The response of *Isidorella newcombi* to copper exposure:

An integrative approach using biochemical, life history and transcriptomic markers to develop a mechanistic understanding of response

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Abstract

The widespread extraction, processing and use of Cu in modern society has caused Cu concentrations to become elevated in the environment. Despite being an essential element, exposure to elevated concentrations of Cu is toxic to aquatic organisms. Multi-generational exposure to sub-lethal concentrations of a contaminant can exert selection pressures on populations that can cause rapid evolutionary change. The aim of this research is to quantify the response of the endemic freshwater gastropod *Isidorella newcombi* to Cu exposure at lethal and sub-lethal concentrations, using acute, chronic and multi-generational exposures.

To investigate response to chronic exposures *I. newcombi* were exposed to 0-120 µg L^{-1} Cu for 28 days. There was complete mortality at concentrations of 60 µg L^{-1} Cu and above. In these treatments there was an exposure concentration-dependent decrease in the time that they survived. In the surviving snails there was an exposure concentration-dependent increase in tissue Cu concentration. In the snails exposed to concentrations above 15 µg L^{-1} Cu, no eggs were produced in the fourth week of exposures. This suggests that populations would not persist at concentrations above 15 µg L^{-1} Cu due to reproductive failure. The general stress biomarker lysosomal membrane destabilisation (LD) indicated organisms exposed to concentrations of 10 µg L^{-1} Cu and above were experiencing Cu-induced stress. This suggests that LD could act as an early warning biomarker for responses at higher levels of biological organisation in *I. newcombi* exposed to Cu.

To investigate the effect of multigenerational exposures and the development of Cu resistance in *I. newcombi,* they were exposed to a range of treatment-specific Cu concentrations in the parental to F_2 generations, and a common Cu concentration in the F_3 generation. In the parental to F_2 generations some general responses to 3 day Cu exposures were seen, including reduced survival and feeding in snails exposed to higher Cu concentrations. This suggested that the snails from the high Cu exposure were experiencing Cu-induced stress that would be likely to apply a selection pressure. In the F_3 generation, when all treatments were exposed to a common Cu concentration, there was an increase in survival that was correlated with the pre-exposure Cu concentration history. The snails that had been pre-exposed to Cu also displayed a reduction in stress at a sub-lethal level as indicated through lower LD. Changes in Cu tissue concentration in the F_3 generation did not follow mortality or LD responses indicating increased tolerance

and reduced stress were not related to changes in Cu bioaccumulation. Total antioxidant capacity (TAOC) increased in the higher pre-exposure treatments which could be associated with lower Cu-induced stress, however, this is not supported by the oxidative damage marker lipid peroxidation (LP) which also increased. Cu tissue concentrations and oxidative stress markers were assessed to determine underlying reasons for increased tolerance in snails from a population with a multi-generational exposure history to Cu, but the results were not conclusive. The mechanisms that led to the increase in Cu tolerance in the treatments that had been previously exposed to high Cu concentrations were not explained by these biomarkers. Despite this, it was demonstrated through the increased survival and reduced LD that Cu resistance can develop over a short evolutionary time scale of single short exposures to elevated Cu concentrations in each generation.

To gain a mechanistic understanding of the response of *I. newcombi* to Cu at the molecular level, transcriptomic responses were investigated using RNA-seq. The transcriptome of *I. newcombi* exposed to Cu for three days was compared to that of un-exposed organisms. Transcriptomic responses to copper were evident in differences in internal transport of copper, metabolic activity, cellular repair and recycling mechanisms and programmed cell death between the two populations. Genes associated with Cu uptake and transport mechanisms such as metallothioneins, Cu ion binding and endocytosis were identified as potential Cu-specific transcriptomic markers. Responses associated with changes in the expression of genes associated with the lysosome, apoptosis and phagocytosis were identified as transcriptomic markers of general stress. An integrated biological response model was developed to provide a framework for the interpretation of complex RNA-seq data sets within the context of ecotoxicological investigations.

RNA-seq was also used to compare the transcriptomic response of two groups of Cu exposed *I. newcombi* from the F_3 generation of the multi-generational study. One group had been exposed to elevated Cu concentrations in the parental to F_2 generations (pre-exposed) and one had not (naïve). There were differences in the transcriptional regulation of genes associated with metabolic activity, protection and repair mechanisms and programmed cell death between pre-exposed and naïve snails. The general increase in expression of genes associated with proteolytic function, immune function, phagocytosis, and other cellular protection and repair mechanisms in the pre-exposed snails indicate that they have an increased ability to protect against and repair Cu-induced damage. The reduced expression of genes associated with ionic transport, transcription, translation and ATP generation in the naïve snails indicate that they

are using a strategy of metabolic depression in response to the Cu exposure. There is also evidence of increased apoptosis occurring in the naïve snails. The evidence from the transcriptomic regulation of genes suggests that the pre-exposed and naïve snails are using different strategies to manage Cu-induced stress. The pre-exposed snails are increasing cellular protection and repair mechanisms to manage the Cu-induced stress, whereas the naïve snails are reducing metabolic activity to avoid cellular damage and have an increased rate of programmed cell death to remove damaged cells.

This project establishes *I. newcombi* as a potential biomonitor of Cu contamination by demonstrating the positive relationship between exposure Cu concentrations and tissue Cu concentrations, as well as linking of LD response in this species to changes at higher levels of biological organisation. The ability to of *I. newcombi* to develop tolerance to Cu over three generations of Cu exposures was demonstrated. Finally, transcriptomic responses explain the mechanistic response of *I. newcombi* to Cu at the molecular level and adaptive differences in response to Cu between pre-exposed and naïve snails.

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Ubrihien, R. P., Ezaz, T., Taylor, A. M., Stevens, M. M., Krikowa, F., Foster, S. and Maher, W. A. (2017). The response of *Isidorella newcombi* to copper exposure: using an integrated biological response model to interpret transcriptomic responses from RNA-seq analysis. *Aquatic Toxicology*, 185, 183-192.

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List of abbreviations

Abbreviation	Explanation
4DPI	Four domain proteinase inhibitor
ABTS	2, 2'-Azino-di-[3-ethylbenzthiazoline sulphonate]
Al	Aluminium
ANOVA	Analysis of variance
ANZECC	Australian and New Zealand Environment and Conservation Council
AOD	Antioxidant defences
AOP	Adverse outcome pathway
ARMCANZ	Agriculture and Resource Management Council pf Australia and New Zealand
As	Arsenic
ATP	Adenosine triphosphate
BCL2	B-cell lymphoma 2
BIRC	Baculoviral IAP repeat-containing protein
CAT	Catalase
cDNA	Complimentary deoxyribonucleic acid
Cd	Cadmium
CMFS	Calcium and magnesium free saline buffer
Cu	Copper
CuATPase	Copper transporting ATPase

$CuSO_4$	Copper sulfate
DEG	Differentially expressed gene
DNA	Deoxyribonucleic acid
FASL	FAS ligand
Fe	Iron
GO	Gene Ontology
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GST	Glutathione *S*-transferase
HSP	Heat shock protein
ICP-MS	Inductively coupled plasms-mass spectrometer
IRE	Serine/threonine protein kinase inositol-requiring protein
KEGG	Kyoto encyclopedia of genes and genomes
KSPI	Kazal-type serine proteinase inhibitor
KTPI3	Kazal type protease inhibitor 3
LC	Lethal concentration
LD	Lysosomal membrane destabilisation
LP	Lipid peroxidation
MDA	Malondialdehyde
MED16	Mediator of RNA polymerase II transcription subunit 16
mRNA	Messenger ribonucleic acid
MTLP	Metallothionein like protein

Ni	Nickel
Pb	Lead
PFA	Polytetra-flouroacetate
PHLPP	PH domain and leucine-rich repeat-containing protein
POD	Peroxidase
PP1D	Protein phosphatase 1D
PRP4	Pre-MRNA processing factor kinase 4
RNA	Ribonucleic acid
RNA-seq	Sequencing of the transcriptome
RIF1	telomere associated protein RIF1
ROS	Reactive oxygen species
RPB1	DNA-directed RNA polymerase II subunit 1
Se	Selenium
SIS	Sodium influx stimulating peptide
SOD	Superoxide dismutase
SP56	Serine protease 56
TAOC	Total antioxidant capacity
TBARS	Thiobarbituric acid reactive substances
TRAIL	Tumour necrosis factor apoptosis inducing ligand
TP53I11	Tumour protein 53 – inducible protein 11
TRAIL-R	Tumour necrosis factor apoptosis-inducing ligand receptor
TRPV6	Transient receptor potential channel subfamily V member 6
TSP9	Transmembrane protease serine 9

XIAP	E3 ubiquitin-protein ligase XIAP
Zn	Zinc

Chapter 1. General Introduction

1.1 Background

This project was based on two major areas of research interest. The first is the need to develop freshwater gastropod species as biomonitors in an Australian context. The second is the history of exposure of the endemic Australian freshwater snail, *Isidorella newcombi* to copper (Cu) in areas of rice cultivation. Of particular interest was the potential for snails to develop resistance to copper over multiple generations. The investigation of the development of resistance required multiple generations of exposures. For biomonitor development, an investigation of the effects of chronic and acute copper exposures was undertaken. An integrated approach was used that included the assessment of transcriptomic responses, cellular biomarkers, behavioural responses and life history traits. Biomarkers and behavioural responses provide an indication of the sub-lethal response of organisms to contaminant exposure. The life history traits measured provide an indication of changes that may occur at the population level. The adoption of an integrated approach allowed responses to be related across several levels of biological organisation. The application of this approach over multiple generations provides an understanding of the rate at which resistance develops under a particular set of exposure conditions. The investigation of transcriptomic responses allows a mechanistic understanding of the response of *I. newcombi* to Cu as well as changes in gene regulation associated with the development of resistance. The general introduction provides a review on the use of freshwater gastropods as biomonitors, with specific reference to *I. newcombi*. The life history, biochemical and genomic techniques that are relevant to the study are discussed.

1.2 Gastropods as biomonitors

It has been reported that freshwater snails can represent six to sixty percent of the biomass of macro-invertebrates in freshwater systems and play an important role in the transfer of energy and materials through freshwater food webs (Habdija *et al.* 1995; Lagadic *et al.* 2007). At a community level, freshwater snails are often grazers and provide major food sources for many birds, fish, reptiles and mammals (Hoang *et al.* 2008). As gastropods form a significant part of the biomass of freshwater systems and play in important role in energy transfer, an understanding of their response to contaminants is essential to an understanding of how contaminants affect these systems. Freshwater gastropods also have several desirable features for use in the investigation of the effects of contaminants, including

- they are easy to culture and test;
- they reach reproductive age early (approximately 3 months);
- reproduction is continuous;
- embryonic development is relatively short (8-10 days);
- embryonic stages are easy to identify;
- they often occupy the littoral zone, which is often the most exposed to contaminants;
- they are sensitive to contaminants; and
- acute effects may be obtained in a short time (Ravera 1977).

Internationally, there have been several studies conducted on developing freshwater gastropods as biomonitors (e.g., Atli and Grosell 2016; Desouky 2006; Gust *et al.* 2011; Hoang and Rand 2009). Despite the evidence of toxicity tests conducted on freshwater gastropods, it has been reported that they are underrepresented in toxicity datasets (Brix *et al.* 2011).

In an Australian context, there is a paucity of studies that investigate freshwater gastropods as biomonitors. The focus on multiple lines of evidence in the revision of the ANZECC/ARCMANZ water quality guidelines demonstrates the need for a wider range of species to be validated for the assessment of freshwater systems in Australia (Simpson *et al.* 2013). There have been several published studies on freshwater bivalve Molluscs in this context (e.g., Marasinghe Wadige *et al.* 2017; Marasinghe Wadige *et al.* 2014; Taylor *et al.* 2016). Although bivalves also come from the phylum Mollusca, gastropods have different feeding strategies, occupy different environmental niches and are likely to have different metal accumulation and toxicity responses compared to bivalves. The development of an understanding of the exposure-bioaccumulation-response relationships in an Australian

gastropod species would fill a knowledge gap in this area and provide a potentially useful resource for environmental assessment and monitoring.

1.2.1 Isidorella newcombi.

Isidorella newcombi belongs to the Order Hygrophila from within the panpulmonata clade of gastropods. Until recently they were classified with the broader clade pulmonata but were reclassified as panpulmonates due to the polyphyletic nature of this clade (Jörger *et al.* 2010). The full taxonomic classification is:

Kingdom: Animalia
Phylum: Mollusca
Class: Gastropoda
Clade: Heterobranchia
Clade: Euthyneura
Clade: Panpulmonata
Order: Hygrophila
Family: Planoribidae
Genus: *Isidorella*
Species: *Isidorella newcombi*

The Order Hygrophila are simultaneous hermaphrodites which have the ability to both self and cross fertilise (Lagadic et al., 2007). In addition to the general characteristics of freshwater gastropods that are desirable in biomonitors, *I. newcombi* are widely distributed within Australia (Figure 1.1; Atlas of Living Australia 2017).

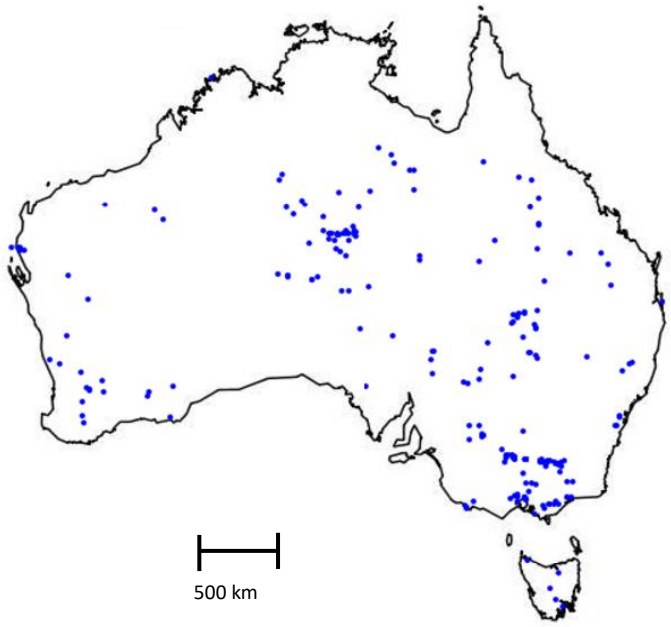


Figure 1-1. Reported records of *Isidorella newcombi* within Australia (Atlas of Living Australia 2017).

In rice growing areas, *I. newcombi* are considered a pest as they feed on juvenile rice plants and significantly reduce rice yields (Stevens 2002). In Australian rice cultivation areas, copper sulfate pentahydrate is used to control *I. newcombi* at applications of up to 12 kg ha^{-1} (Troldahl *et al.* 2016). Copper sulfate ($CuSO_4$) has been used to control *I. newcombi* since 1973 (Stevens 2002). The current published work on the effects of Cu on *I. newcombi* has been focused on testing the efficacy of $CuSO_4$ as a pesticide to remove the snails from rice fields. Stevens *et al.* (2014) reported LC90 values for Cu in *I. newcombi* varied due to the underlying soil type, with LC90 values ranging from 0.41 to 1.14 mg L^{-1} Cu depending on the underlying soil used in the test. Previously, a group of 27 selected pesticides were tested in an effort to find an alternative to Cu, with niclosamide, *n*-tritylmorpholine and nicotinanilide being found the most effective (Stevens *et al.* 1996). Despite these tests, the use of Cu has continued as the preferred method to control the snails in Australian rice fields. The repetitive application of Cu over an extended period leads to the potential for elevated Cu concentrations in soils.

With the long term use of copper as a pesticide in these areas, local populations could become exposed to elevated but sub-lethal concentrations of copper. The potential for pasture plants grown in areas previously subjected to repeated applications of $CuSO_4$ to become toxic to stock is recognised (Stevens 2002; and references therein). This being the case, it is possible for herbivorous organisms in the vicinity of the treated area to be exposed to the toxic Cu

concentrations in plants. In addition, drift and misuse, could lead to the snails receiving sub-lethal exposure to elevated concentrations of Cu. Under these conditions it is likely that local *I. newcombi* populations are exposed to elevated sub-lethal Cu concentrations over multiple generations. Pesticides and metals have been reported to be amongst the most powerful human introduced causes of selective pressure that act on both target and non-target species (Levinton *et al.* 2003). These pesticides may only be transiently available in the water, the use of repeated exposures over time increases the likelihood of genetic adaptation (Boue'tard *et al.* 2014). Under this scenario there is potential for *I. newcombi* populations from rice growing areas to develop increased tolerance to copper.

The broad distribution of *I. newcombi* in Australia in conjunction with the potential for the use of freshwater gastropods in investigating multi-generational responses to contaminants in laboratory studies makes this species ideal for development as a biomonitor. In addition to this, the ease of culture as well as the history of Cu exposure in *I. newcombi* provides an opportunity to use this species to investigate the development of resistance to Cu in gastropods.

1.3 Copper

1.3.1 Copper in the environment

The effect of contamination on the environment is increasing with the increasing global population and level of industrialisation (Moore *et al.* 2004). The intensive use of Cu by modern society has led to an elevated concentration of Cu in the biosphere (Kakkar and Jaffery 2005). Cu is a redox active transition metal that exists in the environment both in an oxidised cupric (Cu^{2+}) or reduced cuprous (Cu^{+}) form (Gaetke and Chow 2003). Cu enters the environment through a range of human activities including mining, smelting, refining, manufacturing and waste disposal (Wright and Welbourn 2002). Copper contamination associated with mining has been reported in Australia (Eriksen *et al.* 2001; Klessa *et al.* 1997). Cu has also used been used as an agricultural pesticide for long periods to control pests in rice crops and orchards in Australia (Merry *et al.* 1986; Stevens *et al.* 2014). It is likely that the increased demand for organic produce could lead to increased use of Cu as a pesticide as crops sprayed with Cu are still considered organic. The environmental persistence of Cu in conjunction with its intensive use can cause it to accumulate to concentrations higher than natural concentrations, especially in areas where it is extracted, processed or used (Kakkar and Jaffery 2005). Elevated concentrations of metals in the environment can have diverse, complex and often unpredictable effects on natural systems (Luoma and Rainbow 2008). Elevated Cu

concentrations have been shown to alter the structure and function of freshwater communities (Gardham *et al.* 2015). Given the elevated concentrations of Cu, in conjunction with its chemical properties and persistence, an understanding of the effects of copper over chronic exposures and across generations in addition to its acute toxicity is required.

1.3.2 Copper as an essential element

Copper is an essential element that is necessary for a wide range of biological processes (Flemming & Trevors, 1989). The essential biological role of Cu includes involvement as a catalytic and structural cofactor for many enzymes that are vital for a broad range of biochemical processes including energy generation, iron acquisition, oxygen transport, cellular metabolism, peptide hormone maturation, blood clotting, signal transduction and other processes (Kim *et al.* 2008). Despite being an essential element for biological function, exposure to Cu concentrations above essential requirements can be toxic to biota (Gaetke and Chow 2003). The homeostatic regulation of Cu is, therefore, essential for metabolic function, limiting toxic effects and survival (Dallinger *et al.* 2005).

1.3.3 Detoxification and storage of copper

When an organism is exposed to high concentrations of bioavailable Cu the detoxification and storage of the biologically active Cu above metabolic requirements plays an important role in minimising adverse effects. Metallothioneins bind free Cu ions and transfer them to the lysosomes where they transported into the lysosomal compartment by the process of endocytosis (Amiard *et al.* 2006). Lysosomes then play a central role in Cu storage and regulating inter-compartmental fluxes of Cu in cells (Polishchuk and Polishchuk 2016). Within the lysosomes of molluscs, excess Cu is accumulated in mineralized granules where it is unavailable for metabolic processes until required (Nott and Nicolaidou 1989).

1.3.4 Toxic effects of copper

When metabolically active Cu in an organism is greater than can be used or detoxified it leads to toxicity through interference with enzymatic function, induction of oxidative stress or through changes to normal metabolic function.

1.3.4.1 Copper interfering with enzymatic function

The binding of excess biologically active Cu to proteins can lead to the functional impairment of a range of biological processes. Metallothioneins and other metal transporters bind Cu through its high affinity to thiol groups associated with cysteine residues. The majority of globular proteins also contain cysteine residues (Letelier *et al.* 2005). Copper ions bind non-

specifically to free thiol groups in the cysteine residues of many proteins, leading to the disruption of protein structure and function (Das and Khangarot 2011). This can lead to the blocking of functional groups of enzymes and other proteins, disruption of transport systems for essential nutrients and ions, and redistribution of essential ions between different cellular components (Kakkar & Jaffery, 2005). Some specific examples of Cu interfering with biological function through non-specific binding of proteins include interference with cytochrome P450, UDP-glucuronyltransferase and glutathione *S*-transferase (Letelier *et al.* 2010).

1.3.4.2 Copper and oxidative stress

Biologically active Cu in organisms above requirements for normal biological function can increase in the rate of reactive oxygen species generation and cause oxidative stress. Most of the molecular oxygen consumed by animals is reduced to water, coupled with the oxidation of food and the production of energy (Figure 1.2, Livingstone 2001). During the process, the partial reduction of molecular oxygen leads to the formation of reactive oxygen species (ROS). ROS are needed for normal cellular processes, however, a deviation from normal levels can cause stress to the organism. Excess ROS that are produced in biological systems are usually detoxified and maintained at an acceptable level by antioxidant defences (Livingstone 2001). Antioxidant enzymes (glutathione peroxidase, catalase, glutathione reductase and superoxide dismutase) and non-enzymatic antioxidant responses (glutathione and oxidised glutathione) are inducible to maintain ROS equilibrium in animals (Di Giulio *et al.* 1989). Oxidative stress occurs when ROS generation is increased beyond the capacity of the antioxidant system, leading to disturbance of cellular metabolism and damage to cellular components (Lushchak 2011).

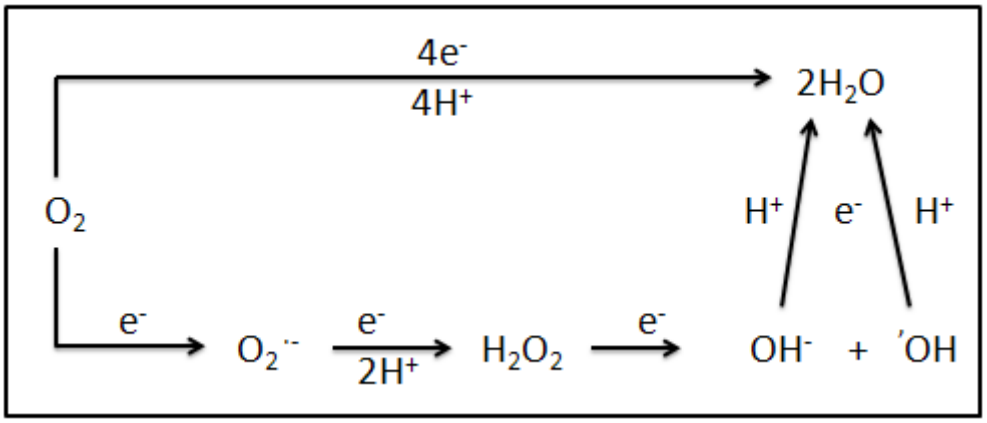


Figure 1-2. Routes of oxygen metabolism in organisms**.** Upper pathway shows four electron oxygen reduction with water formation. Lower pathway shows single electron reduction sequence, leading to the formation of reactive oxygen species (i.e. the superoxide anion radical ($O_2^{\cdot -}$), Hydrogen peroxide (H_2O_2) and hydroxyl radical (´OH)) (adapted from Lushchak, 2011).

Transition metal ions such as Cu can donate electrons to hydrogen peroxide yielding the hydroxyl radical and anions in the Haber-Weiss reaction (Livingstone 2001). Although ROS can be formed through the Haber-Weiss reaction (Equation 1) which is thermodynamically favourable, under normal circumstances it is kinetically quite slow. The presence of excess redox active metals to donate electrons increases the frequency of the Haber-Weiss reaction causing an increase of ROS (Equations 2 and 3) (Di Giulio *et al.* 1989). In aquatic organisms, despite the presence of antioxidant defences, oxidative damage can occur in organisms exposed to contaminants which stimulate ROS production (Livingstone 2001). The oxidative damage can be manifested in the form of lipid peroxidation, protein oxidation and DNA damage (Livingstone 2001).

Haber-Weiss reaction

(1) $O_2^{\bullet -} + H_2O_2 \rightarrow OH. + OH^- + O_2$

Haber-Weiss reaction in presence of transition metal

(2) $O_2^{\bullet -} + Cu^{2+} \rightarrow O_2 + Cu^+$

(3) $H_2O_2 + Cu^+ \rightarrow OH. + OH^- + Cu^{2+}$

1.3.5 Copper-induced changes in metabolic activity

Copper can also affect an organism through the diversion of resources from regular biological functions. Exposure to Cu contamination requires the use of resources for detoxification of Cu and repair of damaged biomolecules. These resources are diverted from important biological processes such as growth and reproduction and can have an adverse effect on these processes. Some examples of energetically expensive processes associated with the storage and

detoxification of Cu are the production of metallothioneins, metal rich granules and chaperone proteins. Increased synthesis of metallothioneins in organisms exposed to high metal concentrations, although often essential for survival, is done at a cost to broader metabolic function (Amiard *et al.* 2006). Production of granules for metal sequestration has been shown to occur at the threshold for growth inhibition, indicating that this detoxification process is at the expense of growth (Lavoie *et al.* 2009). Chaperone proteins such as heat shock proteins that are involved in the repair of metal-induced damage to proteins require the diversion of resources from regular biological processes (Dutta *et al.* 2014). Although these mechanisms may increase survival or reduce damage in organisms exposed to Cu the change in energy allocation leads to reductions in the energy allocated to essential biological functions such as growth and reproduction (De Schamphelaere *et al.* 2007).

1.4 Exposure-bioaccumulation-response relationship

The effects of metal contamination on an organism can be explored through the relationship between external exposure, bioaccumulation of the contaminant and the response of the organism (McCarthy and Shugart 1990; Moore *et al.* 2004). The major benefit of using such a framework is that it allows clear causal links to be established between the contaminant exposure and adverse outcomes in the organisms exposed.

1.4.1 Exposure

In the aquatic environment, organisms can be exposed to contaminants present in water, food and sediment (Luoma and Rainbow 2008). Environmental risk assessment has traditionally focussed on the partitioning of toxic substances in the environment and the potential hazards of these contaminants if they exceed certain threshold concentrations (van der Oost *et al.* 2005). Within each compartment, the concentration of metals can be assessed. Chemical analysis of discrete water and sediment samples, for example, can provide information on the environmental compartments into which contaminants are distributed, the concentrations and chemical form (Taylor 2009). The bioavailability of the metals within each compartment will be influenced by the prevailing physical and chemical conditions. Factors such as salinity, dissolved organics, pH, hardness, mineralogy and sediment grain size all effect chemical form and mobilisation of metals in aquatic systems, which in turn influences the bioavailability and toxicity of these metals (Roesijadi and Robinson 1994; Sadiq 1992).

The majority of aquatic toxicology studies conducted under laboratory conditions use exposures to constant contaminant concentrations (Hoang *et al.* 2007). The use of constant

concentrations may not reflect the concentrations of chemicals in the environment which vary based on many factors. This is particularly true of Cu released into the aquatic environment which adsorbs to organic matter and sediment in a relatively short period and quickly reduces the bioavailable concentration to background levels (Stevens *et al.* 2014). Under this scenario organisms are likely to receive exposure to higher concentrations in pulses associated with contamination events followed by a relatively quick drop to background concentrations. It has been reported that organisms respond differently to the pulse exposures than they do to continuous exposures (Chen *et al.* 2012; Diamond *et al.* 2005; Hoang *et al.* 2007). The response to pulse exposures depends on frequency, magnitude and duration of the pulses as well as the type of chemical (Rogevich *et al.* 2008). An exception is the similar response of algae to time averaged Cu concentrations of pulse exposures to time average Cu concentration of continuous exposure to Cu (Angel *et al.* 2015). This study demonstrates the need to consider recovery from short term exposures when using pulse exposures. It has been suggested that conducting *in situ* toxicity tests in the field to investigate pulse exposures faces several challenges associated with changing environmental conditions and that laboratory studies are a useful way of investigating the effect of pulse exposures (Hoang *et al.* 2007).

