Why reduction of $AgNO_3$ does not occur in the medium is not known but may be due to diffusional constraints, the effects of various ligands or that it is not free Ag (I) ions released from AgNPs that are reduced but ROS intermediates resulting in breaking of the oxidation chain.

The idea that Ag (I) ions are responsible for the de-pigmentation induced by AgNPs directly contradict the assertions of Armstrong *et al*. (2013), who state that de-pigmentation is a particle specific phenomenon. However, it is suggested here that there are several flaws in their logic. Firstly, they state that concentrations of $AgNO_3$ equivalent

to the AgNP concentrations (50 mg/l) they used (and hence the maximum possible Ag (I) ion concentration in their AgNP suspension) was incapable of inducing de-pigmentation because Di Stefano (1943) found that 100 mg/l did not cause de-pigmentation. They did not test whether $AgNO_3$ (50 mg/l) could cause de-pigmentation using their own flies; there are many variables that may affect overall sensitivity to Ag. This is demonstrated by the fact that a strong de-pigmentation effect was observed in this work at ~ 10 mg/l (100 µM) (Section 6.3.1); indeed, a slight de-pigmentation was found at ~ 5 mg/l (50 µM) (data not shown). Secondly, and most significantly, by using an ion selective electrode (Armstrong *et al*. 2013) determined the amount of Ag (I) ions released by AgNPs in *water* and used this to guide the concentrations of $AgNO_3$ that should be used for comparative purposes. However, this work has shown that the behaviour of AgNPs in fly food medium is not the same as plain water owing to the presence of other ions. Indeed, since this work has been completed, Levard *et al*. (2013) have shown that Cl^- ions are able to accelerate the dissolution of AgNPs with atmospheric oxygen as the sole oxidant; the toxicity of the resulting solution to *Escherichia coli* was correlated with the Cl^-, and hence Ag (I), concentration. Lastly, Armstrong *et al*. (2013) found that the de-pigmentation caused by AgNPs was dependent on the monovalent Cu transporters Ctr1A and Ctr1B. Monovalent Cu transporters are known to transport Ag (I) ions (Bertinato *et al*. 2010) but it is highly unlikely that, on a size (Handy *et al*. 2008) and charge basis, that they could transport AgNPs.

6.4.4. Conclusions

Drosophila have been shown to exhibit both $AgNO_3$- and AgNP-induced inhibition of the specific developmental processes of melanization and sclerotization that occur during the final maturation stages. It is suggested that these effects are mediated by Ag (I) ions whether the source of Ag is ionic or AgNPs. The effects of antioxidants appear to be primarily mediated by an *ex vivo* interaction with the AgNPs rather than a reduction of oxidative stress within the organism signifying that care must be taken when interpreting the results of studies that supply antioxidants and AgNPs simultaneously. This represents a considerable challenge when attempting to understand the particle mediated effects that are often ROS dependent and those that result from liberation of free ions.

Chapter 7. General discussion, future work and conclusions

This work has supported the primary hypothesis that Al and Ag are developmental toxicants to *Drosophila*. Figure 52 shows the point of greatest sensitivity to the toxicity of both metals is centred on metamorphosis, and this suggests that the extensive tissue remodelling and growth occurring throughout this period may provide temporally unique toxicological targets. The precise mechanisms are currently unknown but, as the figure illustrates, the toxicity of Al is influenced by its speciation. This difference is seen only during the larval period as Al toxicity though metamorphosis appears largely unaffected by Al speciation, despite differences in tissue uptake between forms. The toxicity of Ag is likely to be partly derived from Ag (I) ions regardless of the form in which Ag is supplied and both molecular oxygen and transition metals are capable mediating the transformation of AgNPs to Ag (I) ions; however, some AgNP specific developmental toxicity appears likely and this requires further investigation. Similarly, the contribution of intact AgNPs to the uptake of Ag remains unclear. Figure 52 also shows that potentially ameliorating interventions for both metals need further scrutiny as, so far, the only robust responses to remedial actions have been for early developmental toxicity and de-pigmentation in Ag exposed *Drosophila*.