1.4.2 Bioaccumulation and biomonitoring

Bioaccumulation is the concentration of a chemical taken up by an organism. Understanding bioaccumulation is important in the field of ecotoxicology as it is only when a contaminant is accumulated that it can exert toxic effects (Luoma 1996). The bioaccumulation of a chemical will be determined by the bioavailable concentration of that chemical that an organism is exposed to. As discussed previously, physical and chemical properties determine the bioavailability of a chemical (Section 1.4.1). Although bioaccumulation is important in understanding toxic effects, it must be considered in the context of the exposure-bioaccumulation-response relationship, as toxic effects resulting from the transfer of metals may occur without changes to overall tissue concentrations. In the marine gastropod *Bembicium nanum* exposed to Cu, lysosomal lysosomal membrane destabilisation significantly increased without change in Cu tissue concentration but the use of isotopically enriched Cu showed that the snails were metabolising the Cu (Ubrihien *et al.* 2017b). Exposure experiments conducted under controlled conditions allow an understanding of bioaccumulation of a chemical under specific conditions, providing a baseline to investigate response.

Biomonitoring involves the use of tissue metal concentrations in organisms to infer the bioavailable concentrations of metals in that environment. These measurements allow a spatial

and time integrated assessment of bioavailability of contaminants (Rainbow and Phillips 1993). The relationship between toxic concentrations and total accumulated body burden is relatively difficult to predict for metals, mainly because of interspecific variability in accumulation patterns and metal-specific modes of toxicity (Morgan *et al.* 2007). This highlights the importance of understanding bioaccumulation in a range of taxa, including gastropods. Despite laboratory exposures under controlled conditions often being less complex than field exposures, they provide an understanding of the exposure-bioaccumulation relationship which forms a baseline from which to interpret more complex field based investigations.

If excess biologically active metals are present in an organism, there is a need to detoxify and store them. Organisms sequester and detoxify metals via the high affinity of metal cations for the sulphydryl groups of metallothioneins, or via their accumulation in membrane-limited granules (Domouhtsidou *et al.* 2004). Once detoxified, there is no limit to the amount of metal that can be stored by an organism, as it represents no danger to the metabolism of the animal (Rainbow 2002)**.** In freshwater gastropods, the digestive gland plays a central role in the accumulation, storage, detoxification and excretion of metals (Moolman *et al.* 2007). Within the digestive gland of gastropods, copper storage has been associated with metal rich granules (Desouky 2006; Ng *et al.* 2011; Snyman *et al.* 2005). As a result of the storage of metals in a detoxified form, information on the concentrations of metals in biota alone does not provide a reliable indication of biological injury, environmental effects or human health consequences (Nicholson 2003; Rank *et al.* 2007). There is also a need to link the concentrations an organism is exposed to and the amount that is bioaccumulated to toxic effects in order to establish clear links between metal exposure, bioaccumulation and resultant responses.

1.4.3 Response

Environmental stress can be defined as the condition where an organism's physiological function is altered by external factors (Miao *et al.* 2015). Stress associated with exposure to metals occurs when the rate of metal uptake into the body exceeds the combined rate of metabolic use, excretion and detoxification of metabolically available metal (Rainbow, 2002). When a stress response is activated in an organism, the potential of the organism to survive may have already started to decline due to the reduced ability of the organism to mount responses to additional challenges (Depledge *et al.* 1995). It is, therefore, necessary to have means of assessing stress responses in organisms in order to evaluate the effect of a metal on the environment.

1.4.3.1 Biomarkers

Historically, impact assessments on freshwater ecosystems have often been based on chemical data and compliance with chemical concentration thresholds such as ANZECC/ARMCANZ (2000) and indicators such as macroinvertebrate biotic indices (Gust et al. 2011). More recently there has been an increased focus on the use of biomarkers and sub-lethal effects as indicated by the move to a multiple lines of evidence approach in the revision of the ANZECC/ARMCANZ sediment quality guidelines (Simpson *et al.* 2013). The move to such an approach requires development of appropriate biomarkers to measure sub-lethal responses.

Biomarkers assess the effect of contaminants on the environment by assessing the condition of organisms exposed to the contaminant. Biomarkers measure responses at the biochemical, cellular, physiological or behavioural level and provide evidence of exposure or effect (Broeg *et al.* 2005). In conjunction with exposure and bioaccumulation measurements, biomarkers provide the evidence of responses that allows links between a contaminant in the environment, its bioaccumulation and responses in an organism to be established. Ideally biomarkers should detect contaminant-induced changes early and at a low level of biological organisation (Izagirre *et al.* 2009). By providing an early indication of contaminant-induced changes occurring in ecological systems, biomarkers can act as early warning systems allowing remediation strategies to be put in place prior to significant damage occurring at higher levels of biological organisation (Bolognesi and Fenech 2012).

Even though biomarkers can play an important role in environmental assessment, there have been criticisms of their effectiveness. Depledge *et al.* (1995) summarised some of the criticisms of biomarkers as being difficult to measure, expensive, not very specific, induced by more than one contaminant, affected by a range of natural environmental stressors, and not sensitive enough to detect contaminant exposures at environmentally realistic concentrations. A further criticism of biomarkers is that they are associated with individual organisms and are unable to reliably predict changes at higher levels of biological organisation (Forbes *et al.* 2006).

It is necessary to undertake a rigorous assessment of biomarkers prior to their use in order to validate their ability to detect responses associated with environmental contamination. van der Oost *et al.* (2005) suggested a range of criteria that should be considered prior to a biomarker being used to assess the condition of natural populations

- the assay to quantify the biomarker should be reliable and preferably cheap and easy to perform;

- the biomarker should be sensitive to pollutant exposure or effects in order to serve as an early warning;
- baseline data on the biomarker should be well defined in order to distinguish between natural variability and contaminant-induced stress;
- the effects of confounding factors to the biomarkers response should be well established;
- the underlying mechanism of the relationship between biomarker response and pollutant exposure should be established; and
- the toxicological significance of the biomarker, such as the relationship between its response and the impact to the organism should be established.

Laboratory studies in a controlled environment provide an opportunity to assess the sensitivity of biomarker, confirm relationships between exposure and response, and provide baseline data. Although it is necessary to understand where biomarkers have limitations, a rigorous assessment process as recommended by van der Oost *et al.* (2005) assists in understanding where it is appropriate to use a particular biomarker. Additionally, as biomarkers can be considered as a single line of evidence in a multiple lines of evidence approach (Taylor and Maher 2016a), additional evidence gained through a broader investigation alleviates many of the prior criticism of biomarkers.

1.4.3.2 Choice of enzymatic and cellular biomarkers

The suite of biomarkers chosen for the assessment of response in this project is based on the expected stress response in *I. newcombi* when exposed to Cu. As discussed earlier (Section 1.3.4.2), exposure to excess Cu can cause oxidative stress, therefore, the biomarkers total antioxidant capacity (TAOC) and lipid peroxidation (LP) were chosen to investigate the effects of oxidative stress. As the lysosome is an important point of storage and detoxification for Cu, lysosomal membrane destabilisation (LD) was used as an indicator of general stress associated with copper exposure. Individual life history traits and differential gene expression were also used to measure response in this project and are reviewed separately in later sections of this review.

1.4.3.2.1 Total antioxidant capacity

Reactive oxygen species (ROS) are produced during aerobic respiration and are always present as part of the cellular respiration process. Exposure to elevated concentrations of Cu can lead

to an increase in ROS (Section 1.3.4.2; Di Giulio *et al.* 1989). The increase in ROS in turn can cause organisms exposed to metal contamination to experience oxidative stress (Lushchak, 2011). Increases in ROS production are counteracted by the antioxidant system (Section 1.3.4.2; Livingstone 2001). The antioxidant system neutralises ROS, however, a continuous oversupply of ROS can lead to the antioxidant system being overwhelmed and result in a reduction in antioxidant capacity. Measurement of reductions in antioxidant capacity provide useful indications of higher order effects, as there may be several consequences including membrane lipid peroxidation, protein damage, DNA alteration and enzyme inactivation (Bocchetti & Regoli, 2006). A summary of studies investigating the antioxidant response to a range of contaminants in gastropods is provided (Table 1-1). The total antioxidant capacity assay measures the overall ability of the antioxidant system overall to neutralise excess ROS.

1.4.3.2.1 Lipid peroxidation

Lipid peroxidation (LP) is widely recognised as a consequence of excess ROS production and associated oxidative stress (Winston and Di Giulio 1991). The attack of membrane lipids by ROS initiates an oxidation process known as LP (Almeida *et al.* 2007). Peroxidation of lipids by ROS results in the formation of unstable hydroperoxides and forms part of a cascade of events that are caused by oxidative stress which can lead to cell death (Reid and MacFarlane 2003). Lipid peroxidation specifically induces disturbance of cellular structures and functional loss of biomembranes, modifies lipoproteins and generates additional toxic by-products (Niki 2009). In laboratory studies, LP has been shown to be effective as a biomarker in molluscs to demonstrate responses to Cd, Pb, Cu, Se, and sediment taken from a known metal contamination gradient (Taylor and Maher 2012a; Taylor and Maher 2012c; 2014; Ubrihien *et al.* 2017b; Viarengo *et al.* 1988). In a field situation lipid peroxidation has been shown to have the required sensitivity to differentiate between contaminated and non-contaminated sites (Almeida *et al.* 2003; Taylor and Maher 2016b; Ubrihien *et al.* 2017c; Wilhelm Filho *et al.* 2001). A summary of studies investigating LP in gastropods is provided (Table 1-2). As one effect of LP is the loss of functionality of cellular and organelle membranes, including lysosomes, the lipid peroxidation process can be a contributing factor to lysosomal membrane destabilisation (Taylor 2009).

Table 1-1. A summary of studies investigating antioxidant defences in marine and freshwater gastropod species exposed to environmental and chemical stressors.

Species	Stressor	Result	Reference
Littorina littorea	Anoxia (6 d) and aerobic recovery (24 h)	Differential regulation of various enzymatic and non-enzymatic AOD in different tissues	Pannunzio and Storey 1998
Austrocochlea porcata	Crude oil (96 h)	GSH and GPx not sensitive	Reid and MacFarlane 2003
Biomphalaria glabrata	Azinphos-methyl (organophosphate) (48 and 96 h)	Variable response of SOD, transient ↓ CAT	Kristoff *et al.* 2008
Dolabrifera brazieri, Bembicium nanum and *Siphonaria denticulata* (embryos)	Temperature and salinity (72 hr)	Temperature, salinity and species-dependent changes in TAOC	Deschaseaux *et al.* 2010
Dolabrifera brazieri, Bembicium nanum and *Siphonaria denticulata* (larvae)	Temperature and salinity (upon hatching)	Temperature, salinity and species dependent changes in TAOC	Deschaseaux *et al.* 2011
Bittium reticulatum	Diet of toxic algae (*Caulerpa taxifolia*) (7 d)	↑ GPx, GR and GsT	Sureda *et al.* 2009
Bembicium nanum	Metal contamination gradient	No change in TAOC	Ubrihien *et al.* 2017b
Bembicium nanum	Cu	No change in TAOC	Ubrihien *et al.* 2017a
Haliotis diversicolor	Tributyltin (30 d)	↓ SOD, ↑ POD,	Zhou *et al.* 2010
Chilina gibbosa	Azinphos-methyl (organophosphate) (48 h)	↑ GSH, ↑ CAT, no effect in SOD or GST	Bianco *et al.* 2013

AOD = antioxidant defences, CAT = catalase, GPx = glutathione peroxidase, GR = glutathione reductase, GSH = glutathione, GST = glutathione *S*-transferase, POD = peroxidase, SOD = superoxide dismutase, TAOC = total antioxidant capacity , ↑ = increase, ↓ = decrease.

Table 1-2. Studies investigating lipid peroxidation in marine and freshwater gastropods exposed to environmental and chemical stressors.

Species	Stressor	Result	Reference
Bittium reticulatum	Diet of toxic algae (*Caulerpa taxifolia*) (7 d)	No effect (MDA)	Sureda *et al.* 2009
Dolabrifera brazier, *Bembicium nanum* and *Siphonaria denticulata* (embryos)	Temperature and salinity (72 hr)	Temperature, salinity and species-dependent changes in MDA	Deschaseaux *et al.* 2010
Dolabrifera brazier, *Bembicium nanum* and *Siphonaria denticulata* (larvae)	Temperature and salinity (upon hatching)	Temperature, salinity and species-dependent changes in MDA	Deschaseaux *et al.* 2011
Littorina littorea	Anoxia (6 d) and aerobic recovery (24 h)	Short-term effects during recovery (conjugated dienes and lipid hydroperoxides) No effect (MDA)	Pannunzio and Storey 1998
Austrocochlea porcata	Crude oil (96 h)	No effect (4-hydroxy-2(E)-nonenal and MDA)	Reid and MacFarlane 2003
Haliotis diversicolor	Tributyltin (30 d)	↑ MDA	Zhou *et al.* 2010
Planorbius corneus	Pentachlorophenol (2, 8 and 13 d)	Transitory and concentration-dependent responses	Klobučar *et al.* 1997
Lymnaea acuminata	Deltamethrin and MGK-264 (96 h)	↑ MDA	Singh *et al.* 2008
Planorbius corneus	Azinphos-methyl and chlorpyrifos (48 h)	↑ MDA	Cacciatore *et al.* 2015
Bembicium nanum	Metal contamination gradient	No change in MDA	Ubrihien *et al.* 2017b
Bembicium nanum	Cu	No change in MDA	Ubrihien *et al.* 2017a
Lymnaea natalensis	Contaminated sediment (30 d)	↑ MDA	Cacciatore *et al.* 2015

MDA = malondiadehyde, ↑ increased induction.

1.4.3.2.2 Lysosomal membrane destabilisation

Lysosomes are membrane-bound intracellular organelles with an acidic interior (Appelqvist *et al.* 2013). They are bounded by a semi permeable lipoprotein membrane and contain approximately 60 hydrolytic enzymes that are involved in the breakdown of endogenous and exogenous material (Lowe and Fossato 2000). A range of important biological functions take place in the lysosome including metal detoxification and storage, degradation of macromolecules, recycling of cellular components and cell death processes (Appelqvist *et al.* 2013; Johansson *et al.* 2010; Ladiges *et al.* 2010). They are responsible for the storage of a broad range of substances including metals (both excess essential and non-essential) and organic chemicals (Da Ros *et al.* 2007; Garmendia *et al.* 2011). The investigation of lysosomal responses for use as biomarkers is based on the premise that lysosomal processes are integral to detoxification, breakdown and storage of contaminants and as such they should be indicative of the functional integrity of cells experiencing contaminant-induced stress (Moore 2002). The accumulation of contaminants into lysosomes along with the associated detoxification processes can alter lysosomal structure, reduce integrity of the membrane and initiate increased autophagy (Broeg *et al.* 2005; Izagirre and Marigómez 2009; Moore *et al.* 2007; Nicholson 2003). Oxidative damage in the form of lipid peroxidation reduces the integrity of the lipid membrane and can cause an increase in lysosomal membrane destabilisation (Taylor and Maher 2010). The onset of apoptotic programmed cell death can also lead to a breakdown of the lysosomal membrane as the hydrolytic enzymes are released from the lysosomal compartment to degrade cellular components (Johansson *et al.* 2010). Once the lysosomal membrane is no longer stable enough to retain the contents of the lysosome, the contents of the lysosome can leak into the cytosol causing cellular injury and death (Viarengo *et al.* 2007). The responses of lysosomes to contaminant exposure range from the earliest detectable alterations to cellular processes through to the induction of cell death and have been widely used as general biomarkers of contamination (Garmendia *et al.* 2011; Moore *et al.* 1985). Lysosomal membrane destabilisation has been broadly recognised as an effective biomarker in many studies (e.g., Broeg *et al.* 2002; Castro *et al.* 2004; Ringwood *et al.* 2003; Taylor and Maher 2016b; Ubrihien *et al.* 2017c). A summary of lysosomal destabilisation studies investigating the effects of stressors in gastropods is provided (Table 1-3).

Table 1-3. A summary of studies investigating lysosomal destabilisation (LD) in marine and freshwater gastropod species exposed to environmental and chemical stressors.

Species	Stressor	Result	Reference
Thais lapillus	Temperature, salinity and aerial exposure	Temporal fluctuations in LD	Stickle *et al.* 1985
Helisoma trivolvis	Cu, Cd and methoxychlor	↑ LD	Molnar and Fong 2012
Bembicium nanum	Metal contamination gradient	↑ LD	Ubrihien *et al.* 2017b
Bembicium nanum	Cu	↑ LD	Ubrihien *et al.* 2017a
Lymnaea stagnalis	Fomesafen	↑ LD	Russo *et al.* 2007

↑ = increased induction.

1.5 Genetic responses to contaminants

In the current environmental context where many populations exist in a modified environment, evolutionary change can be rapid (Boue'tard *et al.* 2014). When exposed to adverse environmental conditions, organisms use specific and in some cases adaptive stress responses in order to cope (Roelofs *et al.* 2010). An organism's ability to survive in a contaminated environment will be determined by its genetic makeup, and the contaminant may in turn affect the genetic makeup of both individuals and populations. When a population is exposed to contamination, genetic effects can be through direct effects of the contaminant in the form of mutations to DNA, or indirect effects in the form of changes to the genotype from microevolution, random genotype effects or phenotypic plasticity (Figure 1.3). The response of populations to contaminants at a genetic level can occur over multiple generations. It has been reported that most populations that live in metal-contaminated environments develop metal resistance (Klerks and Weis 1987). This is particularly true of populations of species with a fully aquatic life cycle and low dispersal ability (Boue'tard *et al.* 2014).

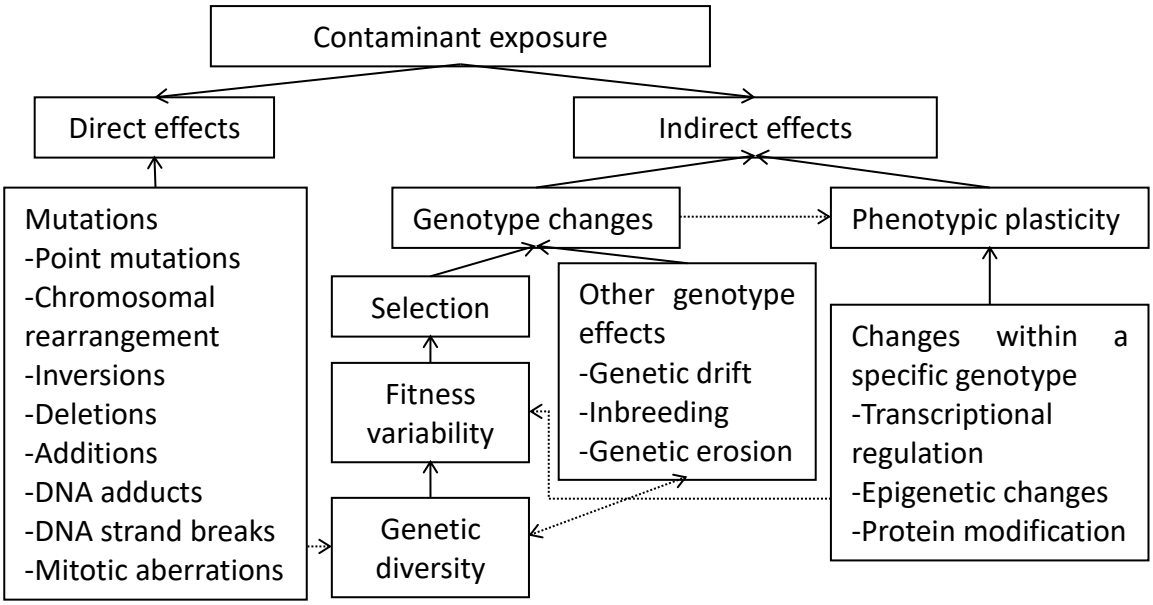


Figure 1-3. Model of genetic based responses across biological levels that occur when an organism/population is exposed to contaminants.

The adaptive responses to contaminants can be classified as acclimation or adaptation responses. An organism's survival in the presence of a chemical stressor may require acclimation within the range of the organism's phenotypic plasticity. This is achieved by altering regulatory mechanisms that affect metabolism or transcriptional profiles (Roelofs *et al.* 2007). Alternatively, exposure to the contaminant can lead to changes in the genetic composition of the population through the process of microevolution. For microevolution to occur there must be sufficient genetic diversity in the population and fitness benefits between individuals within the population experiencing chemical stress that result from the differences in genotypes (Roelofs et al., 2010). Although the processes associated with direct effects on DNA, phenotypic plasticity and microevolution are often viewed as separate they are intrinsically related. Direct effects of contaminants in the form of mutations affect the genotype of the organism and the genetic diversity of the population. The genetic diversity of a population will in turn affect the adaptive potential through the availability of differing alleles that provide the basis for microevolution. Reduction in genetic diversity is a catalyst for the increase of random genetic genotype effects such as inbreeding and genetic drift. Additionally, even though the genetic basis of phenotypic plasticity is generally associated with epigenetics and regulatory elements, the underlying genotype provides the scaffold on which these regulatory differences operate (Figure 1.3).

1.5.1 Effects of contaminants on genotypes

Contaminants can cause direct effects on genotype through the effects of chemical-induced mutations. Contaminants can also have indirect effects on the genetic composition of a population through the processes of genetic drift, inbreeding and directional selection (Medina *et al.* 2007).

1.5.1.1 Direct effects of metals on DNA

The toxic effects of metal exposure can affect DNA through damage to its molecular structure. If a contaminant is mutagenic at sub-lethal concentrations, new genotypes and alleles can be added to a population's genetic make-up (Ribeiro and Lopes 2013). Types of direct damage include point mutations, chromosomal rearrangements, inversions, deletions, additions, DNA adducts, DNA strand breaks and mitotic aberrations (Medina *et al.* 2007). Direct effects of metal toxicity may be limited to one generation, with populations able to recover quickly, however, if direct effects occur in germlines, significant effects to the population can occur (Medina *et al.* 2007). DNA strand breaks and increased micronuclei frequency are some direct effects of Cu exposure that have been reported to occur in molluscs (Gomes *et al.* 2013; Ruiz *et al.* 2015).

1.5.1.2 Genetic drift and inbreeding

Genetic drift is the random change in frequency of an allele in a population and can occur as a result of contaminant-driven population bottlenecks (Ribeiro and Lopes 2013). Inbreeding refers to the process whereby mating among relatives leads to an increase in homozygosity within a population (Keller and Waller 2002). Where populations are driven to low levels by contaminant-induced mortality, genetic diversity is reduced and inbreeding is likely to occur (Coutellec and Caquet 2011). The genetic erosion associated with the effects of genetic drift and inbreeding reduce the adaptive potential of a population (Bijlsma and Loeschcke 2012). The reduced genetic diversity within a population will not only affect the response to the original contaminant but may also cause populations to have reduced ability to cope with other environmental challenges (Coutellec and Barata 2011).

1.5.1.3 Directional selection

Directional selection will change the genetic makeup of a population based on fitness of various alleles in response to a stressor. Rare alleles that are neutral or even slightly deleterious under normal conditions may provide a fitness benefit through increased tolerance when an organism

is exposed to chemical stress (Bell and Collins 2008). Based on the fitness benefit these alleles can accrue they can become common in the population due to natural selection.

1.5.2 Phenotypic plasticity

Phenotypic plasticity is defined as the capacity of a single genotype to produce different phenotypes in response to changing environmental conditions (Hua *et al.* 2015). Phenotypic plasticity is underpinned by the fact that all genomes are intrinsically flexible, with sequence changes in promoter regions or epigenetic adjustments conferring significant phenotypic consequences (Morgan *et al.* 2007). On this basis, cell metabolism is maintained within the presence of the external environment through the modification of gene expression. In addition to changes to gene expression at the transcriptomic level, at the molecular level, allosteric regulation allows the activation and deactivation of proteins that provide a short term response to changes in environmental conditions. Reversible phosphorylation, for example, is a process that can affect the regulation of cellular metabolism including activity levels, protein-protein interactions and subcellular localisation (Storey and Wu 2013).

In contrast to changes associated with genotype, adaptations to the environment associated with phenotypic plasticity are retained for a shorter timeframe. Increases in fitness that result from the regulatory changes associated with phenotypic plasticity are likely to disappear rapidly when organisms are maintained in uncontaminated conditions (Klerks *et al.* 2011). Although this is true in the majority of cases, plasticity can be evolutionarily adaptive and facilitate evolutionary change, especially in rapidly changing environments or the colonisation of novel habitats (Kelly *et al.* 2011, and references therein). An example of this is epigenetic changes associated with the germline that are passed across generations (Head 2014).

The assessment of phenotypic plasticity provides challenges for ecotoxicologists. The relative merits of conducting laboratory experiments versus field studies to investigate phenotypic plasticity have been discussed. It has been suggested that laboratory studies are very labour intensive, time consuming and expensive, that it is difficult to approximate the field situation, that it is hard to capture a long term response and extrapolation is limited as adaptation is only investigated for a very specific set of conditions (Klerks *et al.* 2011). In contrast, when field studies investigating phenotypic plasticity have been conducted there have been difficulties determining differences in population effects associated with different selection pressures linked to lack of sample size, conditions being too benign, or changes being masked by other environmental characteristics (Boue'tard *et al.* 2014). There are benefits and drawbacks to both

field and laboratory studies, laboratory studies provide valuable information on a population's evolutionary trajectory and also provide a predictive measure and information on rate of adaptation under controlled conditions (Klerks *et al.* 2011).

1.5.3 The effect of adaptation and plasticity on risk assessment

In relation to ecotoxicology, the classic dose-response analysis relies on the assumption that the underlying response of a species is consistent. This assumption relies on genetic consistency between populations. In a highly heterogeneous environment where local populations are adapted to natural and anthropogenically introduced variability these assumptions may not hold true. In populations exposed to contaminants over multiple generations, adaptive processes result in those populations showing a different dose-response relationship in comparison to unexposed populations (Coutellec and Barata 2011). As such there is support for the consideration of the adaptation processes and the resultant differentiation between tolerance in species in environmental risk assessment (Boue'tard *et al.* 2014). In order to incorporate allowances for adaptation into environmental risk assessment, there is a need to quantify the adaptive potential of populations (Côte *et al.* 2015). To fully appreciate adaptive potential in the context of ecotoxicology, an understanding of the timeframe over which adaptation occurs, integrated with a measure of the selection pressure as determined by exposure concentrations is required.

1.6 Genomic methods in ecotoxicology

Over the last decade there has been a substantial increase in the use of omic techniques in ecotoxicology. Omic studies include those at the transcriptomic, proteomic and metabolomic level (Figure 1-4). A large part of an organism's response to stress relies on the synthesis of appropriate proteins to participate in mechanisms such as detoxification, cellular repair and a broad range of processes to manage contaminant-induced stress. This involves the transcription of the appropriate genes DNA to RNA and the translation of the RNA to proteins. The activity of the proteins can be confirmed by the presence of metabolites. In ecotoxicology, omic tools have the potential to provide an indication of a response to contaminants at an early stage, investigate response at an organism global level, detect contaminant-specific responses, inform on the level of stress an organism is experiencing, provide a mechanistic understanding of the response and detect novel responses.

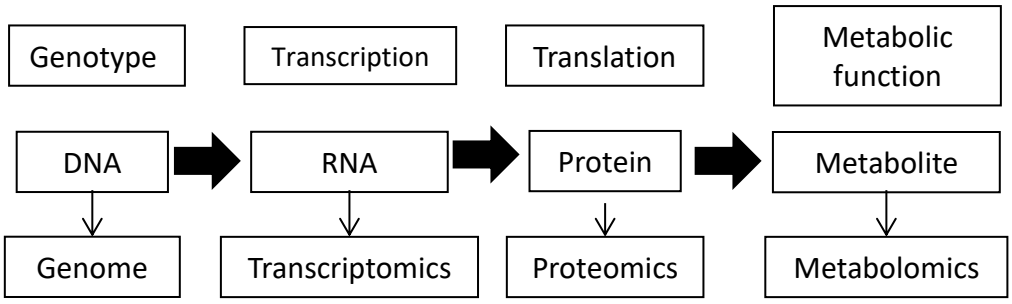


Figure 1-4 Linking genetic processes in response to environmental stimuli from DNA through to metabolites including the molecular feature/process, type of molecule and the relevant omic technique.