Although the toxicity of both metals has been demonstrated in this work, contrary to reports in the literature, the experiments described herein found a relative resistance to Al exposure (Massie *et al*. 1985, Wu *et al*. 2012b) and a markedly greater sensitivity to Ag (Di Stefano 1943, Kroman and Parsons 1960), particularly when the latter was in the ionic form rather than as AgNPs. As indicated in Figure 52, potential causes for these differences in sensitivity include strain differences (Ramel and Magnusson 1992, Ortiz *et al*. 2009) and composition of the food medium (Rapoport 1947, Maroni and Watson 1985). A further possibility is the infection status of the flies with the Wolbachia parasite. Wolbachia infection is common amongst laboratory stocks (e.g. ~30% of Bloomington stocks) of *Drosophila* (Clark *et al*. 2005) infection with this parasite has been shown increase sensitivity to Pb toxicity in *Drosophila* (Wang *et al*. 2012). As also hypothesised, the toxicity of the two metals occurred in tandem with increased body burden. Toxicity varied with the form of the metal although tissue concentration was not an indicator of toxicity in *Drosophila*. This lack of tissue-concentration dependency has also been observed in *L. stagnalis* (Walton *et al*. 2009), suggesting it is something that may be

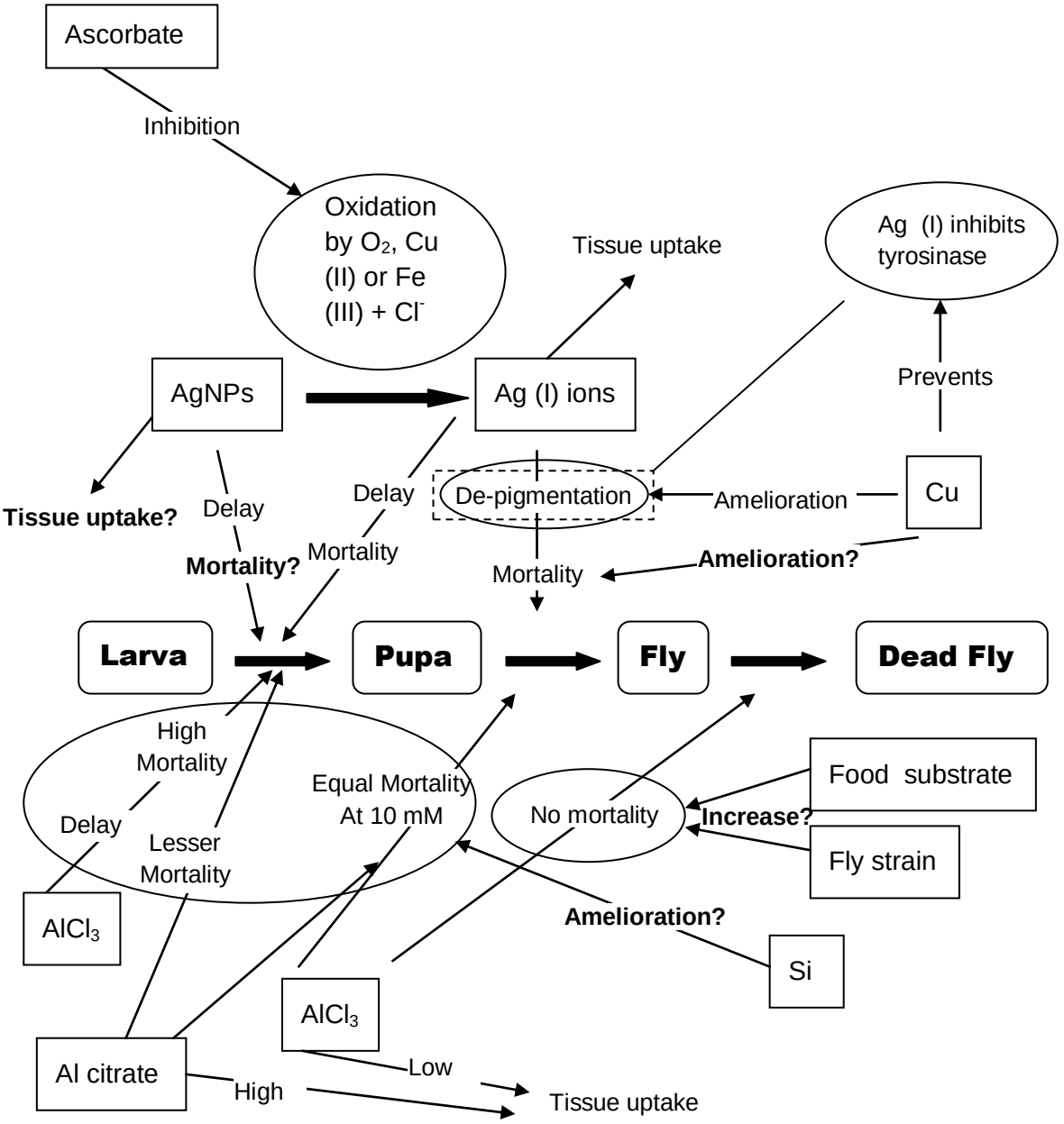

Figure 52. Schematic diagram of developmental toxicity of aluminium and silver to *Drosophila*. Developmental stages are shown in rounded rectangles, treatments or interventions are shown in rectangles whilst possible toxicity targets for interaction are shown in oval boxes. Bold type indicates questions that remain to be addressed. Mechanisms are proposed for Ag toxicity as the data suggest these hypotheses.

common, in invertebrates at least. The resistance of the *Drosophila* used in this study to Al meant that despite impaired development, lifespan was unaffected. This level of tolerance precluded an examination of the effects of Si in ameliorating Al toxicity.

Although speciation of Al appeared to be important to both the uptake and toxicity of Al the experimental setup was limited by different pH values for each ligand and a systematic analysis of pH/ligand combinations is needed to address the contributions of each. In

addition to pH, other unknown components of the medium affected the degree of toxicity as demonstrated by differences seen between exposure of Al via live yeast and standard fly food medium. The Ag experiments were not compromised by variation in medium pH but the evidence strongly suggests that the medium was a significant contributor to the toxicity of AgNPs by facilitating their dissolution. Indeed, this