1.6.1 Early detection of responses

From an ecotoxicological perspective, biomarkers that detect contaminant-induced problems at an early stage allows them to act as an early warning system for effects at higher levels of biological organisation. The use of omic techniques allows contaminant-induced change to be detected at the molecular level, therefore, detecting the earliest alterations associated with the exposure. A change in the transcriptional regulation of genes and biological pathways as a means of maintaining homeostasis is also one of the earliest responses of an organism to an environmental stress (Ankley *et al.* 2006). Metabolites created through interactions between the stressor and cellular targets can provide early indication of contaminant exposure and also the effect of the chemical (Lankadurai *et al.* 2013). Due to the association with early changes to contaminant-induced stress, transcriptomic responses offer high potential for the identification of biomarkers that act as early warning systems of effects at higher levels of biological organisation. The early changes in the transcriptome may not align with changes at the protein level as some mRNAs are not translated or suppressed by regulatory mechanisms (van Straalen and Feder 2012).

1.6.2 Chemical specific responses

The use of traditional biomarkers has been criticised for their lack of chemical specificity (Depledge and Fossi 1994). Different chemicals are taken up, transported, detoxified and exert toxic effects in different ways. The expression signatures at a molecular level that result from the specific uptake, transport and detoxification pathways have the potential to identify the responses that are associated with specific chemicals or groups of chemicals (Schirmer *et al.* 2010). These responses can be identified as stressor-specific induced pathways, transcriptomic patterns, or stressor-specific genetic biomarkers (Brinke and Buchinger 2017). The detection

of individual patterns of altered gene expression that have been linked to specific classes of chemicals has been demonstrated (Bougas *et al.* 2013; Paules 2003).

In addition to the value that chemical-specific responses provide in identifying chemical stressors in one species, they can assist in understanding mechanistic differences in responses to contaminants across taxa and in the assessment of new chemicals. Cross-species genomic comparisons can be made based on existing chemical-specific responses and provide a mechanistic understanding of how the responses to contaminants have diverged across taxa (Snape *et al.* 2004). Knowledge of responses associated with specific groups of chemicals can be used as a comparative basis in the evaluation of new chemicals, especially those with similar characteristics, and assist in understanding uptake, detoxification and modes of action of the new chemical (Brinke and Buchinger 2017).

1.6.3 Multiple single endpoint assays

Outside of omic techniques, assessment methods often focus on the body burden of common contaminants and a limited number of physiological or molecular biomarkers as endpoints for assessing the effects of chemical pollution (Leung *et al.* 2014; Mazzitelli *et al.* 2017). The traditional biomarker analysis focusses on a single measurable endpoint that is associated with changes to an individual biological pathway or a single response. Many current genomic techniques have advantages over traditional single-endpoint assays in that they can provide information on the entire range of biological processes occurring and with further development will allow for a reduction of routine ecotoxicological testing (Snape *et al.* 2004). Although this provides opportunity to obtain data on all pathways, the complexity associated with such a broad range of data and interactions between pathways can be confounding. Care must be taken to ensure that the omic markers are appropriately validated. The use of adverse outcome pathways to link molecular events to adverse outcomes at higher levels of biological organisation is an example of how this challenge is being met (Ankley *et al.* 2010).

1.6.4 Level of stress

In an effort to maintain homeostasis, organisms exposed to chemical stress alter gene expression and protein function as a means of regulating a range of biological processes including contaminant uptake, transport, storage and detoxification, as well as mechanisms that respond to cellular challenge or damage. In addition to contaminant-specific responses, organisms have general stress responses that can be indicative of their level of stress on a continuum from optimal health to highly stressed. At different levels of exposure and associated stress, an organism will initiate different processes that can be detected at the

molecular level (Piña and Barata 2011). As a hypothetical example based on known responses of molluscs to copper, at low, medium and high exposures, responses may move from increased metal transport, to increased antioxidant response and programmed cell death (Figure 1-5).

	Contamination level		
	Low	**Medium**	**High**
Response type	Metal transport	Antioxidant response	Programmed cell death
Associated genes/ proteins	Metallothionein Cu ATPase	Super oxide dismutase Catalase	Cathepsin BCL2

Figure 1-5. Hypothetical example of changes that may be seen as the response to exposure of an organism to low, medium and high levels of copper.

1.6.5 Novel responses

The responses of organisms to contaminants are complex and not fully understood. Existing biomarker techniques generally investigate changes in response that are established and well understood. As omic techniques can inform on the entire range of biological functions occurring, they provide an opportunity to increase the understanding of the response of organisms to contamination through the detection of changes in the regulation of novel transcripts, genes and pathways (Wang *et al.* 2009). Many transcriptomic studies in non-model species have identified differentially expressed genes associated with contaminant exposure and associated stress that have not been annotated (Bougas *et al.* 2013; Hornett and Wheat 2012). As such, the change in their regulation in response to contaminant exposure is established but their function is not. Schirmer *et al.* (2010) recommended that some emphasis be placed on identifying functions of differentially expressed RNA sequences that are not yet annotated.

1.6.6 Mechanistic understanding

Responses made by organisms to contaminants are often seen as behavioural or metabolic, however, these responses are accompanied or driven by the adjustments at the molecular level (Buckley *et al.* 2001). All primary interactions of chemicals with organisms and their responses occur via the biomolecules of which cells are comprised. The key to gaining a mechanistic understanding of responses is through understanding of these interactions (Schirmer *et al.* 2010). Omic techniques can detect responses in organisms from the perspective of changes in the transcriptome associated with genes that are being differentially expressed, the proteome

provides an indication of all proteins present, and the metabolome, the metabolites formed when an organism interacts with the environment. As omic methods can operate at a transcriptome, proteome and metabolite level a complete mechanistic understanding of the response to contaminants can be gained and assist in bridging the gap between cause and effect (Brinke and Buchinger 2017).

1.6.7 Challenges and opportunities

Toxicogenomics holds much promise for ecotoxicologists, but the complex nature of molecular interactions, in addition to the variability in the environment, present substantial challenges to the development of omic tools for ecotoxicology. These complex systems can make linking cause and effect extremely difficult (Brinke and Buchinger 2017). This complexity is compounded in contaminated environments where organisms are exposed to multiple stressors. The major challenge in this highly complex system is to define omic markers that are reliably indicative of contaminant-specific responses at higher levels of biological organisation.

To use mechanistic data from omic methods to support chemical assessments, this information needs to be related to endpoints meaningful for ecological risk (Ankley *et al.* 2006; Leung *et al.* 2014). In response to this challenge, Paules (2003) described phenotypic anchoring, where studies are designed to relate specific alterations in gene expression to specific adverse outcomes of environmental stressors. This concept was extended by Ankley *et al.* (2010) who recommended adverse outcomes pathways (AOP) as a means of integrating omic methods with ecotoxicological understanding. Ankley *et al.* (2010) described an AOP as a conceptual construct that portrays existing knowledge concerning the linkage between a direct molecular initiating event and an adverse outcome at a biological level of organisation relevant to risk assessment. As such, AOPs are a sequential series of linked biological responses that describe the mechanistic response of biological systems to a contaminant, starting at an molecular initiating event through a series of key events to adverse outcomes that are generally indicative of changes at the population or community level (Figure 1.6; Villeneuve *et al.* 2014). An example of the application of AOPs where transcriptomic data has been used to make links to adverse outcomes is in *Daphnia magna* where calcium dependent mechanisms were linked with narcosis induced by lipophilic compounds (Antczak *et al.* 2015)

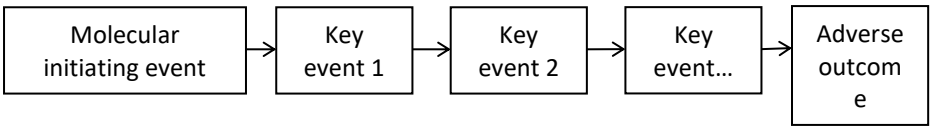


Figure 1-6. Simplified model for an adverse outcomes pathway (Adapted from Villeneuve *et al.* 2014).

Although the challenge of integrating omic methods into the area of ecotoxicology and risk assessment is substantial, the increased generation of data relating to mechanistic effects and the adoption of models such as AOPs to apply findings in a meaningful way is leading to advances. Brinke and Buchinger (2017) suggest that individual studies on a stressor provide an initial understanding. A combination of individual studies on a stressor allows greater information as well as inference through weight of evidence. The combination of data on the diverse range of stressors being studied allows a broader mechanistic understanding of responses. It is only through a concerted effort to combine and build on the existing knowledge that a broad mechanistic understanding of the complex interactions between contaminants and biota will be developed (Brinke and Buchinger 2017). The challenge of successfully integrating omics methods into the field of ecotoxicology lies in understanding of the relationship between the effects of contaminants and changes that can be reliably detected using omic methods. RNA-seq studies, for example, produce large amounts of complex data and improved methods for interpreting the data to detect contaminant-specific responses, determine responses indicative of levels of stress, and link changes to particular biological pathways are required.

1.6.8 Choice of omics technique

Despite the advantages of integrating the results across all omic levels, the time, budget and scope of the current study only allowed for a single omics technique to be used. Proteomics and metabolomics may provide a more realistic understanding of the processes at the cellular level by virtue of occurring later in the sequence of molecular events and, therefore, are subject to less modification (Figure 1-4). Despite this, both proteomics and metabolomics are more diverse and difficult to analyse than transcriptomics (Brinke and Buchinger 2017). It has also been suggested that in order to interpret the functional elements of the genome, it is essential to understand the transcriptome (Wang *et al.* 2009). Based on the applicability to the study and the availability of appropriate techniques that allowed work to be completed within the duration of the project, transcriptomic methods were chosen and as such their use in ecotoxicology will be reviewed in more detail.

1.7 Transcriptomics

The transcriptome consists of all of the RNA molecules present in an organism at a particular point in time. mRNA forms a major part of the total RNA and plays an important role in an organism's response to changes in its environment. mRNA is the link between the genes which reside in the DNA and the proteins which determine biological function in organisms (Figure 1.4; Schirmer *et al.* 2010). The mRNA within a biological sample is, therefore, indicative of the set of genes that are being actively expressed at a given time (Schirmer *et al.* 2010). In organisms experiencing stress, adjustments to cellular processes through transcriptional control allow the organism to cope under the prevailing conditions responsible for the stress and avoid cellular damage that could lead to cell death and ultimately organism mortality. The underlying molecular mechanisms and phenotypic plasticity of an organism responding to contaminant-induced stress can be interpreted from the dynamics of its transcriptome (Leung *et al.* 2014).

Transcriptomics is extremely useful in investigating the responses of an organism to contamination, however, other mechanisms also participate in these responses and not all differences in transcriptomic responses are indicative of stress. Other mechanisms, including the regulation of translation, post-translation modification of proteins, protein degradation and allosteric regulation of proteins, that cannot be investigated using transcriptomics also affect responses (Brinke and Buchinger 2017). In addition, some changes in transcription may not be indicative of stress, but rather indicate that a change to normal biological function within the physiological tolerance levels of the organism have been invoked (Piña & Barata, 2011). For these reasons, the integration of biomarkers or other omic markers in transcriptomic studies is important for appropriate interpretation of the transcriptomic response.

1.7.1 The use of transcriptomics in ecotoxicology

In the area of ecotoxicology the use of transcriptomics can be broadly grouped into the area of transcriptomic biomarkers and pathway analysis for mechanistic understanding of responses.

1.7.1.1 Transcriptomic biomarkers

Genes that are differentially expressed in response to contaminants or are correlated with an adverse outcome may serve as molecular biomarkers (Paules 2003). As well as individual genes, groups or classes of genes which show a reliable pattern of up or down regulation in response to a specific chemical or chemical group are sometimes referred to as transcriptomic fingerprints and can be used as biomarkers (Brinke and Buchinger 2017). If the transcriptomic response can be detected earlier than other effects are evident in the organism, but are clearly

linked to those effects, then they may be used as early warning signals of higher order effects for environmental risk assessment (Brinke and Buchinger 2017). As bivalves are often used for biomonitoring, Miao *et al.* (2015) surveyed 49 comparative transcriptomic studies in bivalves that listed the most commonly differentially expressed genes and categorised the responses into toxicity studies, pathogen related studies, and other studies. This provides a useful background for the selection of potential biomarker genes in targeted studies using bivalves, and as many stress responses are highly conserved, consideration could be given to their use in other taxonomic groups.

A major difficulty in using changes in transcriptomic regulation as biomarkers is the confounding effects of various environmental variables on the reliability of the results. Different responses for light and dark conditions as well as different salinity levels, for example, have been found to alter transcriptomic responses in *Elodea nutalli* exposed to mercury and cadmium that initially showed potential as biomarkers (Regier *et al.* 2013). Like any biomarker, transcriptomic responses need to meet the criteria established for biomarker validation discussed earlier (section 1.4.3.1; van der Oost *et al.* 2005).

1.7.1.2 Pathway analysis

Pathway analysis groups genes that are associated with a specific biological pathway or function. Pathway analysis relies on prior knowledge of the association genes have with particular functions. Two regularly used databases that hold information on the function of genes are Gene Ontology (GO) (Ashburner *et al.* 2000) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa *et al.* 2016). GO terms classify genes at three levels; biological processes, cellular components and molecular functions (Ashburner *et al.* 2000). KEGG pathway maps outline the known association of genes for a particular biological pathway in the areas of metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems, human diseases and drug development (Kanehisa *et al.* 2016). These are powerful resources for determining the biological function associated with changes in transcription in organisms exposed to stress. They are invaluable in determining the mode of action of chemical stressors and also in assisting in the identification of key events in adverse outcome pathways. A key to gaining a mechanistic understanding based on transcriptomic data is being able to associate particular changes in transcriptomic regulation to biological function, and pathway analysis allows this to occur.

Although pathway analysis is useful in assessing mechanistic effects, there can still be difficulty in detecting consistently differentially regulated pathways as a reliable response to a contaminant. Morgan *et al.* (2007) point out that a particular protective pathway can sometimes cooperate with or serve as an alternative to another pathway which can confound the interpretation of dose-response relationships.

1.7.2 Transcriptomic methods

Transcriptomic analyses using quantitative real time polymerase chain reaction and microarray methods have been available for some time. It is only in the last decade since sequencing of the transcriptome (RNA-seq) has become feasible that the much broader application of transcriptomic methods in ecotoxicology has occurred. The increase in the use of transcriptomics in ecotoxicology has coincided with the increased availability of sequencing of the transcriptome (RNA-seq) and is predominantly associated with the ability of this technique to investigate non-model species. An overview of transcriptomic methods is presented below.

1.7.2.1 qPCR and microarrays

Where target sequences are known, transcriptomic analysis can be conducted using quantitative real time polymerase chain reaction (qPCR) and microarray technology. qPCR analysis requires the use of gene-specific primers. A separate reaction is required to quantify each gene, so qPCR studies usually focus on a few target genes (Brinke and Buchinger 2017). Microarray analysis use gene-specific probes attached to a solid surface, that complementary DNA (cDNA) in a sample can attach to and provides a quantitative analysis of the expression of the gene. cDNA is DNA that has been synthesised from a strand of RNA. Microarray technology can quantify the amount of mRNA transcribed for large numbers of genes under exposure to different contaminants and/or stressors (Marioni *et al.* 2008; Piña and Barata 2011). Both of these methods require prior knowledge of the target gene sequences being tested (Ekblom and Galindo 2011). A major limitation for the use of these techniques in ecotoxicology is that often non-model species are used which have no gene sequence information available (Schirmer *et al.* 2010). In more recent times the advent of RNA-seq along with increases in sequencing efficiency more broadly have greatly increased the quantity of sequence information available for many ecotoxicology-relevant species. In this way, while RNA-seq has replaced methods such as qPCR and microarray in some areas, it has also facilitated the use of these methods in gene-targeted studies through the provision of sequence information.

1.7.2.2 Sequencing of the transcriptome (RNA-seq)

Unlike qPCR and microarrays, RNA-seq sequences the entire transcriptome rather than specifically targeted genes. RNA-seq provides sequences for the entire set of transcripts present in a biological sample and, as such, does not require prior knowledge of the sequence. For ecotoxicology purposes, where non-model organisms are frequently used this provides a significant advantage (Ekblom and Galindo 2011). The global nature of RNA-seq also enables the assembly of the whole transcriptome (Schirmer *et al.* 2010). The breadth and number of transcripts provides insight into the regulation of biochemical processes and pathways that more targeted approaches may not provide (da Fonseca *et al.* 2016). This can be useful in situations where there is uncertainty of response, such as when describing the genes involved in physiological adaptation of populations to different environments (da Fonseca *et al.* 2016). RNA-seq also has advantages in that it is robust, highly sensitive and quantitative over a broad range of expression levels (Brinke and Buchinger 2017; Cloonan and Grimmond 2008; Li *et al.* 2013). Despite having many positive attributes, there are shortcomings to using RNA-seq, especially in non-model species. In non-model species where de novo assembly is required there may be inconsistencies in the functional annotation of genes due to evolutionary distance between species involved (Hornett and Wheat 2012). In addition to the uncertainty of annotation, im RNA-seq studies using de novo assembly there is often a large number of contigs sequenced that are not annotated because of the lack of an annotated gene in the database that has sufficient similarity to the sequenced contig (Hornett and Wheat 2012). Another associated issue with RNA-seq is the cost and often associated trade-off between depth of sequencing coverage and replication. It has been shown that while the number of reads sequenced is important, above a threshold increasing the number of reads will provide a diminishing return, but increased replication increases the power to detect differentially expressed genes regardless of sequencing depth (Liu *et al.* 2014). The sequencing depth required will depend on the objectives of the study and the species used, but careful consideration of experimental design and depth of sequencing is required especially as cost is often a limiting factor in the design of RNA-seq studies. RNA-seq has been used in various mollusc and other freshwater species to investigate the effects of contaminants (Table 1-4).

Table 1-4. Selected studies using sequencing of the transcriptome or microarray in freshwater and marine species in ecotoxicology (stusies were selected based on the relevance to gastropods and Cu exposure).

Species	Stressor	Taxonomic group	Method	References
Radix balthica (embryonic stages)	oxazepam	Gastropod pulmonata	Illumina HiSeq 2000	Mazzitelli *et al.* 2017
Nerita melanotragus	NA	Gastropod Nerite	Ion Torrent	Amin *et al.* 2014
Synechogobius hasts	Cu	Fish Goby	Illumina Hiseq 2000	Chen *et al.* 2016
Saccostrea glomerata	Pyrene and fluoranthene	Bivalve Oyster	Illumina Hiseq 2000	Ertl *et al.* 2016
Radix balthica	NA	Gastropod Pulmonata	Illumina/Solexa	Feldmeyer *et al.* 2011
Reishia clavigera	NA	Gastropod Muricidae	Illumina Hiseq 2000	Ip *et al.* 2016
Lymnaea stagnalis	diquat	Gastropod Pulmonata	Roche 454	Bouétard *et al.* 2012
Mytilus gallprovincialis	Heat stress and copper	Bivalve mussel	Microarray	Negri *et al.* 2013

NA = Not applicable as sequence information was reported rather than specific stressor response

1.8 Multiple generation responses

The use of long-term exposures to a toxicant over multiple generations provides the opportunity to investigate changes in evolution and population dynamics (Bal *et al.* 2017). Studies have investigated the multi-generational effects of many stressors including temperature, copper, cadmium, zinc and a synthetic glucocorticoid (Table 1-5). As well as the response to individual contaminants, multi-generational studies have also been used to test the effect of resistance to one stressor on the response to another stressor (Bae *et al.* 2016; Salice *et al.* 2010). There is a large variation in the objectives of these multigenerational studies and therefore experimental designs vary substantially. Two things of note are the timeframes ranging from two to five generations, as well as the choice to start with a common population and expose it to different concentrations over multiple generations or to use organisms sampled from populations from sites that have differing exposure histories. While mortality and responses associated with life

history traits are often investigated, some studies have looked at sub-lethal effects such as oxidative stress, oxidative damage, DNA methylation, shell formation and adaptive potential (Bae *et al.* 2016; Bal *et al.* 2017; Salice *et al.* 2010). The inclusion of biomarker responses with life history traits in multi-generational studies allows links to be made between these responses. These can be valuable, as life history traits can be difficult to measure in the field, but if linked to other easier to measure markers, inferences can be made based on the relationship between the responses.

1.8.1 The development of resistance

Rapid evolutionary changes through the process of directional selection can occur when a population is exposed to a contaminant that exerts strong selection pressure over multiple generations (Hoffmann and Hercus 2000). Directional selection results from the systematic variation in fitness among individuals in a population, associated with variation in a trait or traits, when under pressure from contaminant-induced stress (Hoffmann and Daborn 2007). When exposed to contaminant-induced stress, organisms with the genotype associated with increased fitness traits will provide a greater genetic contribution to the following generations and provide the basis for the genetic changes that result in a resistant population. On this basis, a population's phenotypic level of resistance to a contaminant will be determined by the genetic background and the multi-generational exposure history of the population (Morgan et al., 2007). When microevolution occurs as a result of contaminant exposure, populations can increase their level of tolerance, potentially allowing them to persist as a consequence of the genetic adaptation to the toxic substance (Medina *et al.* 2007). Morgan *et al.* (2007) suggested that increased resistance to a contaminant in populations with multi-generational metal exposure histories results from either the reduction of net accumulation of the metal or increasing the proportion of the accumulated metal that is detoxified. Levinton *et al.* (2003) found that polychaete worms from cadmium-resistant populations produced ten times more metallothioneins than those from non-resistant populations. Copper-tolerant populations of the freshwater gastropod *Lymnaea stagnalis* showed genetic divergence in the expression of genes associated with the Gene Ontology terms "*copper ion binding*" and "*metal ion binding"*(Côte et al. 2015). It was suggested that tolerance may in part due to copper-specific homeostasis mechanisms (Côte *et al.* 2015). These studies demonstrate the importance of understanding the molecular level changes that support the development of resistance for understanding the biological mechanisms involved (Ekblom and Galindo 2011). They also provide further

evidence of the importance of the mechanisms associated with uptake, movement, detoxification and storage of metals in the intraspecific differences in tolerance to metals.

Table 1-5. Studies on marine and freshwater species investigating the intergenerational effects of contamination exposure.

Species	Stressor	Details	result	Reference
Daphnia magna	Cu and temperature	Effect of temp on reproduction, oxidative stress and copper toxicity	↓ reproduction ↑ ROS ↑ LP ↑ copper toxicity	Bae *et al.* 2016
Daphnia magna	Cu	Mobility, reproduction, energy reserves	↑ tolerance	Bossuyt and Janssen 2004
Gammarus pulex	Zn	Mortality, feeding	↑ tolerance to acute exposures	Crane 1995
Tigriopus japonicus	Cu	Reproduction, development, survival	↓ reproduction ↓ development ↑ tolerance	Kwok *et al.* 2009
Physa acuta	prednisolone	Effect on DNA methylation, shell structure, survival	Δ DNA methylation ↓ shell formation, ↓ survival	Bal *et al.* 2017
Pomacea paludosa	Cu	Reproduction, survival	↓ survival	Rogevich *et al.* 2008
Biomphalaria glabrata	Cd – snails from different populations	Population growth rates	Δ population growth rates	Salice and Miller 2003
Biomphalaria glabrata	Cd, temp – snails with different parasite resistance	Tolerance, adaptive potential, population growth rates	Δ Cd tolerance Δ adaptive potential	Salice *et al.* 2010
Nereis diversicolor	Zn and Cu	Fitness cost	↓ scope for growth, ↓ lipids and carbohydrates, ↓ fecundity	Pook *et al.* 2009

ROS = recative oxygen species, LP = lipid peroxidation, Δ = different between treatments, ↓ = decreased, ↑ = increased

1.8.2 Fitness cost of tolerance

The mechanisms that allow organisms to survive and reproduce in the presence of a contaminant may be accompanied by a reduction in fitness in those organisms in uncontaminated environments when compared to populations from the uncontaminated environment. For this reason, in studies investigating the development of resistance, it is important to consider the fitness consequences (Klerks *et al.* 2011). It has been reported that tolerance to oxidative stress leads to trade-offs associated with reduced reproduction, immunity and lifespan (Monaghan *et al.* 2009). Genetic trade-offs between cadmium resistance and growth and reproductive output have been reported for polychaete worms (Levinton *et al.* 2003). Other studies using insects have found a reduction in fitness in metal-tolerant individuals in uncontaminated conditions (Mireji *et al.* 2010; Shirley and Sibly 1999). A review by Medina *et al.* (2007) reported that while biological trade-offs are broadly discussed in the literature they have not been seen in all circumstances and where they have been seen there is often uncertainty in regard to the mechanisms that lead to these biological trade-offs. Three potential mechanisms for the trade-offs associated with the development of resistance are, changes in the allocation of energy and resources, changes in uptake of the contaminant and pleiotropy.

1.8.2.1 Reallocation of energy and resources

A fitness cost can be seen when organisms allocate more energy and resources to mechanisms that are associated with resistance, at the expense of life history traits. Detoxification mechanisms might, for example, use energy and resources that would be otherwise allocated to other fitness traits such as growth and reproduction (Sibly and Calow 1989). The continued allocation of resources to these detoxification mechanisms, when organisms are in an uncontaminated environment, puts these organisms at a comparative disadvantage compared to organisms who are allocating those resources to regular biological functions such as growth and reproduction.

1.8.2.2 Reduction in uptake of the contaminant

Resistance can be associated with reduced uptake of the contaminant. Where the contaminant is an essential element, present at elevated concentrations, the reduced uptake would be a disadvantage when the element is scarce. In populations shown to be resistant to Cu, for example, Agra *et al.* (2011) suggested that reductions in growth and reproduction in uncontaminated environments may be associated with copper deficiencies resulting from the inefficient uptake of Cu.

1.8.2.3 Pleiotropy

As a result of pleiotropy, genetic changes associated with resistance can cause detrimental effects in other traits. Paaby and Rockman (2013) defined pleiotropy as the mapping from one characteristic at a genetic level to multiple characteristics at the phenotypic level. Klerks *et al.* (2011) suggested that when a pleiotropic gene associated with resistance is altered, the development of resistance will be accompanied by a decrease in the fitness related trait. It should be noted that pleiotropic changes may not always be negative trade-offs. It has been pointed out that pleiotropic effects can occur when contaminants such as Cu and Cd share common detoxification pathways and the genetic changes associated with resistance to one will also result in resistance to the other (Shirley and Sibly 1999). Arnaud *et al.* (2005), reported that in flour beetles resistant to malathion, pleiotropic effects conferred increased reproductive capacity through increased sperm numbers and length in the resistant males.

1.9 Life history and behavioural responses

Exposure of a population to contaminats can lead to various changes in life history traits such as mortality, fecundity and offspring viability. These characteristics are of high importance as they directly influence a population's ability to maintain itself under the prevailing environmental conditions. In molluscan species, characteristics such as number of clutches laid, number of eggs per clutch, time to hatching, ratio of hatchlings to eggs and time to reproductive age can provide a measure of a population's reproductive capacity (Coutellec *et al.* 2008; Duft *et al.* 2003; Gust *et al.* 2011). These endpoints, in conjunction with a measure of the mortality in a population, can be incorporated into a population model and provide a measure of the ability of a population to survive in a given set of environmental conditions (Das and Khangarot 2011; Mažuran *et al.* 1999). In addition to life history traits, behavioural changes in organisms, especially in relation to feeding, are valuable in understanding the response to contaminants (Maltby *et al.* 2002).

1.9.1 Mortality

Mortality has been widely used as a response in ecological studies, however, the focus is often on short term studies to determine lethal concentration values to acute exposure (Table 1-6). In some more recent studies on freshwater gastropods, mortality has been used *in situ* to assess a contamination gradient (Gust *et al.* 2011), and over longer chronic type exposures of up to 49 days (Das and Khangarot 2011). These studies have shown that while lethal concentration (LC) values may be relatively high over an acute exposure, longer exposure periods result in a

reduction in LC values that are often more reflective of the organisms survival in the environment.

Table 1-6. Examples of studies investigating the mortality of marine and freshwater gastropods as a response to copper exposure.

Species	Toxicant/s	Result LC_{50}	Authors
Isidorella newcombi (juv)	Cu	260-780 µg L^{-1} Cu (48h)	Stevens *et al.* 2014
Lymnaea stagnalis, Physia gyrina, Pyrgulopsis robusta, Taylorconcha serpenticola, Fluminicola sp., and *Fontigens aldrichi*	Copper, ammonia and pentachlorophenol	Various 13-42 µg L^{-1} Cu (28d)	Besser *et al.* 2016
L. stagnalis (juv)	Cu	30.7 µg L^{-1} Cu (96 hr)	Brix et al., 2011
L. luteola	Cu	63.62 µg L^{-1} Cu (7 d) 9.47 µg L^{-1} Cu (7 d)	Das and Khangarot 2011
Pomacea paludosa	Cu	Increased mortality with sediment and water Co concentration	Hoang and Rand 2009
P. paludosa	Cu	Mortality increased with water Cu concentration	Rogevich *et al.* 2008
L. stagnalis	Cu	24.9 µg L^{-1} Cu (96 hr)	Ng *et al.* 2011
Potamopygrus jenkinsi (juv)	Cu	54 -79 µg L^{-1} Cu (96 hr)	Watton and Hawkes 1984
Bellamya aeruginosa	Cu	480 µg g^{-1} Cu (10 day sediment concentration)	Ma *et al.* 2010

Juv = juveniles used in study

1.9.2 Reproduction

The assessment of reproductive capacity in organisms exposed to contaminant-induced stress is often investigated in ecotoxicology, with several studies describing effects in gastropods (Table 1-7). While some studies separate reproductive output (the number of clutches/eggs

laid) from embryonic development, others combine these, considering only the number of hatched juveniles per adult. Egg production and embryonic development to the point of hatching are, therefore, combined in this review of reproduction. Many changes to reproductive output have been reported for freshwater snails exposed to chemicals. These include changes in the number of embryos, time to hatching, hatching rate, number of clutches, number of eggs, embryonic survival and changes in the time taken to reach reproductive maturity. Exposure of freshwater gastropods to Cu has generally resulted in a decrease in the number of clutches and eggs being produced, as well as delays in hatching and impaired embryonic development (e.g., Das and Khangarot 2011; Khangarot and Das 2010; Rogevich *et al.* 2008). High Cu concentrations can lead to a cessation of embryo development, such as in the freshwater gastropod *Biomphalaria glabrata* where embryos in egg masses exposed to concentrations of 100 µg L^{-1} Cu were unable to complete development (Ravera, 1977). Often the responses occur in a concentration-dependent manner such as in the freshwater snail *Lymnaea luteola* where embryo mortality, malformation and development time all increased with exposure concentration (Das and Khangarot 2011). While exposure to increased Cu concentrations has generally been associated with reduced reproduction, this is not always the case. Peña and Pocsidio (2007) reported no effect on reproduction in the freshwater golden apple snail exposed to concentrations of up to 67.5 µg L^{-1} Cu even though other endpoints such as feeding rate and growth were affected. This suggests that while exposure to Cu can affect the reproduction of gastropods there are interspecific differences and in some species reproduction is not one of the most sensitive endpoints.

Table 1-7. Details and results of studies that investigate the effect of chemical stressors on reproduction in freshwater snails.

Species	Stressor	Response	Author
Potamopyrgus antipodarum	Bisphenol A, 4-*tert*-octylphenol and 4-*n*-nonylphenol	↑ embryos in the brood pouch	Duft et al. 2003
Lymnaea stagnalis	Diquat and nonylphenol polyethyloxylate	↑ time to hatching, ↓hatching rate	Coutellec et al. 2008
Potamopyrgus antipodarum	Metal contamination gradient	↓ embryos in the brood pouch	Gust et al. 2011
Lymnaea palustris	Hexachlorobenzene	↑ clutches, ↑ eggs	Baturo *et al.* 1995

Table 1.7 continued

Species	Stressor	Response	Author
Lymnaea luteola	Cu	↓clutches, ↓ eggs	Das and Khangarot, 2011
Planoribus corneus	CaBr, CaCl and mixture of CaBr and CaCl	↓ clutches	Mažuran *et al.* 1999
Biomphalaria glabrata	Cr,	↓ clutches, ↓ eggs	Ravera 1977
Biomphalaria glabrata	Cd, Pb and As (96 hr)	Δ eggs, Δ hatching time, Δ embryonic survival	Ansaldo *et al.* 2009
Lymnaea stagnalis	Cd (49 d)	↓ clutches, ↑ embryogenic mortality, delayed hatching	Gomot 1998
Biomphalaria glabrata	Cd (56 d)	↓ survival, ↑ time to reproduction, ↓ reproductive output	Jensen *et al.* 2001
Lymnaea luteola	Cu	Impaired development, delayed hatching, ↓ survival	Khangarot and Das 2010
Pomacea canaliculata	Cu	No change in reproduction	Peña and Pocsidio 2007
Pomacea paludosa	Cu (2 generations)	↓ clutch production, ↓ hatch rate	Rogevich *et al.* 2008
Biomphalaria glabrata	Cd (1 generation)	↓ hatch rate, ↓ fecundity, ↑ time to maturity	Salice and Miller 2003

Key: ↑ = increase, ↓ = decrease, Δ = change

1.9.3 Juvenile survival

The juvenile life stages of gastropods are considered the most sensitive to many stressors. In the freshwater snails *Potamopygrus jenkinsi* exposed to Cu and ammonia, juvenile snails were found to be less tolerant than adults (Watton and Hawkes 1984). In the freshwater snail *Biomphalaria glabrata* exposed to Cd, differences in juvenile mortality were greater than the

differences seen in adult mortality (Salice and Miller 2003). It has been suggested the effects on juvenile freshwater gastropods exposed to Cu may be as a result of interference with calcium uptake as gastropods have high calcium requirements for shell formation at early stages of development (Grosell and Brix 2009).

1.9.4 Feeding rates

Changes in feeding rates have been recognised as a general biomarker that reflects behavioural changes induced by a broad range of contaminants (Maltby *et al.* 2002). Studies investigating the effect of Cu exposure on feeding rate have found that exposure to high concentrations of Cu is associated with a reduction in feeding rates. In the freshwater gastropod *Pomacea canaliculata* exposed to Cu, feeding increased at slightly elevated Cu concentrations, however, feeding rates decreased at exposure concentrations of 45 µg L^{-1} Cu and above (Peña & Pocsidio, 2007). The feeding rate of another freshwater snail, *Potamopygrus antipodarum* declined in a concentration-dependent manner in response to both aqueous Cu and Cu nano-particles (Pang *et al.* 2012). During longer term exposures, the effect on feeding rate can be dependent on exposure duration. Das and Khangarot (2011) found that *Lymnaea luteola* exposed to concentrations of 56 µg L^{-1} Cu continued to eat initially but ceased feeding after 2 weeks of exposure.

1.10 Integration of biomarkers

This review includes molecular level responses associated with changes to the transcriptome, through to cellular level responses associated with responses to, and damage caused by, oxidative stress, a general stress biomarker associated with lysosomal function and life history traits that underlie population level changes. These markers provide an indication of changes that occur at the molecular, cellular, individual and population level of biological organisation. It has been noted that in environmental risk assessment, changes at the population and ecosystem levels of biological organisation are the ultimate concern, however, they are too complex and far removed from the causative events to be used as tools for the early detection and prediction of the consequences of environmental stress (Moore *et al.* 2004). As such, the initial responses of organisms to contaminants that occur at lower levels of biological organisation are required as early warning signals for changes at higher levels of biological organisation (Figure 1-7).

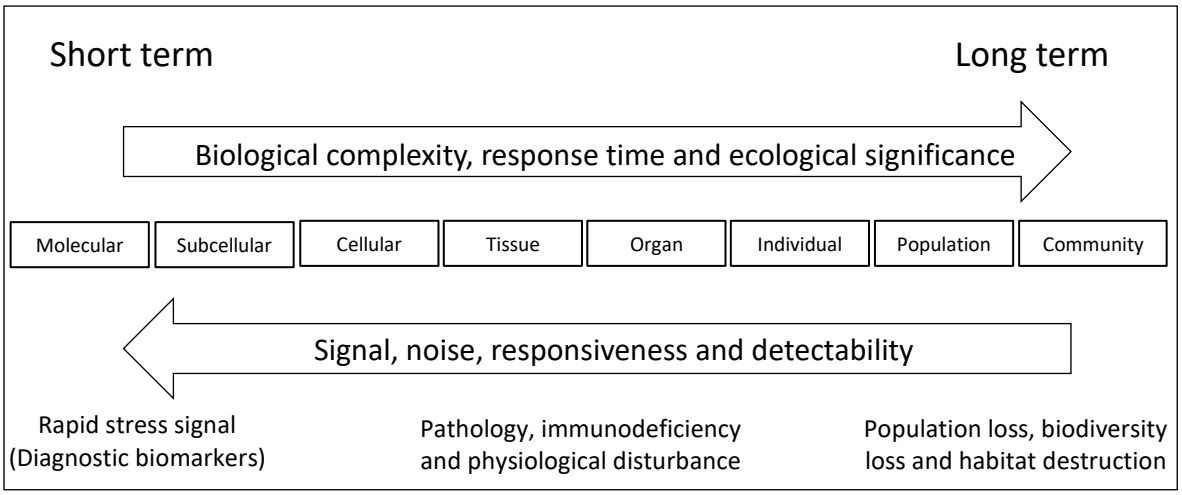


Figure 1-7. Diagrammatic representation of the order of environmental responses and the level of detectability of those responses (modified from Moore *et al.* 2004).

In order to understand the response of an organism to contamination, and establish links between responses at different levels of biological organisation, the integration of biomarkers at different levels of biological organisation is required (Broeg *et al.* 2005). To facilitate this understanding, studies need to collect data on a range of responses at the molecular, subcellular, cellular, individual and community levels (Broeg *et al.* 2005; Viarengo *et al.* 2007). The major links between the exposure to Cu, bioaccumulation of Cu and the resultant effects that have been discussed in this review are presented in Figure 1-8.

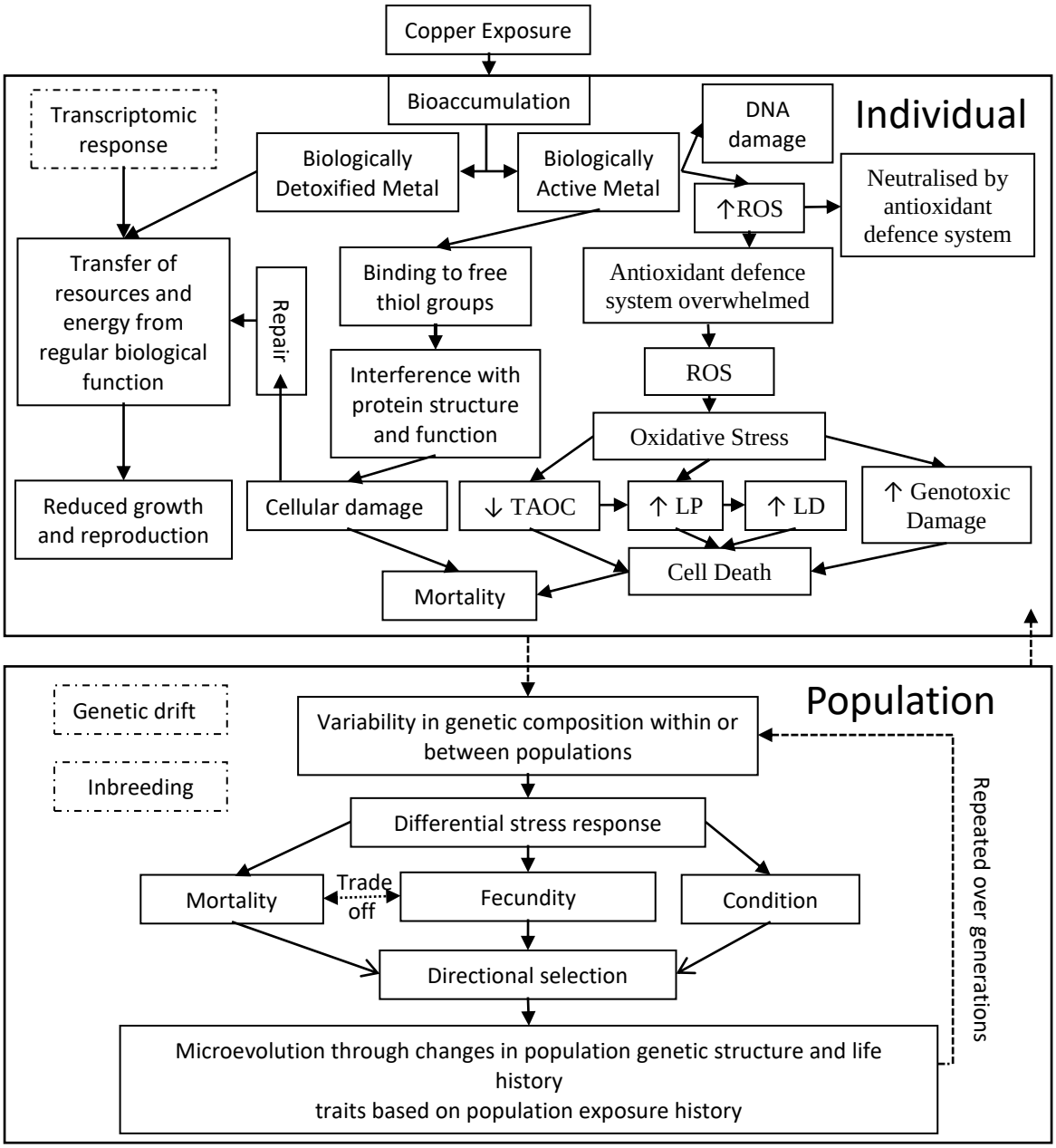


Figure 1-8. Processes linking Cu exposure, bioaccumulation and responses at the individual and population level. Individual responses focus on those associated with oxidative stress, interference with proteins and changes in energy allocation. Population level responses focus on contaminant-induced stress that will lead to directional selection. Large dotted arrows indicate inherent links between individual and population level effects. The small double ended arrow is indicative of biological trade-offs between tolerance and reproductive output. Box with dashed outline indicates general or non-specific effect (an individual transcriptomic level effect that is seen as general, as it will influence and be influenced by all processes listed. At the population level genetic drift and inbreeding are seen as non-specific effects as they will be randomly associated with general population fluctuations resulting from contaminant-induced and other environmental factors). ROS = reactive oxygen species, TAOC = total antioxidant capacity, LP = lipid peroxidation and LD = lysosomal membrane destabilisation.

1.11 Aims, objectives and structure of the thesis

The aim of this thesis was to quantify the response of *Isidorella newcombi* to Cu exposure at lethal and sub-lethal concentrations, using acute, chronic and multi-generational exposures. In describing the responses, a major focus was to use a variety of methods, including transcriptomics, enzymatic responses, cellular level responses, behavioural responses and life history traits. The use of a range of exposures and methods for investigating responses enabled the integration of those responses across different levels of biological organisation. The integration of responses over multiple levels allows responses at lower levels of organisation to be linked to population-level effects. The use of transcriptomics also provided an opportunity to gain a mechanistic understanding of the responses at the molecular level. A schematic diagram of the structure of the thesis is shown in Figure 1-9.

The research focussed on the following objectives:

1. Investigate the bioaccumulation of Cu in *I. newcombi* exposed to a range of Cu concentrations using acute and chronic exposures.
2. Investigate mortality rates of *I. newcombi* exposed to copper at lower concentrations than have been previously investigated.
3. Investigate the effect of copper exposure on *I. newcombi* life history traits including; fecundity, egg viability and juvenile survival.
4. Investigate oxidative and general stress responses of *I. newcombi* to sub-lethal concentrations of Cu with the following biomarkers.
    a) Antioxidant capacity – total antioxidant capacity;
    b) Oxidative damage – lipid peroxidation; and,
    c) General stress - lysosomal membrane destabilisation.
5. Establish Cu exposure-bioaccumulation-response relationships for the sublethal responses outlined in objectives 3 and 4 above.
6. Develop a model of the mechanistic response of *I. newcombi* to Cu based on changes in transcriptomic regulation.
7. Investigate adaptation in *I. newcombi* over multiple generations of Cu exposure.
8. Investigate transcriptomic changes associated with the development of Cu resistance in *I. newcombi*.

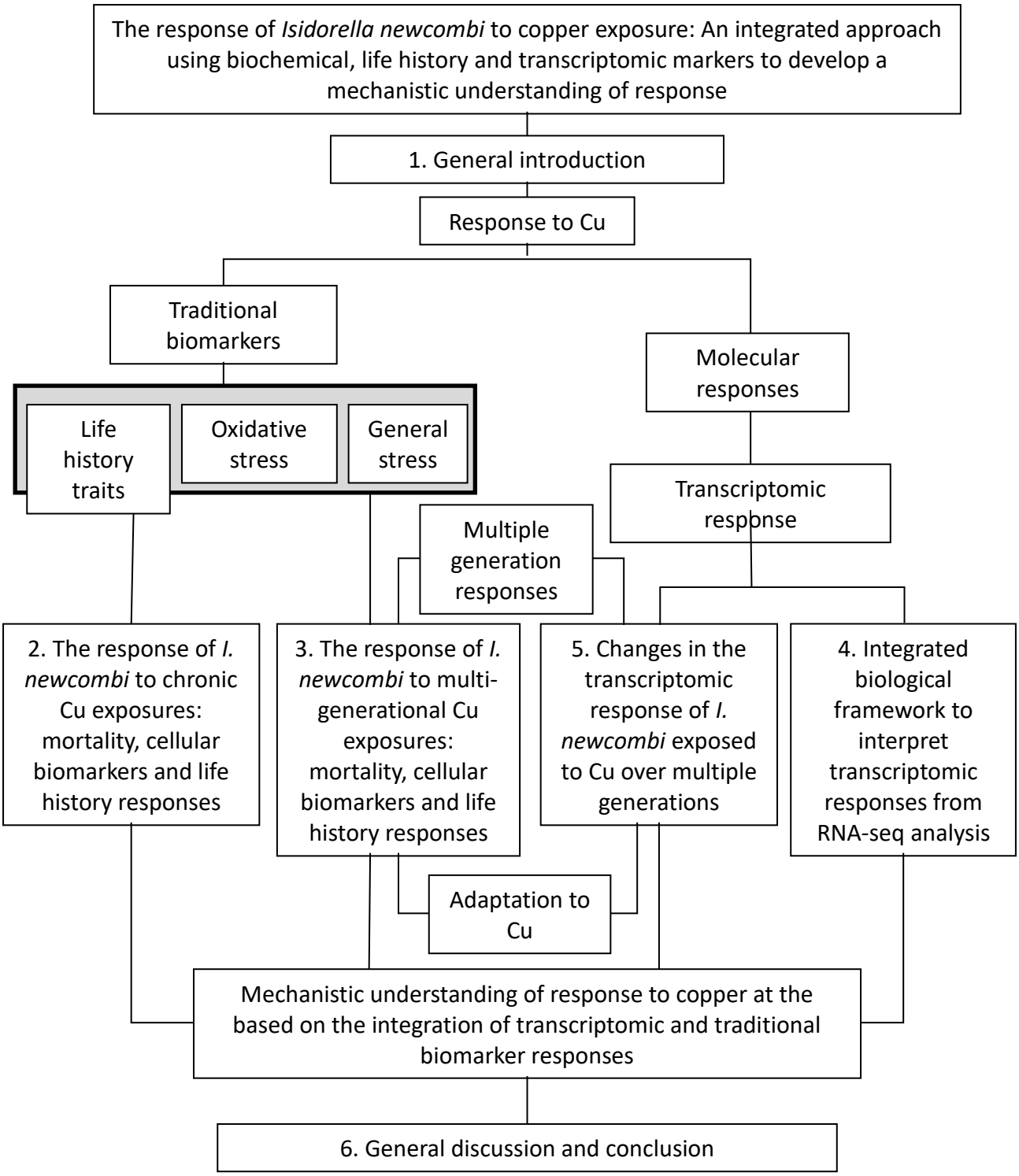


Figure 1-9. Schematic diagram of the thesis.

The chapters included in the thesis and their content are:

Chapter 1. General introduction presents a general background of the methods used in this project with some relevant examples and knowledge gaps identified.

Chapter 2 focusses on biochemical, life history and behavioural responses of *I. newcombi* over a 28 day exposure to Cu.

Chapter 3, the biochemical, life history and behavioural responses of *I. newcombi* to Cu exposure over multiple generations are discussed, with a focus on the adaptations present in the F_3 generations.

Chapter 4 presents an integrated biological response model of *I. newcombi* exposed to Cu developed from changes in gene regulation at the transcriptomic level (published paper (Ubrihien *et al.* 2017a)).

Chapter 5 describes the changes in transcriptional regulation in *I. newcombi* that have developed resistance to Cu.

In Chapter 6 a general discussion of the content of the thesis is presented.

Chapter 2. The response of *Isidorella newcombi* to chronic copper exposures: mortality, cellular biomarkers and life history responses.

2.1 Introduction

The concentration of Cu in the biosphere is elevated through a range of human activities including mining, smelting, refining, manufacturing and waste disposal (Wright and Welbourn 2002). In Australia, Cu contamination has been associated with mining activities (Eriksen *et al.* 2001; Klessa *et al.* 1997). In particular areas, Cu has also been used as an agricultural pesticide in rice crops and orchards (Merry *et al.* 1986; Stevens 2002). The environmental persistence of Cu can cause increased environmental Cu concentrations in areas where it is extracted, processed or used (Kakkar and Jaffery 2005). Copper is an essential element, playing an important role in a wide range of biological functions (Kim *et al.* 2008). Despite being an essential element, exposure to elevated concentrations of Cu is toxic to biota (Gaetke and Chow 2003). Copper toxicity often occurs through non-specific binding to proteins and oxidative stress (Letelier *et al.* 2005; Livingstone 2001).

The management of Cu in the environment requires an understanding of the effects of Cu on biota and the development of appropriate tools for use in monitoring and assessment programs. A multiple lines of evidence approach that incorporates an understanding of the links between exposure, contaminant uptake and chronic and acute effects in biota is recommended (Simpson *et al.* 2013). This type of approach requires the development of appropriate assessment tools and biomonitor species in order to effectively assess the effect of contaminants on the environment. There is currently a shortage of local freshwater sentinel species for the assessment of environmental contamination (Taylor *et al.* 2016). The development of some Australian freshwater bivalve species as biomonitors has been reported (Marasinghe Wadige *et al.* 2017; Taylor *et al.* 2016). While some bivalves have been developed as biomonitors, there has been less focus on gastropods. Gastropods are known to play an important role in freshwater ecosystems by transferring energy and materials through freshwater food webs (Habdija *et al.* 1995; Lagadic *et al.* 2007). Gastropods also have many desirable attributes of biomonitors including being sedentary, tolerant of high contaminant concentrations, widely distributed, abundant, easy to identify and capable of accumulating higher concentrations in contaminated sites than uncontaminated sites (Phillips and Rainbow 1994). Further, gastropods have also have useful characteristics for conducting toxicity tests in that they are easy to culture, reproduce readily in captivity, have a relatively short embryonic development and embryonic

stages easy to identify (Ravera 1977). Despite having desirable attributes, gastropod species have not been developed as biomonitors for the assessment of the effects of contaminants in Australian freshwater systems.

For a species to be useful as a biomonitor, it is necessary to establish relationships between the contaminant in the environment, bioaccumulation of the contaminant and the resultant response in the organism. This requires that appropriate biomarkers be validated to detect contaminant induced responses in the organism. Biomarkers measure responses at the biochemical, cellular, physiological or behavioural level and provide evidence of exposure or effect (Broeg *et al.* 2005). Biomarkers aim to detect contaminant induced changes early and at a low level of biological organisation so they can be predictive of changes at higher levels of biological organisation (Izagirre *et al.* 2009). By providing an early indication of contaminant induced changes occurring in ecological systems, biomarkers can act as early warning systems allowing remediation strategies to be put in place prior to significant damage occurring at higher levels of biological organisation (Bolognesi & Fenech, 2012).

The aim of the current study was to assess relationships between subcellular biomarkers and life history traits in *Isidorella newcombi* exposed to a Cu concentrations ranging from 5 to 120 µg L^{-1}. Life history traits such as fecundity, mortality and offspring viability can provide an indication of population level effects but are difficult to measure in situ. Biomarker measurements such as total antioxidant capacity, lipid peroxidation and lysosomal membrane destabilisation are easier to measure in organisms collected from field populations, but are not directly related to population level effects. Measuring cellular and enzymatic biomarkers as well as life history traits in *I. newcombi* exposed to a broad range of copper concentrations, allowed the relationships between biomarkers and life history traits to be established.

2.2 Materials and methods

2.2.1 *I. newcombi* source and culture

I. newcombi were sourced from an in-house culture maintained at a water temperature of 22 ± 1°C, with a 12/12 day-night light cycle. The original *I. newcombi* were sourced from uncontaminated wild populations at Yanco and were supplied by New South Wales Department of Primary Industries (Yanco Agricultural Institute). The snails were maintained in uncontaminated river water sourced from the Cotter River at Vanities Crossing (pH 6.7,

conductivity 0.044 ms cm^{-2}, turbidity 1.4 NTU, hardness 6.6 mg L^{-1}, salinity 0.02 ppt and total organic carbon 1.46 mg L^{-1}). The snails were fed lettuce leaves washed in the same river water.

2.2.2 Exposure conditions

Two hundred and forty *Isidorella newcombi* (length 7-12 mm across longest shell length) were randomly allocated to 20 treatment replicates of 12 individuals. A control group was maintained in natural river water sourced from the Cotter River at Vanities Crossing that had a background Cu concentration of 4 ± 1 µg L^{-1}. Nine Cu exposure treatments were used with nominal concentrations of 5, 10, 15, 20, 30, 40, 50, 60, 80 and 120 µg L^{-1} Cu added. The control and each treatment had two replicates, with twelve snails in each replicate exposed for 28 days. The solutions for the treatments were made up using the same water as the controls but had additional Cu added from a 1 mg L^{-1} Cu stock solution prepared from $CuSO_4.5H_2O$ (Univar, D3247) in deioninsed water. Exposures were conducted in 770 mL polypropylene containers (Chanrol, O1C30) with 600 mL of solution. The solution was aerated throughout the exposure to maintain ≈ 100% oxygen saturation. Each container had 12 g of lettuce (wet weight) added at the start of the experiment. Treatments were monitored daily, with a) dead snails recorded and removed, and b) the number of egg clutches counted and marked to record the date of oviposition. Every three days snails were transferred to a new container with new solution and lettuce. In replicates where mortality occurred the volume of solution was reduced by 50 mL and lettuce allocated was reduced by 1 g per dead snail.

2.2.3 Fecundity, embryo viability and juvenile survival

At each change of solution, the number of clutches and the number of eggs in each clutch were counted. The totals were normalised to the number of snails surviving in the treatment to determine the number of clutches and eggs per snail per day. Individual clutches form a gelatinous mass, and each clutch was transferred to individual 30 mL polypropylene containers (Chanrol, 01PC1) with solution at the same Cu concentration as the treatment they were taken from. Each individual clutch was inspected daily until there was evidence that there were hatched juveniles in the container. If embryo development in a clutch was not evident for 5 days and no movement of embryos in the egg capsules was observed, they were considered dead. Once snails had hatched, a piece of lettuce sufficient to feed the snails for 1 week (approximately to 5-10 mm^2) was added to the container. One week after hatching, the containers were inspected, and the number of surviving offspring was counted. Embryo

viability and juvenile survival was calculated as the number of surviving offspring at 1 week after first hatching from the clutch divided by the total number of eggs in the clutch.

2.2.4 Estimation of feeding rate

Unconsumed lettuce was collected from the exposure vessels at each water change. The leaves were blotted dry with a paper towel prior to being weighed.

The feeding rate of *I. newcombi* for each copper concentration was calculated using the following formula adapted from Das and Khangarot (2011):

$F = C_i - C_{fnt}$

Where F = feeding rate gram/snail/day, C_i = initial leaf weight (g), C_f = final leaf weight (g), nt = sum of the total number of snails alive in the treatment at each day of the experiment. Results were expressed in grams of wet mass of lettuce consumed per snail per day.

2.2.5 Metal analysis

Snails for metal analysis were depurated for 24 hours. Organisms were dissected and soft tissues removed from the shells. Soft tissues were lyophilised using a freeze dryer (Labconco, Freezone plus 6) and digested as per the procedure outlined in Baldwin *et al.* (1994). Total dry mass was recorded for all samples. If the samples had a dry mass greater than 0.07 g, the samples were homogenized and a subsample of 0.07 g was used for the digestion. The sample was placed into a 7 mL polytetra-flouroacetate (PFA) digestion vessel with 1 mL of nitric acid (Aristar BDH, VWR, USA). For every 20 samples, 2 certified reference materials (National Institute of Science and Technology 1566b) and 2 blanks were prepared, digested and analysed with the samples. The samples were digested at 600 W for 2 minutes, 0 W for 2 minutes and 450 W for 45 minutes in a microwave oven (MDS-2000, CEM Corporation, USA). Samples were then diluted to 1% v/v concentration of acid/tissue to deionised water using an auto dilutor (Gilson GX 271, USA), for inductively coupled plasms-mass spectrometer (ICP-MS) analysis. Water used for dilution contained an ICP-MS mixed 7-element internal standard (EM Science) to monitor for variations due to instrument drift or matrix effects.