work has shown for the first time that AgNPs can be dissolved in the presence of Cl^- ions by transition metals. The co-administration of AgNPs and ascorbate suggests that in particulate form AgNPs are considerably less toxic than the equivalent concentration of Ag (I) ions. However, precisely how toxic AgNPs are to *Drosophila* during development, in the absence of dissolution, is still unclear. The results presented here strongly suggest that failure to produce a mature cuticle, following exposure to AgNPs is due to elevated levels of Ag (I) ions but the developmental delay and life stage specific mortality appear to result from both particle specific and ionic form of Ag.

A further aim of this study was to assess the suitability of *Drosophila* as potential model for human toxicity arsing from Al and Ag exposure. The issues described above complicate the application of *Drosophila* for this purpose but do not prohibit it. For *Drosophila* to be successful as a model the most pressing issue to be resolved is the delivery of the metal in a consistent and well characterised form. Resolving this problem will also address some of the knowledge gaps that remain, concerning the effects of speciation of Al and Ag on *Drosophila* development. It is therefore necessary to devise experiments that allow for more precise control of metal speciation. The data presented here allow the following two hypotheses to be formulated. The first is that the citrate chelate of Al is less toxic to *Drosophila* during larval development than the free ion. The second is that AgNPs are developmentally less toxic than the Ag (I) ion at the same concentration. It is now clear that simple addition of these metals to the food substrate is inadequate for testing these hypotheses. Mammalian studies are able to overcome changes in metal speciation caused by the feed by directly administering the metals intravenously in a simple aqueous carrier. Although intravenous introduction of toxicants such as Al and Ag is not a realistic model of exposure it will help to establish the mechanisms behind differences observed during enteral administration. Although more laborious than administration through the fly food medium, direct introduction of metals into the embryo, or via the larval or pupal haemocoel is feasible even when using the large numbers of individuals required to gain superior statistical power to mammalian studies. It is therefore

proposed that these hypotheses are tested following microinjection at different developmental stages. This method would have the further advantage of precisely controlling doses, whereas administration via food will be influenced by the rate of consumption by the organism.