Diluted samples were analysed using a Elan DRC-e ICP-MS (Perkin-Elmer, USA) following the protocol described by Maher *et al.* (2001). Certified reference materials (National Institute Standards and Technology Oyster tissue 1566b) and blanks were analysed with samples to determine the recovery of metals. Certified value for NIST1566b is 71.6 ± 1.6 µg g^{-1} Cu dry

mass and the measured value was 68.3 ± 0.5 µg g^{-1} Cu dry mass (n=6) was and within the acceptable range. External calibration standards used for quantitation were made up from a 10 mg L^{-1} Reference Standard, ICP-MS Calibration Multi Element Standard 2 (AccuTrace, AccuStandard, USA) in 1 % (v/v) HNO_3 acid as 1, 0.1, 0.01 and 0.001 mg L^{-1} solutions. Recalibration was performed every 15 samples during analysis.

2.2.6 Measurement of Total antioxidant capacity

Tissue preparations for total antioxidant capacity (TAOC) and thiobarbituric acid reactive substances (TBARS) analysis were prepared at the end of the exposure period. Snail tissues were removed from their shells. The digestive tract of the organism was then separated from the remaining tissue. The digestive tract tissue was homogenized in 500 µL of a 5 mM potassium phosphate buffer containing 0.9% sodium chloride and 0.1 % glucose, pH 7.4 (1:5 w/v). The tissue was homogenized on ice using a motorized microcentrifuge pellet pestle, sonicated on ice for 15 seconds at 40 V and centrifuged (5804R centrifuge, Eppendorf, Austria) at 10,000 × g for 15 min at 4°C. A 50 µL aliquot of supernatant was removed for TAOC and a 250 µL aliquot of supernatant was removed for TBARS, with the pellet and remaining supernatant being reserved for protein analysis. All three samples were stored at -80°C until analysed.

The TAOC of tissue lysates was measured using a Cayman chemical assay (Sapphire Bioscience #709001). This assay is based on the ability of the antioxidants in the sample to inhibit the oxidation of 2, 2'-Azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) to $ABTS^{\cdot+}$ by metmyoglobin. The samples were thawed at room temperature and 10 µL of each pipetted into a 96 well plate with 10 µL of metmyoglobin and 150 µL of ABTS®. Reactions were initiated with 40 µL of a 441 µM solution of hydrogen peroxide. The plate was shaken for 5 min at 25°C and absorbance read at 750 nm on a Bio Rad Benchmark plus microplate spectrophotometer. The suppression of absorbance under reaction conditions in the sample is proportional to the concentration of combined antioxidants in the sample. The capacity of the antioxidant in the sample to prevent ABTS® oxidation was compared to Trolox (Cayman chemicals, Michigan, USA), a water soluble tocopherol analogue. Sample antioxidant capacity is quantified as millimolar Trolox equivalents calculated from a 7 point Trolox standard curve.

2.2.7 Lipid peroxidation

Lipid peroxidation (LP) was determined by measuring the thiobarbituric reactive substances (TBARS) present in the tissue lysates. The Oxitek® TBARS assay (Zeptometrix corporation,

#0801192) used is based on specificity of malondialdehyde (MDA), which is a by-product of lipid peroxidation, for TBARS. The MDA present in the sample forms a 1:2 adduct with the thiobarbituric acid in the reaction. Tissue lysates were thawed at room temperature and 100 µL of each was pipetted into pre-labelled glass test tubes. 100 µL of a 0.28 mol sodium dodecyl sulphate solution was added and tubes gently shaken to homogenize the solution. 2.5 mL of TBA / Buffer reagent (0.5 g thiobarbituric acid dissolved in 50 mL acetic acid and 50 mL of sodium hydroxide) was added to each test tube. Samples were incubated in a water bath at 95°C for 60 min with glass marbles placed on top of the test tubes to prevent evaporation. After cooling in an ice bath for 10 minutes, samples were centrifuged at 3000 rpm for 15 minutes at room temperature. Supernatants absorbances were read at 532 nm on a Bio Rad Benchmark plus microplate spectrophotometer. Absorbances were compared to those of an MDA standard prepared from 100nmol mL^{-1} malondialdehyde bis (dimethyl acetal) made up in buffer. TBARS for individual samples were calculated as MDA nmol mL^{-1} equivalents from a 5 point MDA standard curve.

2.2.8 Protein analysis

Protein in samples was measured to provide a baseline for the normalisation of TAOC and MDA. Protein was quantified using the Fluoroprofile protein quantification kit (#FP0010; Sigma Aldrich, USA), a fluorescent assay based on epicocconone. Fluorescence was read at 485 nm excitation and 620 nm emission wavelengths on a microplate spectrophotometer (Benchmark Plus, BioRad, USA). A bovine serum albumin (BSA) calibration curve was used to calculate protein concentrations.

2.2.9 Lysosomal membrane stability

The methods used for the lysosomal stability test were based on procedures developed by Ringwood *et al.* (2003). Each organism was dissected to isolate the digestive gland and all attached gonadal tissue was removed. The digestive gland was rinsed with calcium and magnesium free saline buffer (CMFS) (20 mM HEPES (Thermo-Trace C07133), 360 mM NaCl (UNIVAR F2A021), 12.5 mM KCl (M and B Laboratory Chemicals 20116) and 5 mM tetrasodium EDTA (Merck 10093.5V)) pH 7.35-7.4. The digestive gland tissue was then homogenised with the use of a scalpel and glass Petri dish (inverted and filled with ice). Samples were then placed into a 24 well plate with 600 µL of CMFS on ice and shaken for 20 min at 100 rpm on an orbital shaker. A trypsin solution was made up using 1 mg of trypsin (Sigma 1426-1G) to 1 mL CMFS. 400 µL of the trypsin solution was then added to the sample

and the sample was shaken for a further 20 minutes at 100 rpm on an orbital shaker. Samples were then sheared with a glass pipette and transferred to a microcentrifuge tube/filter apparatus. Samples were initially centrifuged at 200-225 × g (5 min, 15 °C). The filter was then removed, the supernatant discarded and the pellet resuspended in 1 mL CMFS. This solution was then centrifuged at 200-225 × g (5 min, 15 °C). If required a further resuspension and centrifugation step was added to remove debris from the cells. After the final rinse the supernatant was discarded and the pellet was resuspended in 50-300 µL of CMFS dependent on the size of the pellet. A neutral red (Sigma Aldrich, N-7005) stock solution was made up in dimethyl sulphoxide (Merck 10323) at a concentration of 4 mg mL $^{-1}$. A working solution was then prepared using the stock solution at a concentration of 10 µL mL^{-1} in CMFS. The working solution was added in 1:1 v/v ratio to the final addition of CMFS in the microcentrifuge tube and the cells were incubated in the dark for 60 min. After the incubation period a wet mount slide was prepared and observed at 400× magnification under a light microscope. At least 50 cells were counted for each sample.

2.2.10 Data analysis

Initial data organisation and preparation of some figures was completed using Microsoft Excel 2010. Mortality data were corrected for deaths due to natural causes based on mortalities in the control treatment (Abbott 1925). All data analysis was conducted using R version 3.1.2 (R Core Team 2014). LC_{50} values were calculated using the "ecotoxicology" package (Gama 2015). Where comparisons among multiple treatments were required for non-parametric data, a Kruskal-Wallis test was used, with a Dunn's tests for multiple pairwise comparisons (Dinno 2016). The "ggplot2" package was used to produce figures (Wickham 2009).

2.3 Results

2.3.1 Mortality

Mortality of *I. newcombi* generally increased with exposure Cu concentration and exposure period. The snails exposed to 120, 80 and 60 µg L^{-1} Cu had 100% mortality, with all snails being deceased in these treatments by days 5, 13 and 28 of exposure respectively (Figure 2-1). The LC_{50} value decreased with increased exposure time, with the LC_{50} values being 99, 64, 59 and 44 µg L^{-1} Cu for 7, 14, 21 and 28 days respectively. At concentrations 50 µg L^{-1} and below, there was less than 85 % mortality in all treatments. The mortality in these treatments was not related to Cu exposure concentration, indicating that *I. newcombi* have the capacity to survive at concentrations ≤ 50 µg L^{-1} Cu for 28 days under the experimental conditions.

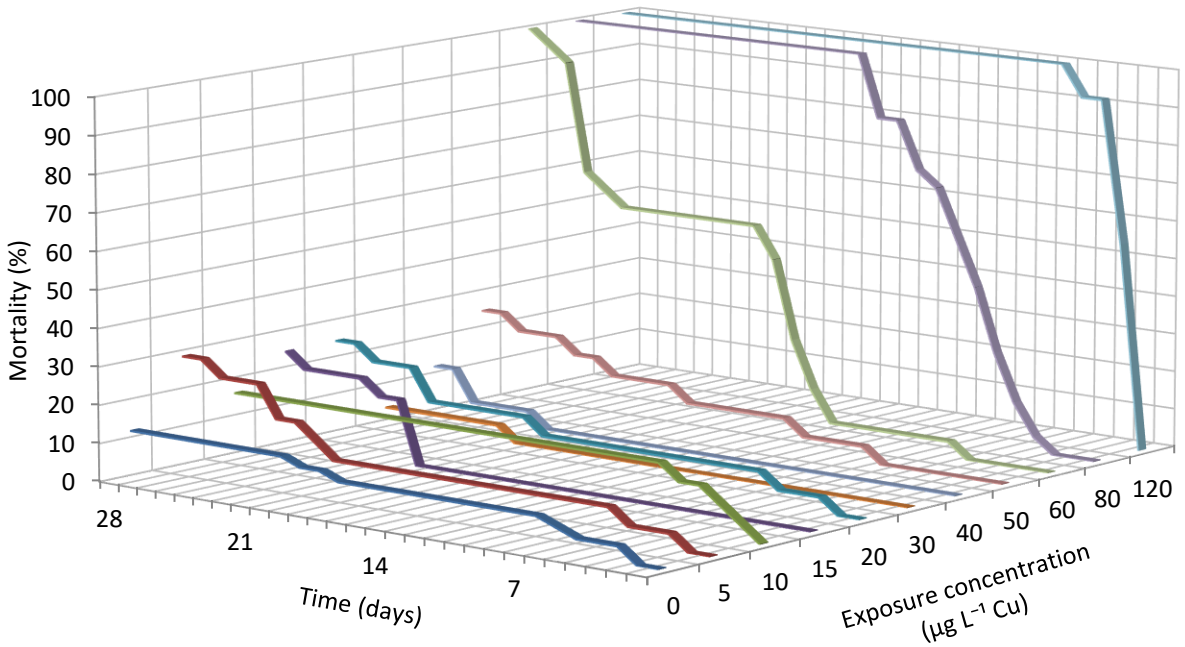


Figure 2-1. Mortality of *Isidorella newcombi* exposed to 0 - 120 µg L^{-1} Cu (nominal concentrations) in water over a 28 day exposure period. Data for each exposure concentration are based on mean mortality across 2 replicates.

2.3.2 Bioaccumulation of copper

Tissue copper concentrations were significantly different among treatments (F = 24.72, $d.f.$ = 7,47, p < 0.001). The tissue Cu concentrations in *I. newcombi* increased as the Cu exposure concentrations increased. The snails from the 50 µg L^{-1} Cu exposures had significantly higher Cu tissue concentrations than the snails from other treatments in which snails survived to the end of the exposure period (Figure 2-2).

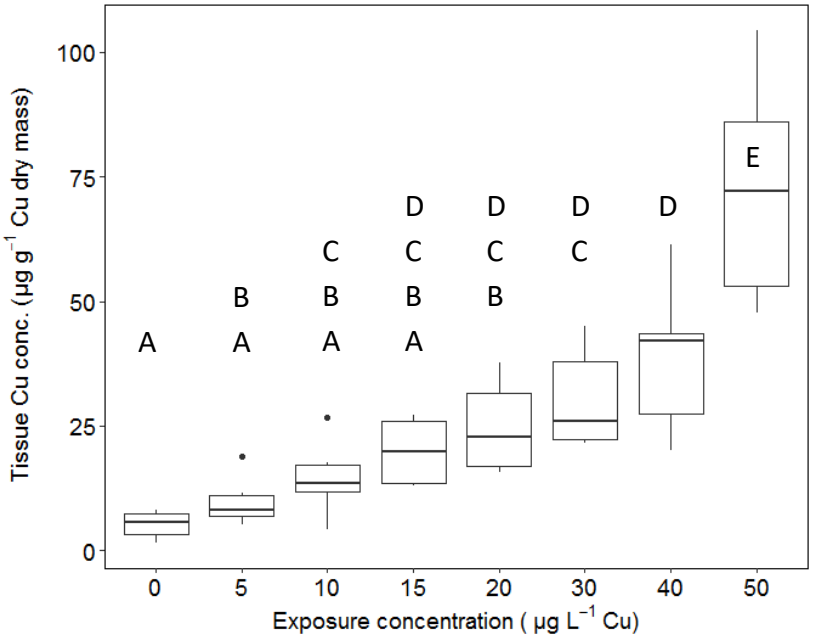


Figure 2-2. Boxplot of tissue copper concentration in copper exposed *Isidorella newcombi* after 28 days. Exposure concentrations are nominal concentrations. Letters indicate significant differences among means (Tukey HSD test, $p < 0.05$). Middle line in box is median, the lower and upper lines of the box are 25th and 75th percentiles respectively, whiskers are 0 and 100th percentiles with dots representing outliers ($n = 6$).

2.3.3 Feeding rate

The snails in the higher exposure treatments generally consumed less lettuce per day than the snails from the lower exposure treatments (Figure 2-3). There was no significant difference among treatments for the consumption of lettuce for those treatments that had individuals surviving at the end of the exposure period (0 to 50 µg L^{-1} treatments) ($\chi^2 = 7.953$, $d.f. = 7$; $p = 0.337$). Linear regression analysis performed using the exposure concentrations and the mean daily lettuce consumption per snail indicated there was a significant relationship between the two variables ($r^2 = 0.788$, $F = 29.73$, $p < 0.001$). The data indicates that there is little change in the consumption of lettuce per snail at Cu exposure concentrations of up to 40 µg L^{-1} Cu, but at higher concentration there was a dose-dependent reduction in the feeding rate of the snails (Figure 2-3). The 120 µg L^{-1} Cu exposure is not included in the analysis as there was no lettuce consumed in this treatment.

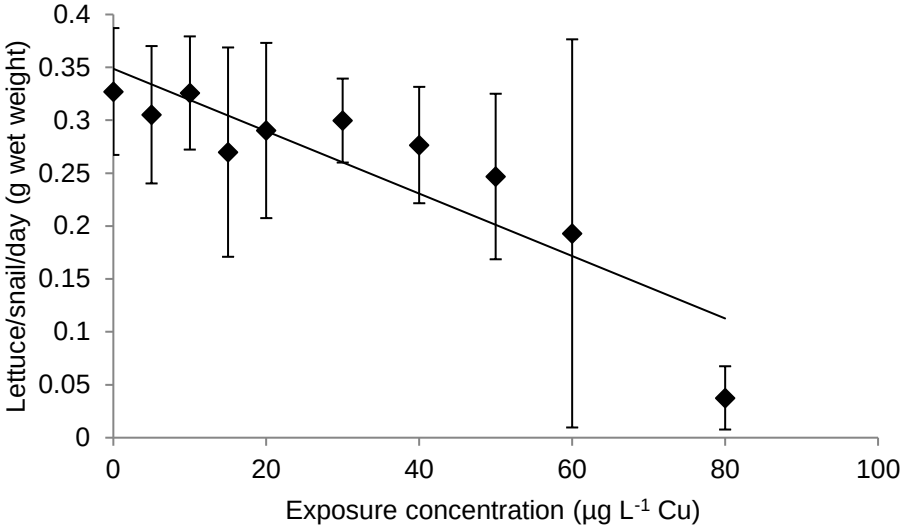
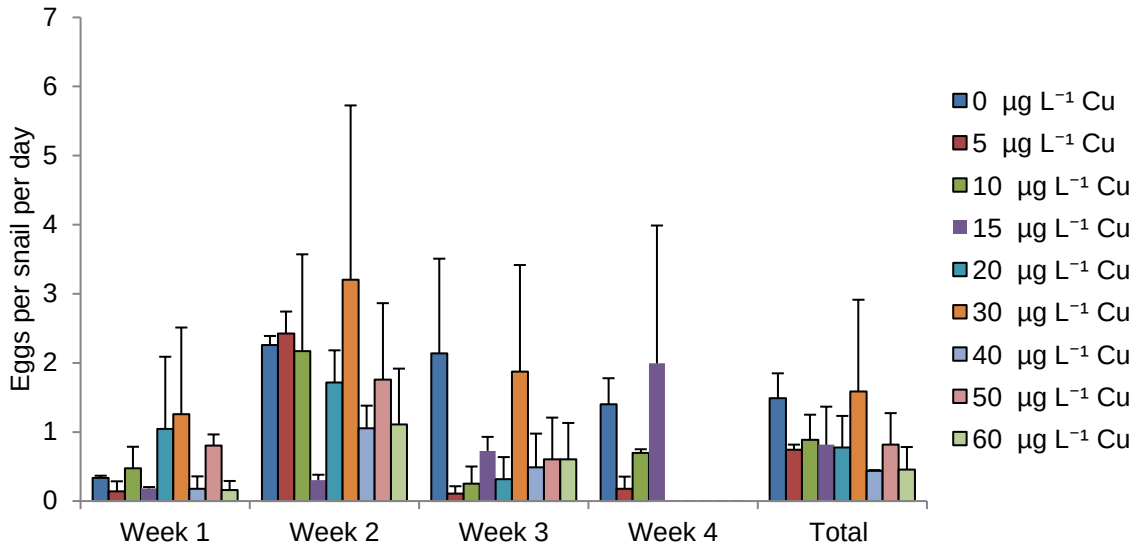


Figure 2-3. Consumption of lettuce by *I. newcombi* exposed to Cu over a 28 day exposure period. Exposure concentrations are nominal concentrations. Mean and standard deviation are calculated from the average lettuce consumption per snail per day at each water change.

2.3.4 Fecundity

There was no egg production from the snails from the 80 and 120 µg L^{-1} Cu treatments. Over the duration of the experiment the snails from 30 µg L^{-1} treatment laid both the most clutches and eggs per snail (Figure 2-4 a & b). Despite the snails from the 30 µg L^{-1} treatment laying the most eggs overall, after day 15 of the exposure, this group laid no more eggs. A trend of cessation of egg production in snails exposed to Cu above a threshold concentration was observed more broadly, with snails exposed to greater than 15 µg L^{-1} Cu ceasing oviposition in week 4 of the exposures. This was in contrast to the snails from treatments exposed to 15 µg L^{-1} or below where clutch production was observed throughout the duration of the experiment.

a)



b)

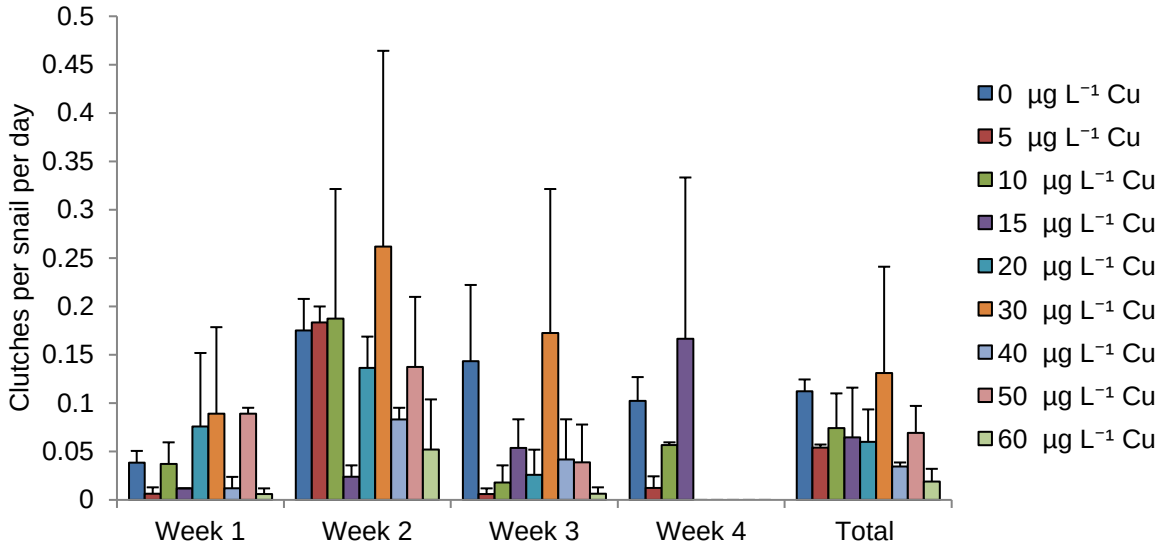


Figure 2-4. **N**umber of (a) eggs and (b) clutches laid per snail per day by *Isidorella newcombi* exposed to Cu. Exposure concentrations are nominal concentrations. Data are means and standard deviations of the replicates and are separated for individual weeks and the total for the 28 day exposure period.

2.3.5 Embryo viability and juvenile survival

There was a significant difference between the proportions of live juveniles to the number of eggs laid among the differing copper concentrations (χ^2 = 27.49, d.f. = 8, p < 0.001). At exposure concentrations of 30 µg L^{-1} and above, there was a trend of Cu exposure concentration-dependent reduction in juvenile survival, however, post hoc analysis indicated that there were only significant differences between the survival of juvenile offspring from the 50 and 60 µg L^{-1} Cu and those from lower treatments (Figure 2-5).

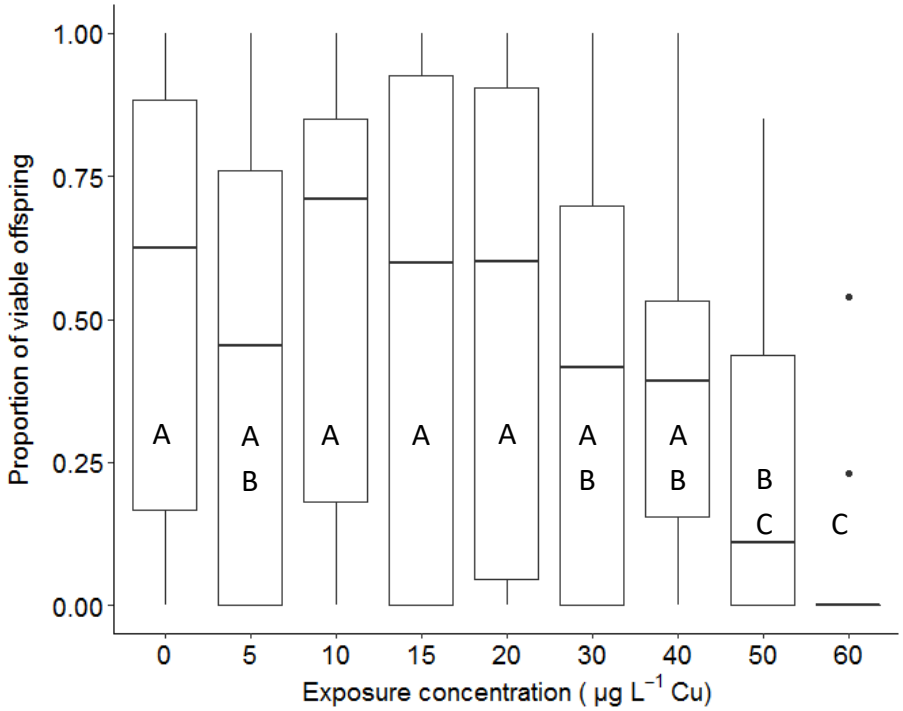


Figure 2-5. Boxplot of the proportion of live snails at one week after hatching to the number of eggs laid by *Isidorella newcombi* exposed to Cu. Exposure concentrations are nominal concentrations. Middle line in box is median, the lower and upper lines of the box are 25th and 75th percentiles respectively, whiskers are 0 and 100th percentiles with dots representing outliers. Letters (A, B and C) on the boxplot represent homogenous subsets as determined by Dunn's test. Data used are based on individual egg masses so the number of observations for each Cu exposure concentration varied; *n* (0) = 65, *n* (5) = 23, *n* (10) = 28, *n* (15) = 16, *n* (20) = 31, *n* (30) = 44, *n* (40) = 21, *n* (50) = 34 and *n* (60) = 9.

2.3.6 Total antioxidant capacity

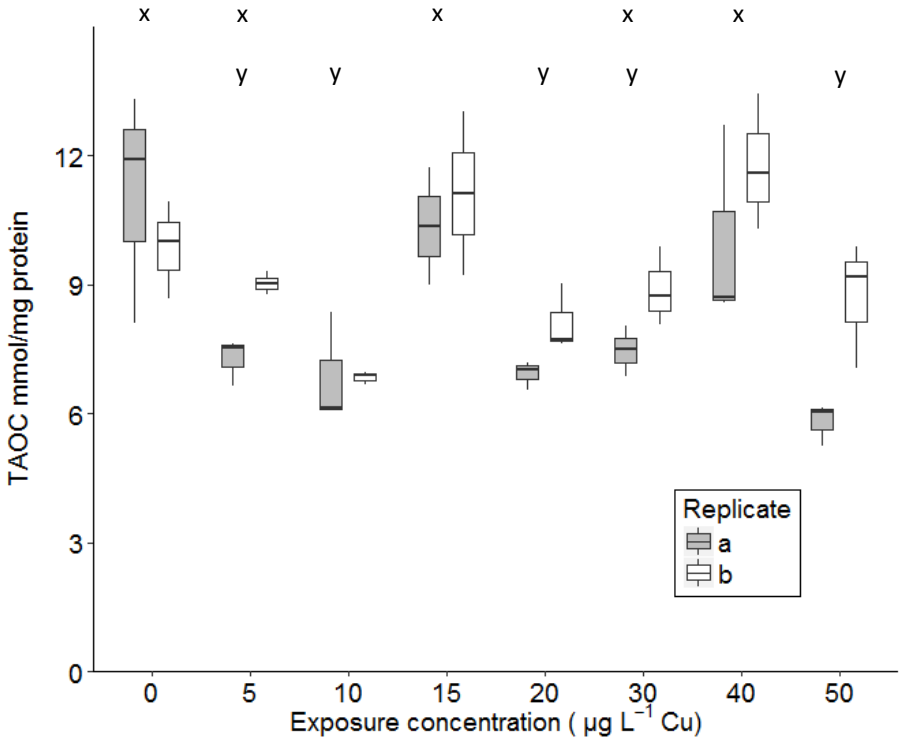
The total antioxidant capacity (TAOC) of *I. newcombi* was significantly different among treatments (F = 6.753, $d.f.$ = 7, 37; p < 0.001). There were two separate groups, with *I. newcombi* exposed to concentrations of 0, 15, and 40 µg L^{-1} Cu having significantly higher total antioxidant capacity than those exposed to 10, 20 and 50 µg L^{-1} Cu treatments. The 5 and 30 µg L^{-1} Cu treatments were included in both groups (Figure 2-6a). While TAOC varied among the treatments, there was not clear trend of TAOC increasing or decreasing with increased Cu concentration but rather the response fluctuated up and down as copper exposure increased (Figure 2-6a). As this pattern was unusual, the TAOC results for the individual treatment replicates are displayed separately. The similar pattern seen in both replicates

provides support that the response seen is a legitimate response and not an anomaly associated with a single replicate.

2.3.7 Lipid peroxidation

There was no significant difference in the concentration of MDA among the treatments (F = 1.686; df = 7,37; p = 0.143). While this was the case, a dose-dependent increase can be seen in the concentration of MDA from the 0 to 15 µg L^{-1} Cu treatments, followed by a decrease to the 20 µg L^{-1} Cu treatment, an increase to the 30 µg L^{-1} Cu treatment and a large amount of variability in the 40 and 50 µg L^{-1} Cu treatments. While there is some variability in individual replicates within treatments, the general trends seen at treatment level were also apparent in the individual replicates supporting the unusual pattern seen as being real and not an artefact associated with an individual anomalous replicate (Figure 2-6b).

a)



b)

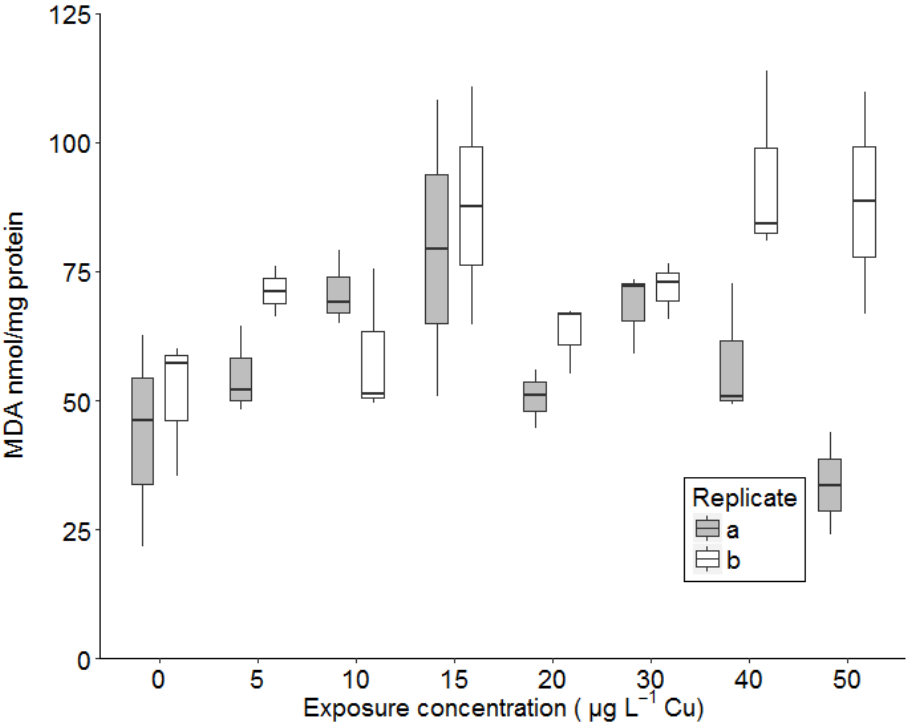


Figure 2-6. Box plot of a) total antioxidant capacity (x and y indicate homogenous subsets) and b) maliondialdehyde (MDA) in *Isidorella newcombi* exposed to Cu for 28 days (n = 3). Exposure concentrations are nominal concentrations. Middle line in box is median, the lower and upper lines

of the box are 25th and 75th percentiles respectively, whiskers are 0 and 100th percentiles with dots representing outliers.

2.3.8 Lysosomal membrane destabilisation

There were significant differences in the percent of cells that had unstable lysosomes among the treatments (χ^2 = 17.20, *d.f.* =7, p = 0.02). *I. newcombi* exposed to 0 and 5 µg L^{-1} Cu had significantly lower lysosomal destabilisation (LD) than those exposed to higher concentrations, with the exception of the 30 µg L^{-1} Cu exposure which was not different from any other treatment (Figure 2-7).

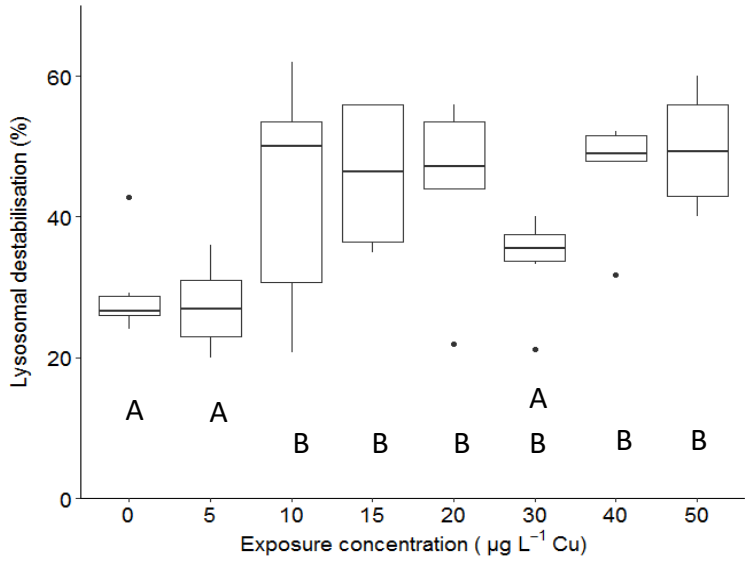


Figure 2-7. Box plot of percent of unstable lysosomes in *I. newcombi* exposed to copper in water for 28 days. Exposure concentrations are nominal concentrations. Middle line in box is median, the lower and upper lines of the box are 25th and 75th percentiles respectively, whiskers are 0 and 100th percentiles with dots representing outliers. Letters (A and B) on the boxplot represent homogenous subsets as determined by Dunn's test.

2.3.9 Relationships between cellular biomarkers

Linear regression analysis shows there is a significant relationship between TAOC and MDA (t =24.480, p < 0.001; Figure 2-8a). As the constant was not significantly different from 0 (t = 1.541, p = 0.131) the intercept was set to 0. The relationship between the variables can be explained by the equation:

MDA = 7.31 × TAOC

As the intercept is set to 0 no r^2 value is reported but the fit of the line can be seen in Figure 2-8a.

Linear regression analysis showed that there was no significant relationship between TAOC and LMS ($t = 0.449$, $p = 0.660$; Figure 2-8b).

Linear regression analysis showed that there was a significant relationship between MDA and LD ($t = 14.497$, $p < 0.001$) (Figure 2-8c). As the constant was not significantly different from 0 ($t = 2.032$, $p = 0.062$) the intercept was set to 0. The relationship between the variables can be explained by the equation:

$LD = 0.59 \times MDA$.

As the intercept is set to 0 no r^2 value is reported but the fit of the line can be seen in Figure 2-8c.

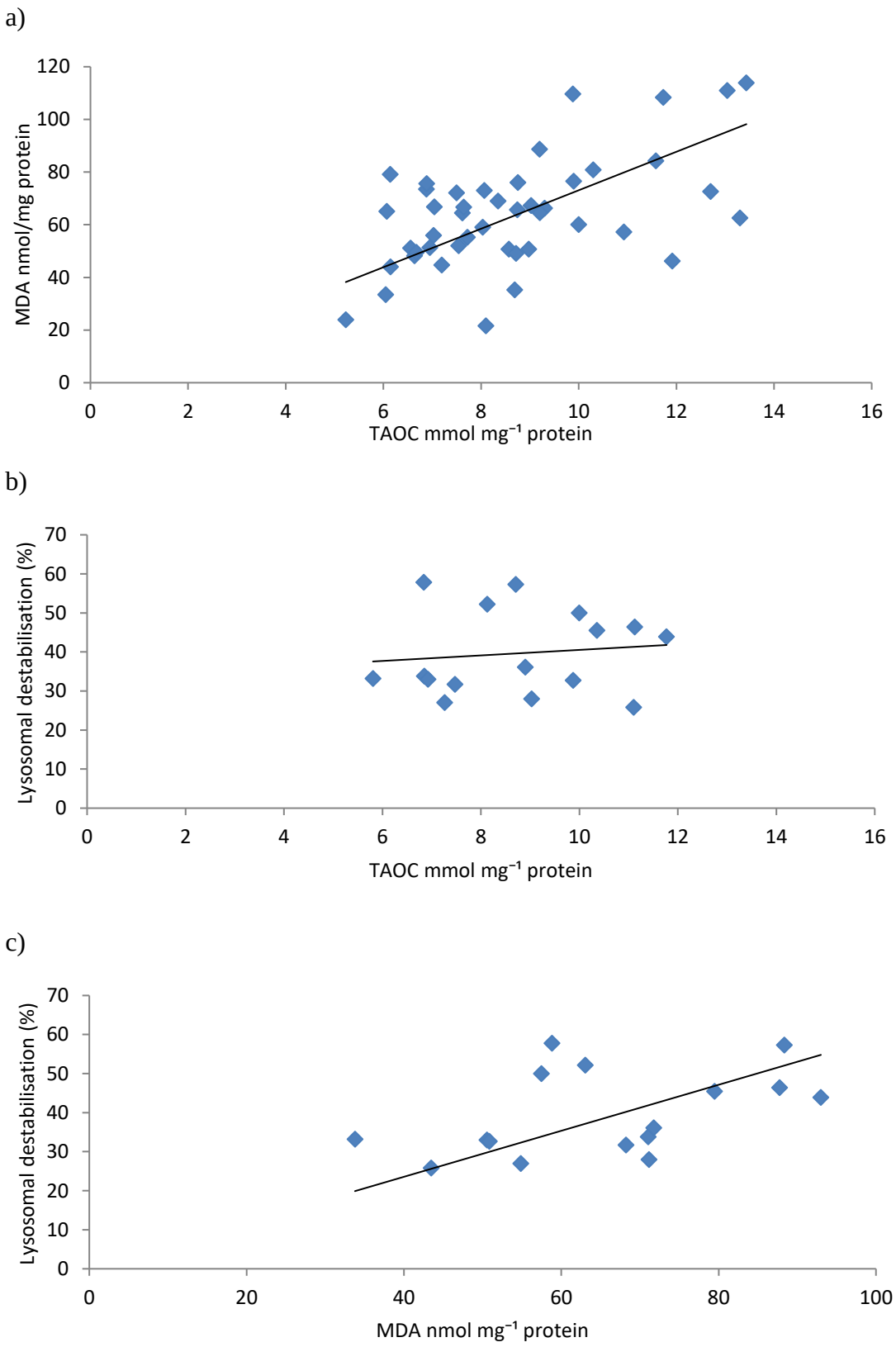


Figure 2-8. Scatterplots showing the relationship between oxidative stress biomarkers in *Isidorella newcombi* exposed to Cu (0-50 µg L^{-1}) for 28 days; a) total antioxidant capacity (TAOC) and Maliondialdehyde (MDA), b) TAOC and lysosomal destabilisation and c) MDA and lysosomal destabilisation. Data in a) are individual snails, data in b) and c) are means from replicates.

2.3.9.1 Relationship between cellular biomarkers and individual effects

There were no significant correlations between the cellular level biomarkers (TAOC, MDA and LD) and the individual effects (feeding rate, mean number of clutches per snail, mean number of eggs per snail and offspring viability) in *I. newcombi* (Table 2-1).

Table 2-1. Correlation analysis between cellular biomarkers (total antioxidant capacity (TAOC), malondialdehyde (MDA) and lysosomal membrane destabilisation (LD) and individual effects (feeding rate, mean number of clutches per snail, mean number of eggs per snail and offspring survival) in *Isidorella newcombi* exposed to 0-50 µg L^{-1} Cu for 28 days.

Variables		*p*
TAOC	Feeding rate	0.87
TAOC	Mean clutches	0.83
TAOC	Mean Eggs	0.95
TAOC	Offspring viability	0.68
MDA	Feeding rate	0.25
MDA	Mean clutches	0.43
MDA	Mean Eggs	0.34
MDA	Offspring viability	0.84
LD	Feeding rate	0.15
LD	Mean clutches	0.21
LD	Mean Eggs	0.13
LD	Offspring viability	0.80

2.3.10 Summary table of results

The results from all endpoints are summarised (Table 2.2).

Table 2-2. Summary of results from all endpoints

Concentration	**0**	**5**	**10**	**15**	**20**	**30**	**40**	**50**	**60**	**80**	**120**
Mortality	Low mortality rates								100 % mortality		
Bioaccumulation of copper	Exposure concentration dependent increase								NA	NA	NA
Feeding rate	Consistent feeding rate							Exposure concentration dependent decrease			NA
Egg production	Layed eggs throughout exposure				No oviposition in week 4 of exposure					No egg production	
Juvenile survival	High juvenile survival					Trend of reduced juvenile survival				NA	NA
TAOC	High	NSD	Low	High	Low	NSD	High	Low	NA	NA	NA
Lipid peroxidation	No significant difference								NA	NA	NA
Lysosomal membrane destabilisation	Low	Low	High	High	High	NSD	High	High	NA	NA	NA

2.4 Discussion

2.4.1 Mortality

All snails exposed to 60 µg L^{-1} Cu and above died during the 28 day exposure period. There were no differences in mortality in snails exposed to concentrations of 50 µg L^{-1} Cu and below, indicating snails were able to survive at these concentrations. Previous studies have found that mortality is related to Cu exposure concentration in gastropods (Brix *et al.* 2011; Hoang and Rand 2009; Rogevich *et al.* 2008). In the current study, at concentrations ≤ 50 µg L^{-1} Cu, there was an exposure-dependent increase in tissue Cu concentrations, but no resultant increase in mortality. Gastropods are known to produce metallothioneins and granules for the detoxification and storage of excess Cu (Dallinger *et al.* 1997; Desouky 2006; Roesijadi and Robinson 1994). It is likely that metallothioneins and granules were being used by the surviving snails and that at least some of the Cu had been stored in a detoxified form. As such, at exposure concentrations of ≤ 50 µg L^{-1} Cu, even though the *I. newcombi* were accumulating Cu, they had the capacity to detoxify the excess accumulated Cu at least to the extent where biologically available Cu was not lethal over the exposure period. The ability of molluscs to survive under exposure conditions that increase tissue metal concentrations, especially when there is an associated increase in metallothionein production has been demonstrated (Andrès *et al.* 1999; Baudrimont *et al.* 1999)

In the snails exposed to concentrations above 50 µg L^{-1} Cu, there was a concentration-dependent increase in mortality, as indicated by a reduction in LC_{50} values over time. The LC_{50} values were 99, 64, 59 and 44 µg L^{-1} Cu at 7, 14, 21 and 28 days respectively. The study design for this experiment aimed to investigate a wide range of concentrations and link trends in cellular level biomarker responses with individual level responses. The workload involved required a trade-off in a smaller number of replicates per treatment. Also, a focus on Cu concentrations that resulted in sub-lethal responses rather than a focus of treatments at Cu concentrations close to those causing mortality was required. This restricted the use of a full probit model that would allow confidence levels to be calculated for the LC_{50} values. The limitations of the calculated LC_{50} values based on the experimental design are acknowledged, however, it was still considered worthwhile to include LC_{50} values as they are used broadly in ecotoxicology and their inclusion allows comparisons with other studies. It is reported that in juvenile freshwater snail *Lymnaea stagnalis,* LC_{50} values of 24.9 µg L^{-1} Cu and > 18.2 µg L^{-1} Cu were calculated at 96 h and 28 d respectively (Ng *et al.* 2011). Another study on *L.*

stagnalis showed that it was particularly sensitive with 100% mortality at a concentration of 13 µg L^{-1} Cu over a 30 day exposure period (Brix *et al.* 2011). Based on these results, *I. newcombi* is less sensitive to copper than some other freshwater gastropods. In *Lymnaea luteola*, it was found that the snails had LC_{50}s of 64, 41, 21 and 16 µg L^{-1} Cu at 7, 14, 21 and 28 days respectively, indicating a time dependent mortality response to Cu, with values broadly comparable to those from this study (Das and Khangarot 2011). While these values are lower than those calculated in the current study, they show a similar reduction in LC_{50} values over time. The reasons for the lower LC_{50} values between tests may be interspecific differences or the ages of snails used in the test, as it was stated in the study by Das and Khangarot (2011) that young snails were used, but their age was not specified.

2.4.2 Copper bioaccumulation

The exposure concentration-dependent increase in tissue Cu concentrations demonstrated that *I. newcombi* is a net accumulator of Cu and accumulates Cu relative to the bioavailable concentrations in its environment. Copper accumulation has been reported to increase in an exposure-dependent manner in other freshwater gastropods (Das and Khangarot 2011; Hoang and Rand 2009; Ng *et al.* 2011; Pyatt *et al.* 2003; Rogevich *et al.* 2008). The ability to accumulate Cu relative to bioavailable concentrations present in the surrounding environment is a desirable characteristic of a potential bio-monitor species, as it provides a time-integrated measure of bioavailable concentrations in the environment (Rainbow 1995).

2.4.3 Feeding rate

As Cu exposure concentration increased there was a decrease in the feeding rate of *I. newcombi*. This relationship was particularly evident at exposure concentrations greater than 40 µg L^{-1} Cu. In a 28 day exposure of the pulmonate freshwater snail *Lymnaea stagnalis* to Cu, it was found that there was no reduction in feeding rate, but the highest concentration used was 18.2 µg L^{-1} Cu, lower than the Cu concentration where feeding rate was reduced in the current study (Ng *et al.* 2011). In *L. luteola* the feeding rate reduced in an exposure concentration-dependent manner with feeding ceasing at 32 µg L^{-1} Cu after 2 weeks (Das and Khangarot 2011). This was similar to the pattern seen in *I. newcombi* in this study. For *Pomacea canaliculata* there was also a similar pattern, with no significant differences in the food consumption of snails exposed at concentrations up to 30 µg L^{-1} Cu, but snails exposed to 45 and 67.5 µg L^{-1} Cu for 99 days having a reduction in food consumption (Peña and Pocsidio 2007).

2.4.4 Fecundity

The snails exposed to 30 µg L^{-1} Cu laid the most clutches and eggs for the entire experimental period. There was high variability in the clutches and eggs laid, both between the replicates at treatment level and between the treatments. Large amounts of variability in reproductive output of snails have been reported in other studies. In the freshwater snail *Potamopygrus antipodarum* exposed to copper as well as to copper oxide nano particles it was noted that similar trends were evident but, differences were not statistically significant because of high inter-snail variability in reproductive output (Ramskov *et al.* 2014). Even in species from the same genus fecundity results can vary widely. In *Pomacea paludosa* exposed to 8 and 16 µg L^{-1} Cu, a significant reduction in clutch production and eggs was recorded (Rogevich *et al.* 2008), while for *P. canaliculata* exposed to concentrations of up to 67.5 µg L^{-1} Cu there was no effect on clutch production or the total number of eggs (Peña and Pocsidio 2007).

Despite high variability in fecundity within and among treatments, Cu exposure led to a cessation of egg production that occurred immediately at high exposure concentrations but was delayed at lower concentrations. In treatments exposed to concentrations ≥ 80 µg L^{-1} Cu no egg production was recorded, a similar finding has been observed in the pulmonate gastropod *Biompahalaria glabrata* with no eggs produced by snails exposed to concentrations of 100 µg L^{-1} Cu and above (Ravera 1977). Additionally, in the current study, all snails exposed to greater than 15 µg L^{-1} Cu had ceased egg production by week four. In *L. luteola* exposed to copper for seven weeks there was a similar trend, with snails exposed to copper concentrations above 10 µg L^{-1} laying far fewer clutches of eggs after 3 weeks of exposure (Das and Khangarot 2011). Both in the current study and in the literature discussing fecundity in freshwater gastropods exposed to Cu, it can be seen that as exposure concentrations increase there are threshold levels where the Cu-induced stress causes discernible changes to fecundity. At low Cu concentrations where the snails are not stressed, fecundity levels are maintained over time. At intermediate Cu concentrations where snails are able to survive in a stressed state, reproductive function persists for a period then ceases. The cessation of egg production after multiple weeks of exposure at intermediate concentrations is likely to be associated with the need to transfer biological resources from reproductive functions to functions associated with detoxification and cellular repair. Biological trade-offs in the form of reduced reproduction in response to stress have been reported in molluscs (Petes *et al.* 2008). At high Cu concentrations where mortality is likely to occur within four weeks of exposure, egg production ceases immediately upon Cu exposure.

This suggests an extreme level of stress causing the organisms to either enter a metabolically depressed state or divert all biological resources away from reproductive functions.

2.4.5 Embryo viability and juvenile survival

At Cu exposure concentrations greater than 30 µg L^{-1} Cu there was a trend of reduced embryo viability and juvenile survival with increased Cu exposure concentrations. In the current study the number of eggs hatched and the number of offspring surviving was combined as a single endpoint. Many studies assess only eggs hatched or juvenile survival or they are divided into eggs hatched and juvenile survival as separate endpoints. In *L. luteola* significant reductions in the percent of eggs hatching were seen in snails exposed to 10 µg L^{-1} Cu and above (Das and Khangarot 2011). The proportion of eggs hatched in *Pomacea paludosa* was significantly reduced at 16 µg L^{-1} Cu but not at 8 µg L^{-1} Cu, however survival of the juveniles was not altered (Rogevich *et al.* 2008).

2.4.6 Total antioxidant capacity

While there were significant differences in the TAOC in *I. newcombi* exposed to different Cu concentrations, these changes did not occur in a concentration-dependent manner. This may be due to the regulation of specific enzymatic and non-enzymatic antioxidant responses changing at different Cu exposure concentrations. In a previous study examining antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) and non-enzymatic antioxidant responses (glutathione and oxidised glutathione) in the hepatopancreas, foot and mantle of *L. stagnalis*, the responses of individual enzymatic and non-enzymatic components of the antioxidant system varied among specific tissues and Cu concentrations (Atli and Grosell 2016). This indicates that different parts of the antioxidant system are being regulated at different levels of reactive oxygen species (ROS) production. The TAOC measurement used in the current study examined the ability of the antioxidant system overall to eliminate ROS. The changes in TAOC at different Cu concentrations may relate to changes induced in specific enzymatic and non-enzymatic components of the antioxidant system based on different levels of ROS being produced at different Cu concentrations. Despite fluctuations seen in antioxidant capacity in this study, the TAOC at 40 µg L^{-1} Cu was not significantly different from the controls. This indicates that at least up to 40 µg L^{-1} Cu the antioxidant system has not become overwhelmed by any increased production of ROS occurring as a result of copper exposure, but the changes seen at concentrations below this level are likely associated

with changes in the regulation of individual enzymatic and non-enzymatic components of the antioxidant system.

TAOC in the mollusc phylum appears to differ between gastropods and bivalves. Other studies on gastropods have found little reduction in TAOC associated with; copper, metal contamination, temperature and salinity (Deschaseaux *et al.* 2011; Ubrihien *et al.* 2017b; Ubrihien *et al.* 2017c). Conversely, bivalves have shown a reduced TAOC when exposed to various metals (Marasinghe Wadige *et al.* 2014; Taylor *et al.* 2016; Taylor and Maher 2012c). This may indicate that the antioxidant system of gastropods has a higher capacity to manage ROS than bivalve molluscs.

2.4.7 Lipid peroxidation

There was no significant difference in LP among the treatments. LP is damage associated with oxidative stress. Despite TAOC differing among treatments, there was no reduction in TAOC that would be indicative of the antioxidant system being overwhelmed. Based on these results there is no evidence that *I. newcombi* were experiencing oxidative stress at the exposure concentrations and duration tested in this study. As the snails were not experiencing oxidative stress it is not surprising that no oxidative damage in the form of lipid peroxidation was observed. In exposures of *L. stagnalis* to Cu over a 28 day exposure period at concentrations up to 18.2 µg L^{-1} Cu there was no increase in MDA, indicating that oxidative damage was not occurring (Ng *et al.* 2011). Ng *et al.* (2011) also measured an increase in metallothionein like proteins (MTLPs) and it was suggested that the increase in MTLPs may have had a sufficient detoxifying effect to prevent toxicity through the oxidative stress pathway at the Cu concentrations tested. In a study on the marine gastropod *Littorina littorea* exposed to anoxic conditions for 6 days followed by aerobic recovery there were substantial changes in enzymatic and non-enzymatic antioxidant defences, however, there was no change in MDA (Pannunzio and Storey 1998). There were changes in earlier stages of lipid peroxidation measured as lipid hydroperoxide levels and conjugated dienes (Pannunzio and Storey 1998). It was hypothesiszed that antioxidant defence systems were able to adjust to rapid changes in free radical generation and that preliminary peroxidative damage products were repaired, avoiding the accumulation of terminal breakdown products. Exposures of *Planiborus corneus* to 450 and 800 µg L^{-1} pentachlorophenol led to increases in MDA at 2 and 8 days, but by 13 days lipid peroxidation had returned to control levels (Klobučar *et al.* 1997). It was suggested that while LP was seen as an initial response it was reduced over time by the activation of

compensatory adaptive mechanisms. In other studies investigating the marine gastropods *Austrocochlea porcata* (Reid and MacFarlane 2003) and *Bembicium nanum* (Ubrihien *et al.* 2017c) exposed to oxidative stress inducing conditions, no change in LP was detected. It is likely that under high oxidative stress inducing conditions LP is initiated, but in organisms that have the capacity to survive the initial effects of the toxicant, adaptive mechanisms such as enzymatic and non-enzymatic antioxidants and metallothionein-like proteins are able to manage ROS and cellular mechanisms are able to repair oxidative damage that has occurred in the short term.

2.4.8 Lysosomal membrane destabilisation

Isidorella newcombi exposed to concentrations of 10 µg L^{-1} Cu and higher had increased levels of LD. In previous studies where gastropods have been exposed to copper as well as metal contamination gradients there has been evidence of concentration-dependent increases in LD (Ubrihien *et al.* 2017a; Ubrihien *et al.* 2017c). For the interpretation of LD results a framework has been developed that classifies organisms with below 30% of cells having LD is within the normal range, 30-40% is considered a cause for concern where levels are marginally outside of the normal range, and above 40% organisms are considered stressed (Ringwood *et al.* 2003). Based on these criteria, organisms from the treatments exposed to 10 µg L^{-1} Cu and above could be considered as in the cause for concern or stressed groups.

Reductions in the stability of the lysosomal membrane can occur through the accumulation of contaminants in the lysosome leading to alterations in lysosomal structure (Moore *et al.* 2007) and oxidative stress-induced LP (Company *et al.* 2004). Alternatively, changes in the permeability of the cell membrane can occur as part of apoptotic, necroptotic or autophagic cell death (Johansson *et al.* 2010). In the current study there is no evidence of the organisms experiencing sufficient oxidative stress to cause differing levels of LP among treatments. It is suggested that the changes in LD are caused by sequestration of excess Cu into the lysosome or the initiation of programmed cell death mechanisms in response to Cu-induced damage.

2.4.9 Relationship between biomarkers

2.4.9.1 Relationship between cellular biomarkers

The positive relationship between TAOC and MDA in the current study along with the lack of lipid peroxidation detected in other gastropod studies (Klobučar *et al.* 1997; Pannunzio and Storey 1998; Reid and MacFarlane 2003; Ubrihien *et al.* 2017b; Ubrihien *et al.* 2017c) suggests

that when gastropods come under sustained challenge from increased ROS generation they have the capacity to increase antioxidant capacity to compensate. Only one of these studies detected increased MDA, and that was at up to 8 days of exposure, but after 13 days of exposure MDA had returned to normal levels (Klobučar *et al.* 1997). Based on the evidence in the literature, when gastropods are exposed to ROS-inducing chemicals in the short term there is potential for oxidative damage, but over longer exposure periods they have the capacity to increase antioxidant capacity to an extent where oxidative damage is not occurring. In contrast, molluscs from the Class Bivalvia exposed to metals have shown a negative relationship between TAOC and MDA (Marasinghe Wadige *et al.* 2017; Taylor and Maher 2012c; 2014). It has been hypothesised that when metal exposure significantly increases the rate of ROS production in bivalves, the antioxidant system can be overwhelmed, resulting in excess ROS and oxidative damage. Thus, these two classes from within the mollusc phylum appear to have differing antioxidant responses and resulting oxidative damage when exposed to ROS-inducing chemicals for an extended period.

There was no significant relationship between TAOC and LD, however, there was a significant positive relationship between MDA and LD. While changes in MDA were not significantly different among treatments, there is evidence that changes in lipid peroxidation are related to changes in LD. Other studies in molluscs exposed to metals have shown a relationship between MDA and LD (Marasinghe Wadige *et al.* 2014; Taylor *et al.* 2016). It is known that the peroxidation of lipids in the lysosomal membrane leads to an increase in the permeability of the membrane, leading to LD (Fong *et al.* 1973). Despite the significant relationship between MDA and LD, as MDA was not significantly different among treatments and LD was, it is likely that there were other causes of LD such as copper sequestration and programmed cell death as discussed previously.

2.4.9.2 Relationship between biomarkers and individual characteristics

In order to relate effects on individuals to higher levels of biological organisation there is a need to relate biomarker responses to responses that are indicative of a reduction in fitness, such as growth, reproductive output or metabolic function (Depledge and Fossi 1994). Directly measuring endpoints such as reproductive output or growth in field studies can be difficult, but the establishment of relationships between biomarkers and population-relevant endpoints in laboratory studies allows inferences to be made on populations based on biomarker responses. In this study there were no significant correlations between the cellular biomarkers measured

and fecundity or offspring viability. Despite this, there was a significant increase in LD in snails exposed to greater than 10 µg L^{-1} Cu and after 3 weeks of exposure organisms exposed to greater than 15 µg L^{-1} Cu ceased egg production. This indicates that the LD in *I. newcombi* increases above a thirty percent baseline which represents the upper limit of the normal range, at a slightly lower Cu concentration than that which causes the inhibition of reproduction. Based on these findings, increased LD could be used as an early warning signal that populations are approaching a level of stress that would cause loss of reproduction.