Clearly, considerably more effort would be required for such a study of toxicity following injection but, the advantages of statistical power, genetic tractability and wealth of physiological knowledge regarding the organism repay the additional efforts required. Therefore, the most important studies to follow from this work would be the use of the developmental screens as applied previously but following a parenteral administration programme. Once the methodology and approach has been verified, a more in depth analyses of Al and Ag toxicity could be performed of which one of the most important would be determination of the contribution of oxidative stress. Although not tested for Al in this work, oxidative stress has been implicated in the pathology associated with Al toxicity (Zatta *et al*. 2002, Wu *et al*. 2012b). The paraquat assay used here gave an indication that oxidative stress may be involved in the developmental delay induced by both AgNPs and Ag (I) ions and gene expression, antioxidant level and *in vivo* ROS probe studies described by Owusu-Ansah *et al*. (2008) could be employed for establishing in what tissues and at what developmental time-points, oxidative stress may be occurring. Since AgNPs would be administered parenterally there would be no problems resulting from metal-antioxidant interactions in the food medium allowing antioxidants such as ascorbate or tocopherols to be administered via dietary means. Similarly, there could be no *ex vivo* interaction between Si and Al so the effects of Si on Al toxicity could be examined using the developmental delay assay described above.

The ultimate aim of these investigations would, however, be to gain an understanding of how Al and Ag affect development when administered by mouth. Although, changes in speciation through interaction with the medium cannot be prevented it would be possible to control for such changes. The simplest way to do this would be to use a chemically defined medium (e.g. as described by Troen *et al*. (2007)) that would remove any variability caused by differences resulting from biologically derived feedstuffs. In the case of AgNPs this would be especially useful in determining if the observed effects after oral administration may be attributed to absorbed particles or the ion.

Further suggestions for future work include gaining a deeper understanding of the dissolution of AgNPs in the presence of transition metal ions, with or without Cl^-. This is important as many media that could become contaminated with AgNPs may contain Cu or Fe and significant quantities of Cl^-. Clearly such work would also have environmental relevance as similar interactions between AgNPs and other anions and cations would occur following release from industry and consumer products. Natural sources of the oxidising species used in this work may be much lower than those used in this work (e.g. Fe^{3+} (aq) (50 μM)), but certain environments may nevertheless provide significant concentrations of Fe^{3+} (aq). For example, water from peat-bog draining rivers that contains chelating ions and is of low pH has an ultra-filterable Fe (III) concentration of ~15 μM of which 3.3 μM does not precipitate even at high ionic strength (Krachler *et al*. 2010). Krachler *et al*. (2010) define this non-precipitating fraction as "truly soluble". However, as much of the Fe (III) in the precipitating fraction will be bound by organic material it is possible that it would retain its capacity to oxidise AgNPs. It is suggested that the dissolution of AgNPs by Cu (II) and Fe (III) ions be studied at lower concentrations but over longer time scales, perhaps using material from natural sources as a supply of Fe (III). An investigation could be performed to provide a definitive answer to the question regarding the role of AgNPs in de-pigmentation as although the data here suggest that Ag (I) ions are the mediator of this effect, Armstrong *et al*. (2013) contend that AgNPs are responsible. They performed a tyrosinase assay using haemolymph from AgNP exposed *Drosophila* and found inhibition; this, however, does not prove AgNPs are the inhibiting species nor does it mean that the enzyme is simply de-metalated. It is proposed that the tyrosinase assay be performed with the enzyme incubated with AgNPs or Ag (I) ions. Native and SDS polyacrylamide gel electrophoresis with western blotting could discriminate between apo-, holo- and inappropriately metalated forms of the enzyme.

7.1. Conclusions

Both Ag and Al are developmentally toxic to *Drosophila* although the medium in which they are administered significantly influences toxicity. The form of the metal is critical to the degree of toxicity observed although the interactions with the medium make it difficult to define exactly the effects of chemical speciation on the target organism. In this work *Drosophila* were found to be quite resistant to Al toxicity, but this may be caused by the food medium or a particularly resistant strain of fly. It appears that when administered to

Drosophila, via the food medium, AgNPs exert a large proportion of their toxicity through dissolution and release of Ag (I) ions. The evidence suggests that *Drosophila*, with carefully designed experiments, would be a good model for assessing and understanding the developmental toxicity of Ag. With further investigation it is possible that *Drosophila* will form a useful model for Al developmental toxicity, but it is critical that the reasons for the differential susceptibilities seen when the literature is compared to this work are identified, understood, and accounted for.

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