2.5 Conclusions

While individuals were able to survive at concentrations up to 50 µg L^{-1} Cu there was evidence of sub-lethal responses to Cu exposure at much lower concentrations. Possibly the most important response measured was the cessation of egg production when *I. newcombi* was exposed to Cu concentrations above 15 µg L^{-1}. The general biomarker LD was able to detect a response at concentrations of 10 µg L^{-1} Cu. This indicates that LD could act as an early warning biomarker for responses at higher levels of biological organisation in *I. newcombi* exposed to Cu. In addition, *I. newcombi* has been shown to accumulate Cu in a manner that directly relates to the Cu concentration in its environment. The current water quality guidelines trigger values for Cu are set at 1-2.5 µg L^{-1} Cu depending on the level of protection (ANZECC/ARMCANZ 2000). The concentrations at which the endpoints used in this study detected responses were higher than these trigger values. The trigger values indicate the percentage of species expected to be protected but don't take into account sublethal responses and chemical mixtures in the environment. The validation of a reliable biomarker that acts as an early warning system of higher level effects, along with a known relationship between tissue concentrations and Cu exposure, makes *I. newcombi* a potentially valuable bio-indicator of Cu contamination in Australian freshwater systems. Given that LD is a general biomarker and has been validated for Cu exposure in *I. newcombi*, it would be a useful candidate for use in detecting sublethal responses to other chemicals as well as investigating the response to mixtures of chemicals.

Chapter 3. The responses of *Isidorella newcombi* to multi-generational Cu exposures: mortality, cellular biomarkers and life history responses.

3.1 Introduction

The concentration of Cu is elevated in the biosphere due to mining, processing and transport as well as the widespread industrial, domestic and agricultural use of Cu (Wright and Welbourn 2002). In Australia, there are reported instances of Cu contamination associated with mining activity and with the use of Cu as an agricultural pesticide over extended periods (Eriksen *et al.* 2001; Klessa *et al.* 1997; Merry *et al.* 1986; Stevens 2002). The persistence of Cu in the environment can cause high Cu concentrations to accumulate in areas where it is extracted, processed or used (Kakkar and Jaffery 2005). The widespread use of Cu and its persistence in the environment provide the potential for plant and animal populations to be exposed to elevated concentrations over multiple generations.

A population's phenotypic level of resistance to a contaminant will be determined by the genetic background and multi-generational exposure history (Morgan *et al.* 2007). When populations are exposed to contaminants that exert selection pressures over multiple generations, rapid evolutionary change can occur (Hoffmann and Hercus 2000). The microevolution which can occur as a result of multi-generational exposure can lead to populations with an increased tolerance to the contaminant (Medina *et al.* 2007). While adaptations associated with multi-generational contaminant exposure can result in increased tolerance in contaminated environments, biological trade-offs in resistant populations in the form of reduced fitness in uncontaminated environments have also been reported (Mireji *et al.* 2010; Monaghan *et al.* 2009).

The classic dose-response framework often used in risk assessment relies on the assumption of a consistent underlying response that would also assume genetic consistency between populations. Populations previously exposed to contaminants, however, often show a different dose-response to unexposed populations (Coutellec and Barata 2011). To account for adaptation in risk assessment, there is a need to quantify the adaptive potential of populations (Côte *et al.* 2015). This is particularly true for contaminants such as Cu that are persistent in the environment and have the potential to exert toxic effects over multiple generations.

While experiments have been conducted to investigate the development of resistance to Cu in aquatic organisms, these studies have used exposure to consistent concentrations over entire lifecycles (Peña and Pocsidio 2007; Rogevich *et al.* 2008). In aquatic environments, excess bioavailable Cu resulting from contamination events is only present for a short period due to chemical complexation, precipitation and adsorption (Flemming and Trevors 1989). As a result, the exposure of organisms in certain Cu-contaminated environments may be in short pulses that are associated with specific contamination events. An understanding of the effects of Cu over multiple generations of short exposures would increase the understanding of the multi-generational effects of Cu on organisms exposed to environmentally realistic scenarios.

The Australian endemic freshwater gastropod *Isidorella newcombi* has been identified as a pest in Australian rice growing areas and is controlled by application of $CuSO_4$ (Stevens *et al.* 2014). Under this scenario there is potential for multi-generational exposure of this snail to elevated Cu concentrations and a resultant increase in Cu tolerance. Freshwater snails form important links within freshwater ecosystems by transferring energy and materials through food webs (Habdija *et al.* 1995; Lagadic *et al.* 2007). Freshwater snails also have desirable characteristics for investigations on the multigenerational effects of contaminants, as they are easy to culture, reach reproductive age early, reproduce continuously and have a short embryonic development period (Ravera 1977). The association with Cu in rice growing areas, as well as its desirable characteristics for investigating multi-generational exposures, make *Isidorella newcombi* an ideal model species for the investigation of the effects of multigenerational Cu exposures.

Isidorella newcombi were exposed to Cu over four generations. The aim of the study was to investigate the development of resistance and associated biological trade-offs over these generations. The effects of Cu on survival, fecundity, embryo development and juvenile survival were assessed across multiple exposure concentrations in the parental, F_1 and F_2 generations. In the F_3 generation all treatments were exposed to a common Cu concentration, and the same responses were compared with the specific objective of determining the extent to which differing pre-exposure histories led to increased tolerance to Cu. The bioaccumulation of Cu and the oxidative stress response were also investigated in the F_3 generation to determine if the biological mechanisms associated with differential resistance among treatments could be identified. Potential biological trade-offs were also investigated through an investigation of reproductive capacity of the F_3 generation and the viability of the F4 eggs and juvenile snails.

3.2 Methods

3.2.1 Snail cultures and experimental organisms

The snails were sourced from an in-house culture maintained at a water temperature of 22 ± 1°C, with a 12/12 day-night light cycle. The original *I. newcombi* were sourced from uncontaminated wild populations at Yanco New South Wales and were supplied by New South Wales Department of Primary Industries (Yanco Agricultural Institute). The snails were maintained in uncontaminated river water sourced from the Cotter River at Vanities Crossing (pH 6.7, conductivity 0.044 ms/cm, turbidity 1.4 NTU, hardness 6.6 mg L^{-1}, salinity 0.02 ppt and TOC 1.46 mg L^{-1}). At all stages during the study aerators were used to maintain oxygen levels in the experimental solutions at ≈ 100% oxygen saturation. The only exception to this was in the small containers used for the individual egg masses and juveniles to six days old, as they were too small for aeration. The snails were fed lettuce leaves washed in the same river water in which they were maintained.

3.2.2 Exposure conditions and experimental design

Prior to the experiment, adult snails were kept in a large plastic aquarium for one week. All snails were then removed from the aquarium. The aquarium was refilled with water and clutches of eggs attached to the side of the tank were allowed to hatch. Once the juveniles were large enough to be handled they were collected and divided into multiple three litre aquariums (PLA-House 1013, Oscar Enterprises, Gardena, CA, USA) at equal densities. After 56 days, 450 individuals were selected and separated into 15 three litre plastic aquariums (PLA-House 1013, Oscar Enterprises, Gardena, CA, USA) containing 2 L of solution and 30 snails per aquarium. Each aquarium was randomly allocated to one of five treatment groups with 3 replicates per treatment. After separation into replicate-specific aquariums, the snails were acclimated to the aquariums under control conditions for 1 week prior to exposure. Snails were exposed just prior to reproductive maturity, as juvenile life stages are typically the most sensitive, and for the purposes of this study the snails were exposed to relatively high Cu concentrations. In addition, exposing snails prior to reproductive maturity ensured that all offspring were from snails that survived the treatments. Prior to the experiment, the time to first reproduction observed in other cultures maintained under experimental conditions was approximately nine weeks. On this basis, the snails were exposed at 60 days immediately prior to reproductive maturity. The experimental design is represented in Figure 3-1. The snails were exposed to treatment Cu concentrations for 3 days. The control snails were kept in Cotter River

water. The treatment groups had nominal concentrations of 25, 50, 75 and 100 µg L^{-1} Cu added. Water samples were taken at the start of the exposure period and analysed to confirm water concentrations. Fifteen grams of lettuce (wet weight) was added to each replicate at the start of the three day exposure period. During the exposure period all aquariums were checked regularly, and dead snails removed to minimise the chance of fouling. Due to high mortality in the 100 µg L^{-1} Cu treatment this treatment was discontinued after the parental generation. After the exposure period the snails were transferred to a clean aquarium containing Cotter River water for 14 days to allow them to recover prior to fecundity and juvenile survival assessment. The exposures of the F_1 and F_2 generations were conducted in the same manner, with the juvenile snails from each treatment grown to 60 days of age and again exposed to treatment-specific Cu concentrations. In the F_3 generation the same design was used, with the exception of the Cu exposure concentrations. All treatments were exposed to 75 µg L^{-1} Cu in the F_3 generation. An F_4 generation was produced to allow an assessment of hatching rates and juvenile survival but no further exposures were conducted.

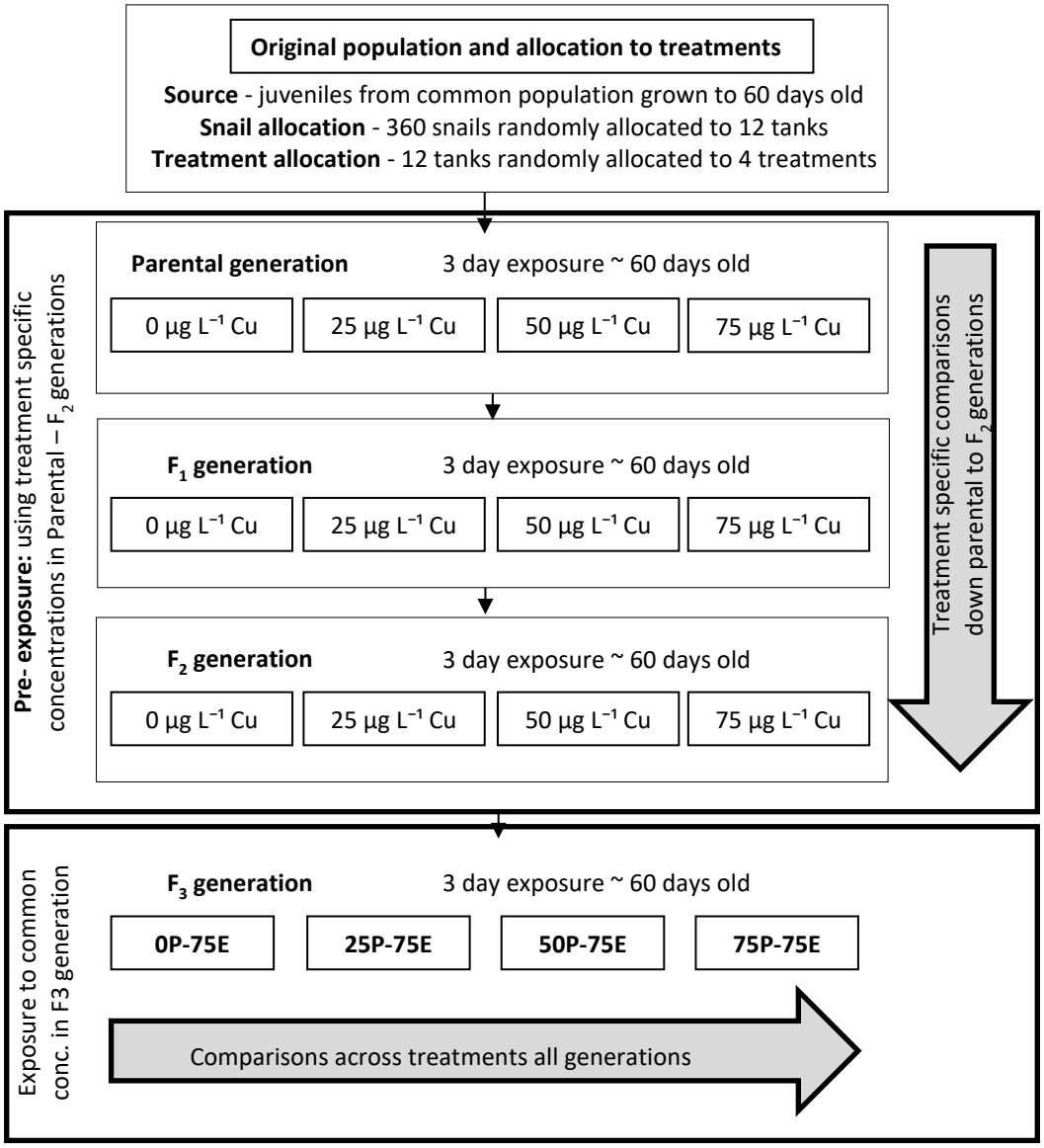


Figure 3-1. Experimental design for the multigenerational exposure of *Isidorella newcombi* to Cu. Treatment specific concentrations are included in the boxes within the figure for the parental, F_1 and F_2 generations. In the F_3 generation all treatments were exposed to 75 µg L^{-1} Cu. In the F_3 generation, P = pre-exposure (µg L^{-1} Cu treatment the snails were exposed to in the parental, F_1 and F_2 generations), and E = exposure, which is the concentration in µg L^{-1} Cu that the snails were exposed to in the F_3 generation.

3.2.3 Food consumption

As the wet mass of lettuce increases when put in water for 72 hours, an average mass for 15 g initial mass of lettuce left in water for 72 hours was determined. Four aquarium tanks were set up identical to the exposure aquariums, but no snails were added. The post-immersion mass of the lettuce from these four aquariums was used as a starting point to calculate lettuce consumed by the snails. Lettuce consumed in each replicate was calculated by subtracting the lettuce

remaining in the replicate at the end of the exposure from the average post-immersion mass of the lettuce from these four aquariums. There was some variability in the data, and if the calculated value for a replicate was negative, zero lettuce consumption was assumed. The lettuce consumption was normalised to lettuce consumption per snail per day to adjust for mortality during the exposure period.

3.2.4 Fecundity

After the exposure, surviving snails were given a 14 day recovery period, prior to being transferred to 770 mL polypropylene containers (Chanrol C30). The organisms from each treatment replicate were divided between two containers to reduce density. The containers were inspected daily, the egg clutches were marked and any adult mortality recorded. Every three days snails were transferred to new containers, the number of eggs in each clutch was counted, and the clutches were transferred to individual polypropylene containers (Chanrol 01PC1). All treatments were maintained under these conditions for 12 days (day15 to day 26 following exposure) for the purposes of recording fecundity data and harvesting clutches for the assessment of offspring viability. While some treatments were maintained under these conditions for a longer period to ensure there were sufficient numbers for the next generation, only data from egg masses laid in the first 12 days were included in fecundity and offspring viability calculations.

3.2.5 Hatching success and juvenile survival

Once in their individual containers, the clutches were monitored every day until they hatched, the number of hatched individuals from each clutch was recorded, and a small piece of lettuce (5 to 10 mm^2) was added. Hatching success was calculated as the number of hatched snails divided by the total number of eggs in the clutch. Six days after hatching the number of juvenile snails surviving in each container was counted. Juvenile survival to 6 days was calculated as the number of snails alive in the containers at 6 days divided by the total number of hatched snails. After they were counted at six days of age, all juvenile snails were then transferred to a treatment replicate specific tank for ~ 54 days prior to the next Cu exposure.

3.2.6 Exposures for biomarkers and genomics

In the F_1, F_2 and F_3 generations, excess snails that were additional to requirements for breeding stock were used for biomarker and genomic exposures. These snails were exposed to the same concentrations and under the same conditions as the experimental treatments described in section 3.2.2, the only difference being that the snails were exposed at ~ 100 days old rather

than at 60 days of age. This was to ensure that there were sufficient numbers in each replicate for the next generation.

3.2.7 Metal analysis

Copper concentrations were measured in the experimental media and the tissues of the F_3 snails. The methods used for metal analysis are described in Chapter 2 (section 2.2.5).

3.2.8 Total antioxidant capacity, lipid peroxidation and lysosomal membrane destabilisation

In the F_3 generation total antioxidant capacity (TAOC), lipid peroxidation (LP) and lysosomal membrane destabilisation (LD) were assessed. The methods used for these analyses are described in Chapter 2 (sections 2.2.6, 2.2.7, 2.2.8 and 2.2.9).

3.2.9 Data analysis

Initial data preparation was completed in Microsoft Excel 2010. All data analysis was completed using R 3.1.2 (R Core Team 2014). If assumptions of normality and homoscedasticity were met, single factor ANOVA with Tukey HSD post hoc analysis was used to compare among treatments. If data did not meet assumptions, a Kruskal-Wallis test was used with a Dunns test used for multiple pairwise comparisons (Dinno 2016).

3.3 Results

Within both the results and discussion sections the parental, F_1 and F_2 generations will be presented separately prior to F_3 generation results and discussion. This is because the parental to F_2 generations used treatment specific exposure concentrations in order to establish different pre-exposure histories across the treatments. Conversely, all treatments were exposed to a common concentration in the F_3 generation as a means of testing the effect of the different pre-exposure histories on the response of the snails to Cu (see experimental design Figure 3-1). The measured Cu concentrations in the control, 25, 50 and 75 µg L^{-1} Cu treatments were 4±1, 28 ± 1, 53 ± 2 and 80 ± 1 µg L^{-1} Cu respectively.

3.3.1 Parental to F_2 generations

Note that while this section focusses on the parental to F_2 generations some analyses for the 75 µg L^{-1} Cu treatment includes data from the F_3 generation. As the exposure concentrations remained consistent from parental to F_3 generation for the 75 µg L^{-1} Cu treatment, where appropriate for comparisons across generations within the 75 µg L^{-1} Cu treatment the F_3 data were included.

3.3.1.1 Food consumption

In the parental and F_1 generations, there was no significant difference in food consumption among treatments (parental $\chi^2 = 6.44$, *d.f.* = 3, $p = 0.09$; F_1 $\chi^2 = 7.51$, *d.f.* = 3, $p = 0.06$). In the F_2 generation, there was a significant difference in the food consumed by *I. newcombi* exposed to different Cu concentrations ($\chi^2 = 10.65$, *d.f.* = 3, $p = 0.01$). There was a strong negative correlation between the concentration of Cu *I. newcombi* were exposed to and the amount of food consumed in the parental ($r_s = -0.67$, $n = 12$, $p < 0.05$), F_1 ($r_s = -0.80$, $n = 12$, $p < 0.01$) and F_2 ($r_s = -0.93$, $n = 12$, $p < 0.001$) generations (Figure 3-2).

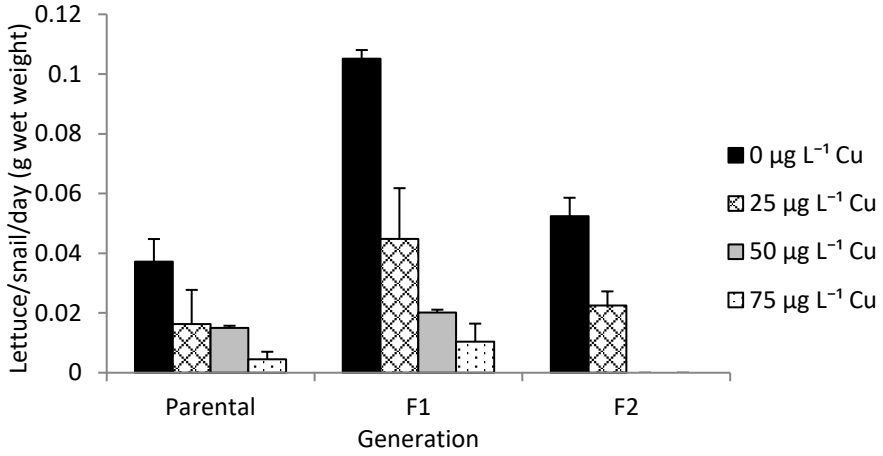


Figure 3-2. Lettuce consumed by *Isidorella newcombi* exposed to Cu for 3 days. Data are means and one standard error (n=3).

3.3.1.2 Adult survival

Only 6 of the snails from the 100 µg L^{-1} Cu treatment survived the parental exposure so this treatment was discontinued and is not included in further analysis.

Comparison between treatments within each generation

There was significant difference in the survival of *I. newcombi* exposed to Cu for three days in the parental generation (parental, $F = 16.46$, *d.f.* = 3,8, $p < 0.001$). In the parental generation the 75 µg L^{-1} treatment had significantly lower survival rates than all other treatments ($p < 0.05$). In the F_1 and F_2 generations there was no significant differences in survival among the treatments (F_1, $\chi^2 = 4.91$, *d.f.* = 3, $p = 0.18$; F_2, $\chi^2 = 7.03$, *d.f.* = 3, $p = 0.07$). Despite the differences not being statistically significant, there was a trend of reduced survival in the 75 µg L^{-1} treatment compared to other treatments (**Error! Reference source not found.**).

Comparison between generations within each treatment

There were no significant differences in adult survival among the generations for the 0, 25 and 50 µg L^{-1} Cu treatments, but there was a significant difference in adult survival among generations in the 75 µg L^{-1} Cu treatment (Table 3-1). In the 75 µg L^{-1} Cu treatment the survival in the parental generation was significantly lower in the parental generation than in the F_3 generation.

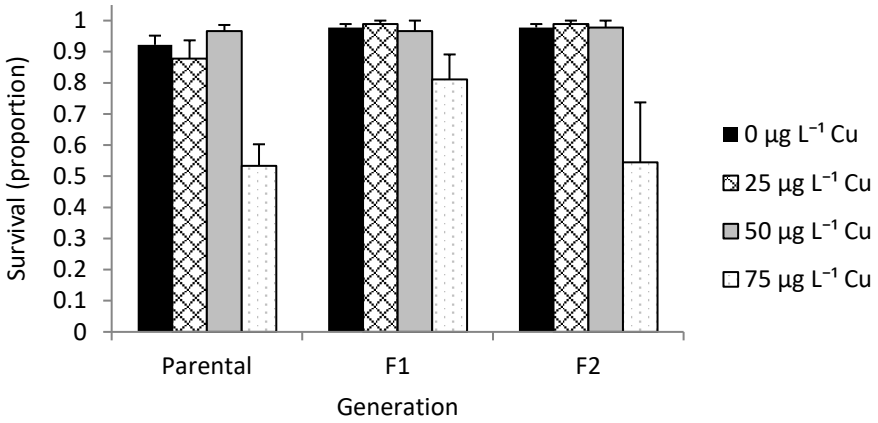


Figure 3-3. The proportion of *Isidorella newcombi* surviving when exposed to Cu for 72 hours. Data are means and standard errors (n = 3).

Table 3-1. Results from analysis of survival data comparing survival in each generation of *Isidorella newcombi* exposed to specific Cu concentrations over multiple generations. Results for 0, 25 and 50 µg L^{-1} Cu treatments are Kruskal-Wallis tests and compare three generations (parental, F_1, and F_2). Results for 75 µg L^{-1} Cu is single factor ANOVA and compare four generations (parental, F_1, F_2 and F_3).

Treatment (µg L^{-1} Cu)	F or χ^2	*d.f.*	*p*
0	3.96	2	0.14
25	4.83	2	0.09
50	0.24	2	0.89
75	5.17	3, 8	0.03

3.3.1.3 Fecundity

Comparison among treatments within each generation

There were no significant differences in the number of clutches or eggs laid by *I. newcombi* during days 15 to 26 after exposure to 0 – 75 µg L^{-1} Cu in any of the generations (Table 3-2).

There was a lot of variability in the clutches and number of eggs laid per adult among the treatments in the parental, F_1 and F_2 generations (Figure 3-4). There was also no regular concentration-dependent trend of increasing or decreasing clutch or egg production per adult in the parental, F_1 and F_2 generations.

Table 3-2. Comparison of differences in the number of clutches laid per adult and the number of eggs laid per adult *Isidorella newcombi* using a Kruskal-Wallis test. Clutches and eggs were counted on day 15 to 26 after snails were exposed to 0 – 75 µg L^{-1} Cu for 72 hours in the parental, F_1 and F_2 generations.

Generation	Variable	χ^2	*d.f.*	*p*
Parental	Clutches	4.54	3	0.21
F_1	Clutches	2.74	3	0.43
F_2	Clutches	0.86	3	0.84
Parental	Eggs	5.21	3	0.16
F_1	Eggs	6.08	3	0.11
F_2	Eggs	0.74	3	0.86

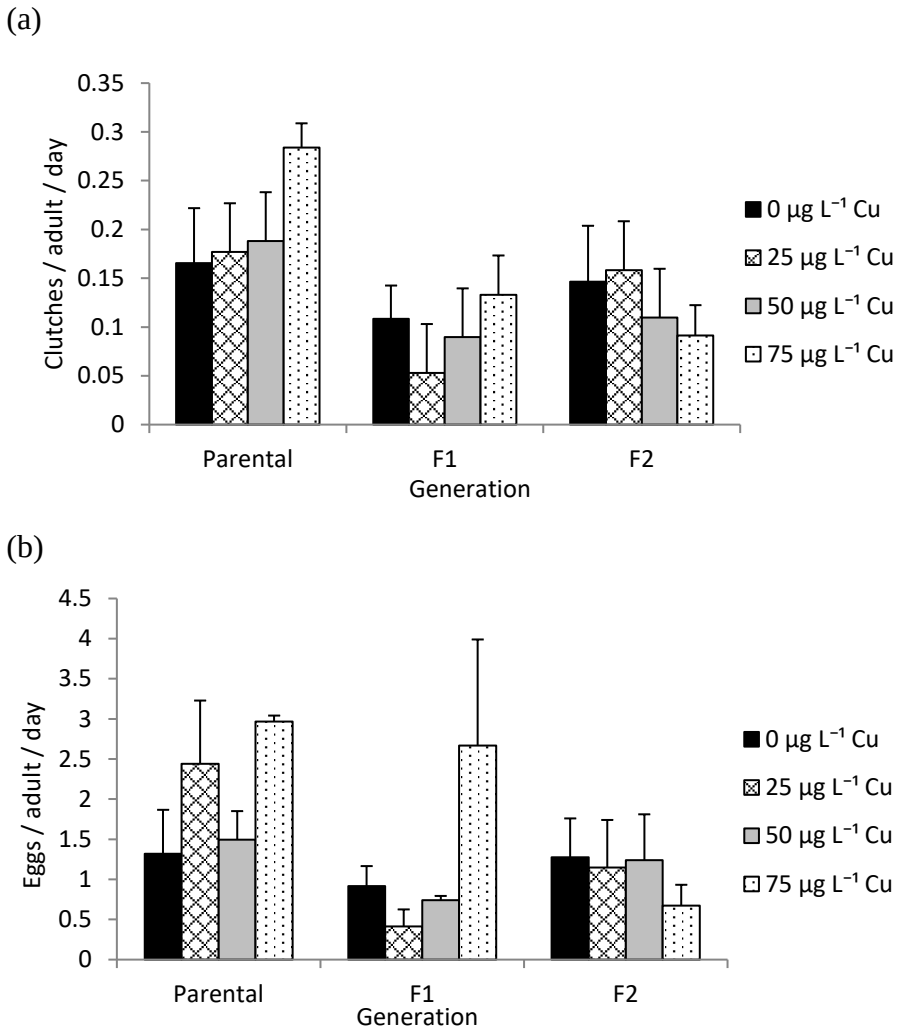


Figure 3-4. The number of clutches (a) and eggs (b) laid by *Isidorella newcombi* on days 15 to 26 days after exposure to 0 to 75 µg L^{-1} Cu for 72 hours in the parental, F_1 and F_2 generations. Data are means and standard error (n = 3).

Comparison among generations within each treatment

There were no significant differences in the number of clutches laid among generations for the 0, 25 and 50 µg L^{-1} Cu treatments, but there was a significant difference in the number of clutches laid among generations in the 75 µg L^{-1} Cu treatment (Table 3-3). In the 75 µg L^{-1} Cu treatment significantly more eggs were laid in the parental generation than in the F_2 generation. In the 75 µg L^{-1} Cu treatment there was a trend of a reduced number of clutches being laid after the parental generation, even though this was not statistically significant in the F_1 and F_3 generations (α = 0.05). Thus, in this treatment while not all generations laid statistically significantly fewer clutches of eggs then the parental generation, there was a general trend of

a reduction in clutch production in all subsequent generations (Figure 3-4a). Despite the differences in the number of clutches there were no significant differences in the number of eggs laid among generations in any of the treatments (Table 3-3).

Table 3-3. Values for single factor ANOVA test used to compare differences in the number of clutches laid per adult and the number of eggs laid per adult in different generations of specific treatments for *Isidorella newcombi* exposed to Cu. Clutches and eggs were counted on days 15 to 26 after exposure to 0 – 75 µg L^{-1} Cu for 72 hours.

Treatment	Variable	*F*	*d.f.*	*p*
0	Clutches	0.33	2, 6	0.73
25	Clutches	1.99	2, 6	0.22
50	Clutches	3.28	2, 6	0.11
75	Clutches	5.291	3, 8	0.03
0	Eggs	0.244	2, 6	0.79
25	Eggs	3.117	2, 6	0.12
50	Eggs	0.971	2, 6	0.43
75	Eggs	2.42	3, 8	0.14

3.3.1.4 Hatching success

Comparison among treatments within each generation

There were no significant differences in the hatching success of *I. newcombi* juveniles among treatments in any of the F_1, F_2 or F_3 generations eggs laid by parents exposed to 0 – 75 µg L^{-1} Cu (Table 3-4, Figure 3-5).

Table 3-4. Comparison of differences in the hatching success of juveniles from eggs laid by *Isidorella newcombi* exposed to Cu using a Kruskal-Wallis test. Parents were exposed to 0, 25, 50 or 75 µg L^{-1} Cu for 72 hours, left to recover for 14 days and eggs were collected during the following 12 days.

Generation	Variable	X^2	*d.f.*	*p*
F_1	Hatching success	6.69	3	0.08
F_2	Hatching success	0.54	3	0.91
F_3	Hatching success	3.47	3	0.32

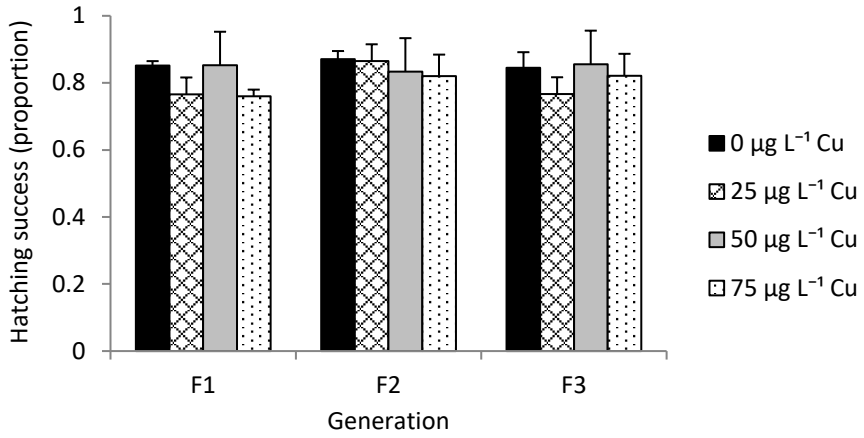


Figure 3-5. Hatching success of *Isidorella newcombi* juveniles from eggs laid 15 to 26 days after parents were exposed to 0 to 75 µg L^{-1} Cu for 72 hours in the F_1, F_2 and F_3 generations. Data are means and standard error (n = 3).

Comparisons among generations within each treatment

There were no significant differences in the hatching success of juveniles from eggs laid by *I. newcombi* among generations for any of the treatments (Table 3-4, Figure 3-5).

Table 3-5. Differences in juvenile hatching success across multiple generations from eggs laid by *Isidorella newcombi* exposed to 0 to 75 µg L^{-1} Cu using a single factor ANOVA.

Treatment	Variable	*F*	*d.f.*	*p*
0	Hatching success	0.18	2, 6	0.84
25	Hatching success	4.95	2, 6	0.054
50	Hatching success	0.11	2, 6	0.89
75	Hatching success	0.54	3, 8	0.54

3.3.1.5 Juvenile survival

Comparison among treatments within each generation

In the F_1 generation, there was significant difference in the juvenile survival of *I. newcombi* (Table 3-6). *post hoc* analysis indicated that there were no significant differences between any of the treatment pairs (α = 0.05). The pairwise comparisons between the 75 µg L^{-1} treatment and the 0 and 50 µg L^{-1} treatments were closest to being significantly different with p = 0.1 in both cases. There were no significant differences among treatments in the juvenile survival rate for the F_2 and F_3 generations (Table 3-6, Figure 3-6).

Table 3-6. Comparison of differences in the juvenile survival of *Isidorella newcombi* until 6 days of age using a Kruskal-Wallis test. Parents were exposed to 0 – 75 µg L^{-1} Cu for 72 hours in the Parental, F_1 and F_2 generation 15 days prior to the 12 day egg collection period.

Generation	Variable	X^2	*d.f.*	*p*
F_1	Juvenile survival	7.82	3	0.049
F_2	Juvenile survival	3.62	3	0.31
F_3	Juvenile survival	1.56	3	0.67

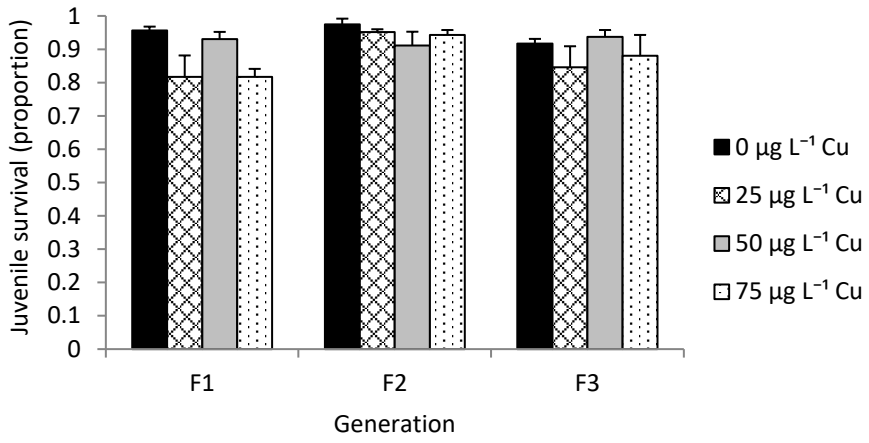


Figure 3-6. Juvenile survival of *Isidorella newcombi* hatched from eggs laid on days 15 to 26 after exposure to 0 to 75 µg L^{-1} Cu for 72 hours in the parental, F_1 and F_2 generations. Data are means and standard error (n = 3).

Comparisons among generations within each treatment

There were no significant differences in the juvenile survival of *I. newcombi* among generations within specific treatments (Table 3-7, Figure 3-6).

Table 3-7. Differences in juvenile survival across multiple generations in juveniles from *Isidorella newcombi* exposed to 0 to 75 µg L^{-1} Cu using single factor ANOVA.

Treatment	Variable	*F*	*d.f.*	*p*
0	Juvenile survival	4.03	2, 6	0.08
25	Juvenile survival	1.84	2, 6	0.24
50	Juvenile survival	0.22	2,6	0.81
75	Juvenile survival	3.75	3, 8	0.06

3.3.2 Section 2 – F_3 generation

3.3.2.1 Survival (72 hour exposure)

In the F_3 generation when all snails were exposed to 75 µg L^{-1} Cu there was a significant difference in survival among the treatments based on their pre-exposure history (χ^2 = 9.43, *d.f.* = 3, p = 0.02). The 0P-75E had significantly lower survival than the 75P-75E. There was a trend of increased survival with increased pre-exposure concentration history (Figure 3-7). A Spearman's correlation confirmed that there was a strong correlation between F_3 survival rates and pre-exposure concentration histories (r_s = 0.93, n = 12, p < 0.001).

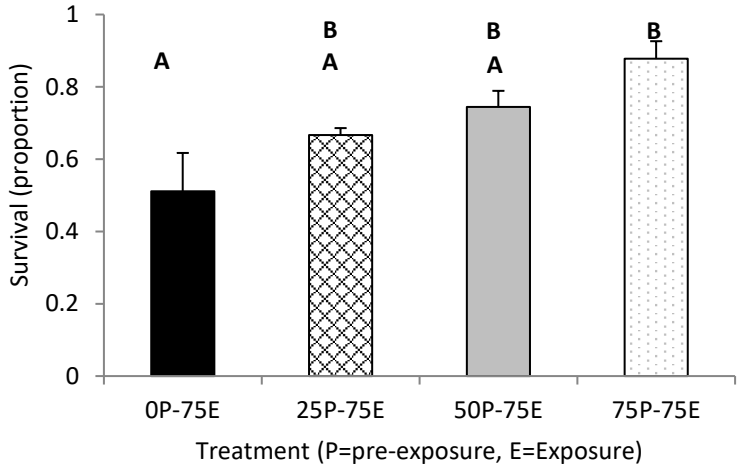


Figure 3-7. Proportion of *Isidorella newcombi* with differing pre-exposure concentration histories surviving when exposed to 75 µg L^{-1} Cu for 72 hours in the F_3 generation. The labels on the x-axis indicate pre-exposure history and experimental exposure concentrations (P = pre-exposure, indicating the Cu concentration that the snails were exposed to in the parental, F_1 and F_2 generations, E = the Cu concentration that the snails were exposed to in the F_3 generation. Letters indicate homogenous subsets). Data are means and standard errors (n = 3).

3.3.2.2 Survival: two weeks post-exposure

There was no difference in the survival rates of the snails in the two weeks after exposure to 75 µg L^{-1} Cu for 72 hours in the F_3 generation based on pre-exposure history (F = 2.25, *d.f.* = 3,8, p = 0.16). Despite no significant difference being detected among treatments, there is a trend of increasing survival in the treatments with a higher pre-exposure history (Figure 3-8). A Spearman's correlation confirmed that there was a strong correlation between F_3 post exposure survival rates and pre-exposure concentrations (r_s = 0.73, n = 12, p < 0.01).

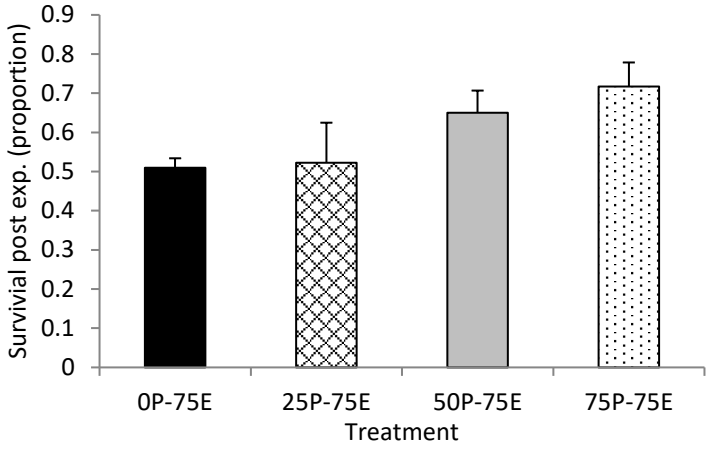


Figure 3-8. Proportion of *Isidorella newcombi* surviving for two weeks following exposure to 75 µg L^{-1} Cu for 72 hours in the F_3 generation. The labels on the x-axis indicate pre-exposure history and experimental exposure concentrations (P = pre-exposure, indicating the Cu concentration that the snails were exposed to in the parental, F_1 and F_2 generations, E = the Cu concentration that the snails were exposed to in the F_3 generation). Data are means and standard errors (n = 3).

3.3.2.3 Food consumption

There was no significant difference in the food consumption of *I. newcombi* exposed to different Cu concentrations in the F_3 generation (χ^2 = 7.31, *d.f.* = 3, p = 0.06) (Figure 3-9).

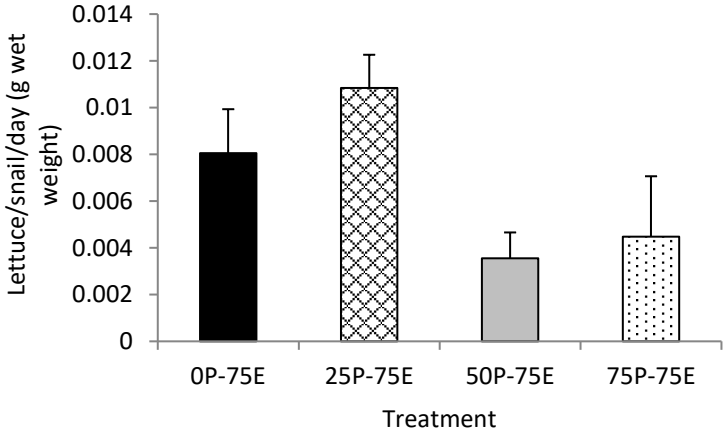


Figure 3-9. Lettuce consumed by *Isidorella newcombi* exposed to 75 µg L^{-1} Cu for 72 hours in the F_3 generation. The labels on the x-axis indicate pre-exposure history and experimental exposure concentrations (P = pre-exposure, indicating the Cu concentration that the snails were exposed to in the parental, F_1 and F_2 generations, E = the Cu concentration that the snails were exposed to in the F_3 generation). Data are means and standard errors (n = 3).

3.3.2.4 Fecundity

In the F_3 generation, there were no significant differences in fecundity among treatments as assessed by the number of clutches (χ^2 = 0.23, *d.f.* = 3, p = 0.97) or eggs (χ^2 = 0.03, *d.f.* = 3, p = 0.99; Figure 3-10). As there were insufficient snails to run a control (0P-0E) in the F_3 generation the number of clutches and number of eggs per snail per day from each treatment

in the F_3 generation was compared to the controls from each of the parental, F_1 and F_2 generations as a means of comparison against normal reproductive output. There were no significant differences in the number of clutches or eggs laid per adult per day in any of the treatments in the F_3 generation and the control snails from the parental, F_1 and F_2 generations (clutches, $\chi^2 = 1.0043$, *d.f.* = 6, $p = 0.99$; eggs $\chi^2 = 0.329$, *d.f.* = 6, $p = 0.99$).

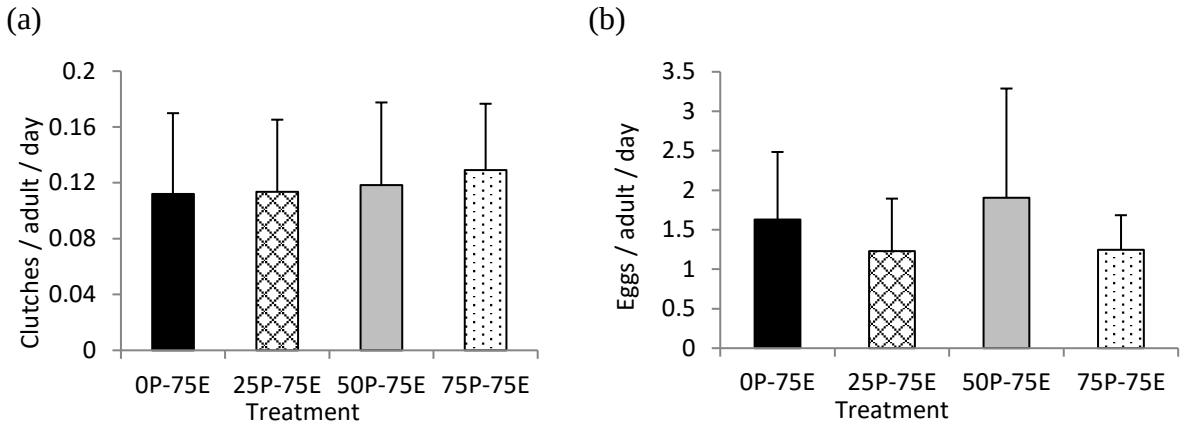


Figure 3-10. The number of clutches (a) and eggs (b) laid by *Isidorella newcombi* 15 to 26 days after being exposed to 75 µg L^{-1} Cu for 72 hours in the F_3 generation. The labels on the x-axis indicate pre-exposure and exposure concentrations (P = pre-exposure, indicating the Cu concentration that the snails were exposed to in the parental, F_1 and F_2 generations, E = the Cu concentration that the snails were exposed to in the F_3 generation). Data are means and standard errors ($n = 3$).

3.3.2.5 Hatching success

In the F_4 generation, there were no significant differences in juvenile hatching success among treatments ($\chi^2 = 5.47$, *d.f.* = 3, $p = 0.14$; Figure 3-11). As no F_4 control data was available due to insufficient snails, the F_4 generation was compared against the controls for each of the F_1, F_2 and F_3 generation. There was no significant difference in the hatching success of juveniles in any of the treatments in the F_4 generation and the controls from any of the F_1, F_2 and F_3 generations ($\chi^2 = 7.08$, *d.f.* = 6, $p = 0.31$).

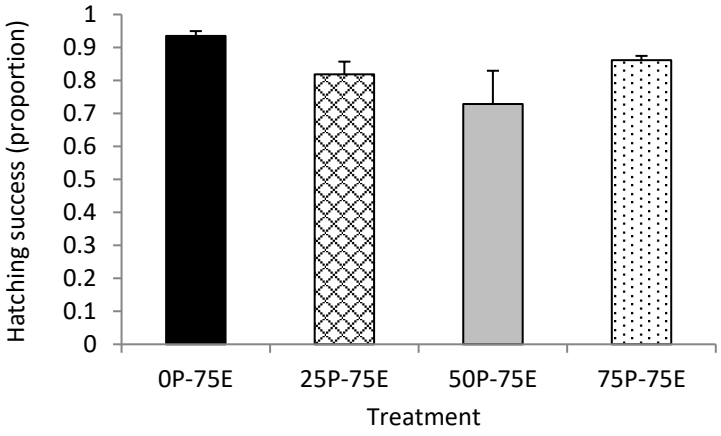


Figure 3-11. Hatching success of F_4 *Isidorella newcombi* eggs laid on days 15 to 26 after exposure to 75 µg L^{-1} Cu for 72 hours in the F_3 generation. The labels on the x-axis indicate pre-exposure and exposure concentrations (P = pre-exposure, indicating the concentration µg L^{-1} that the snails were exposed to in the parental, F_1 and F_2 generations, E = exposure, indicating the concentration the snails were exposed to in the F_3 generation). Data are means and standard error (n = 3).

3.3.2.6 Juvenile survival

In the F_4 generation, there were no significant differences in juvenile survival among treatments (χ^2 = 2.50, *d.f.* = 3, p = 0.48; Figure 3-12). As no F_4 control data is available due to insufficient snails, the F4 juvenile survival was compared against the controls for the F_1, F_2 and F_3 generation. There was no significant difference in the proportion of juvenile survival in any of the treatments in the F_4 generation and the controls from any of the F_1, F_2 and F_3 generations (χ^2 = 6.90, *d.f.* = 6, p = 0.33).

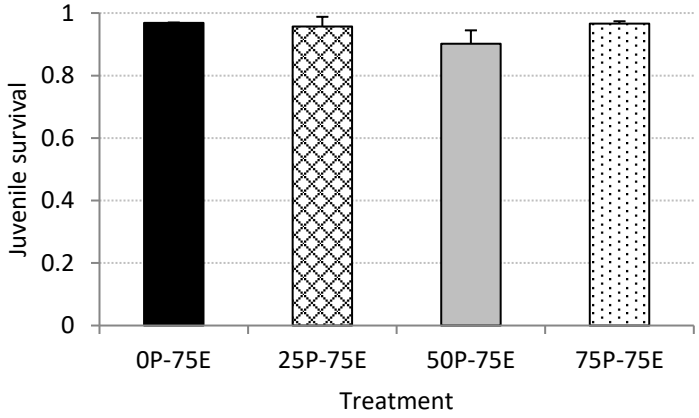


Figure 3-12. Juvenile survival of F_4 *Isidorella newcombi* hatched from eggs laid on days 15 to 26 after parents had been exposed to 75 µg L^{-1} Cu for 72 hours in the F_3 generation. The labels on the x-axis indicate pre-exposure and exposure concentrations (P = pre-exposure, indicating the Cu concentration that the snails were exposed to in the parental, F_1 and F_2 generations, E = exposure, indicating the Cu concentration That the snails were exposed to in the F_3 generation). Data are means and standard error (n = 3).

3.3.2.7 Bioaccumulation of copper

When all treatments were exposed to 75 µg L^{-1} Cu for 72 hours in the F_3 generation there was a significant difference in whole tissue Cu concentrations among the treatments (F = 10.83, $d.f.$ = 3, 26, $p < 0.01$; Figure 3-13). The only treatments that were significantly different from each other in pairwise comparisons were 0P-75E and 25P-75E with the treatment with no pre-exposure history to elevated Cu concentrations accumulating higher concentrations of Cu (Figure 3-13). While they were not significantly different from each other, in the treatments that had pre-exposure to Cu there was a consistent increase in the concentration of Cu accumulated with increasing Cu pre-exposure concentration histories.

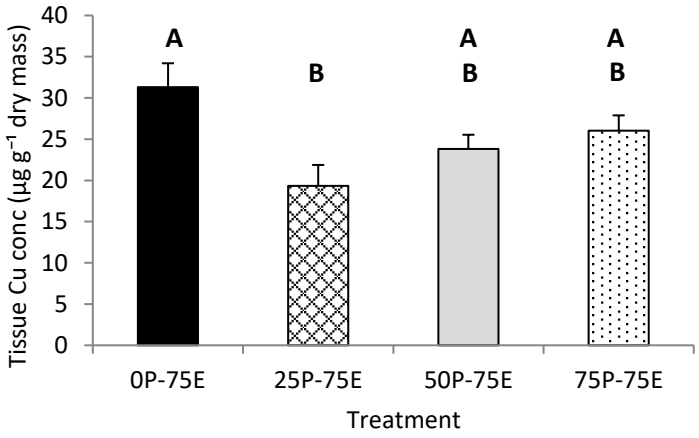


Figure 3-13. Cu tissue concentrations in *Isidorella newcombi* exposed to 75 µg L^{-1} Cu for 72 hours in the F_3 generation. The labels on the x-axis indicate pre-exposure history and experimental exposure concentrations (P = pre-exposure, indicating the Cu concentration that the snails were exposed to in the parental, F_1 and F_2 generations, E = the Cu concentration that the snails were exposed to in the F_3 generation. Letters on graphs indicate homogeneous subsets). Data are means and standard errors (n = 9).

3.3.2.8 Total antioxidant capacity

When all treatments were exposed to 75 µg L^{-1} Cu for 72 hours in the F_3 generation there was a significant difference in TAOC among the treatments (χ^2 = 10.83, $d.f.$ = 3, p = 0.01). The treatments with lower pre-exposure history generally had lower TAOC than those that had been exposed to higher Cu concentrations in the parental, F_1 and F_2 generations (Figure 3-14a).

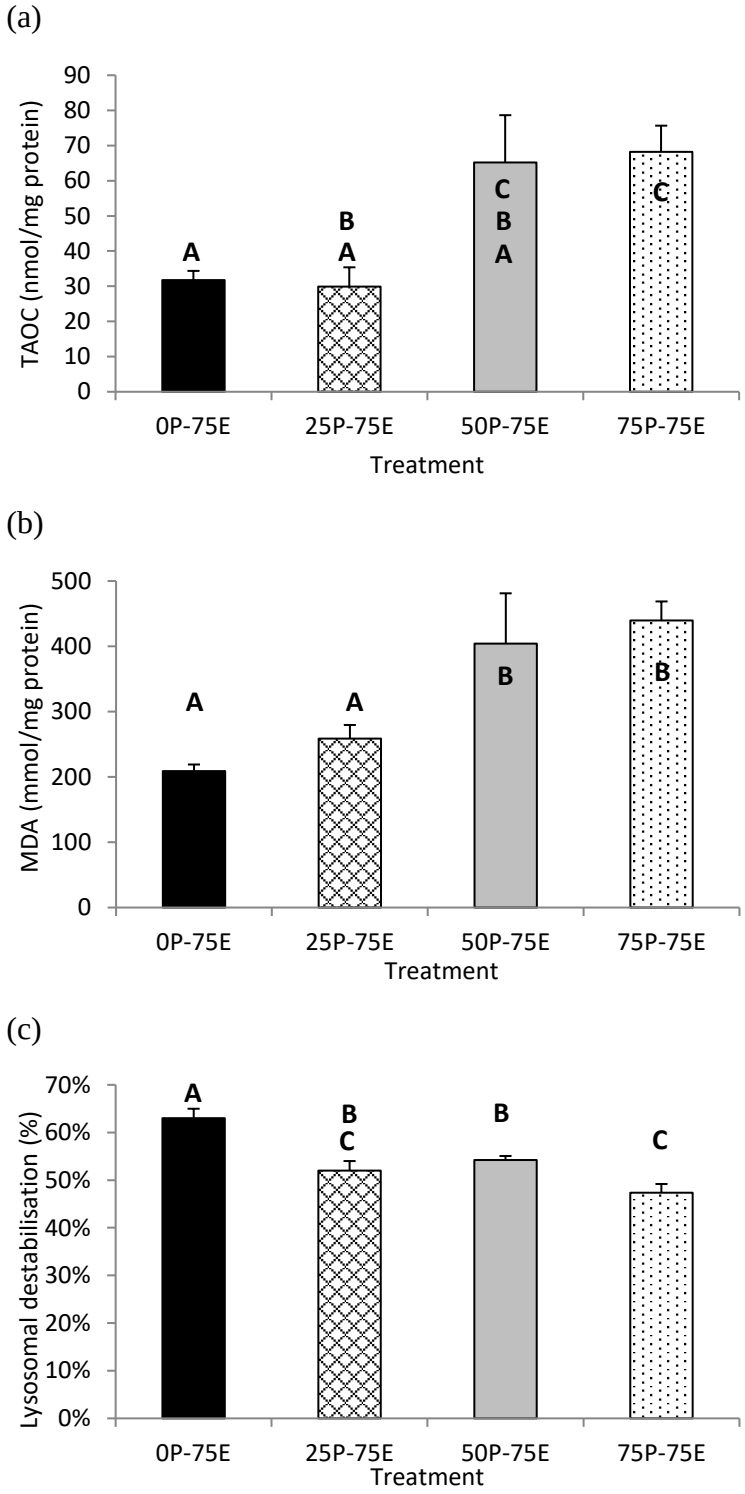


Figure 3-14. Biomarker responses, (a) total antioxidant capacity, (b) lipid peroxidation and (c) Lysosomal membrane destabilisation in *Isidorella newcombi* exposed to 75 µg L^{-1} Cu for 72 hours in the F_3 generation. The labels on the x-axis indicate pre-exposure history and experimental exposure concentrations (P = pre-exposure, indicating the cu concentration that the snails were exposed to in the parental, F_1 and F_2 generations, E = the Cu concentration that the snails were exposed to in the F_3 generation. Letters on graphs indicate homogeneous subsets). Data are means and standard errors (*n* = 9).

3.3.2.9 Lipid peroxidation

When all treatments were exposed to 75 µg L^{-1} Cu for 72 hours in the F_3 generation there was a significant difference in malondialdehyde (MDA) among the treatments ($\chi^2 = 16.71$, *d.f.* = 3, $p < 0.001$). As with the TAOC, the treatments with lower Cu pre-exposure history generally had a lower TAOC than those that had been exposed to higher Cu concentrations in the parental, F_1 and F_2 generations (Figure 3-14b).

3.3.2.10 Lysosomal membrane destabilisation

In the F_3 generation where all treatments were exposed to 75 µg L^{-1} Cu for 72 hours there was a significant difference in LD among the treatments ($F = 14.7$, *d.f.* = 3, 26, $p < 0.001$). The organisms that had no history of pre-exposure to Cu had the highest levels of LD, while the organisms that had been exposed to 75 µg L^{-1} in each generation of the study had the lowest levels of LD (Figure 3-14c). The organisms that had been pre-exposed to 25 and 50 µg L^{-1} Cu did not follow the pre-exposure concentration-dependent decrease in LD in the F_3 generation, however, the levels of LD in these treatments were very similar and there was no significant difference among these treatments.

3.3.2.11 Relationships between biomarkers

3.3.2.11.1 Total antioxidant capacity and lipid peroxidation

A Pearson product moment correlation coefficient was computed to assess the relationship between TAOC and malondialdehyde (MDA) in the F_3 generation *I. newcombi* from all treatments. There was a strong positive correlation between the two variables ($r = 0.87$, $n = 27$, $p < 0.001$). A scatterplot summarizes the results (Figure 3-15a).

3.3.2.11.2 Total antioxidant capacity and lysosomal membrane destabilisation

A Spearman's rank-order correlation was run to determine the relationship between mean TAOC and mean LD in *I. newcombi* from the individual replicates in the F_3 generation. There was no relationship between the variables ($r_s = 250$, $p = 0.13$). A scatterplot summarizes the results (Figure 3-15b).

3.3.2.11.3 Lipid peroxidation and lysosomal membrane destabilisation

A Spearman's rank-order correlation was run to determine the relationship between mean MDA and mean LD in *I. newcombi* from the individual replicates in the F_3 generation. There was no relationship between the variables ($r_s = 261$, $p = 0.07$). A scatterplot summarizes the results (Figure 3-15c).

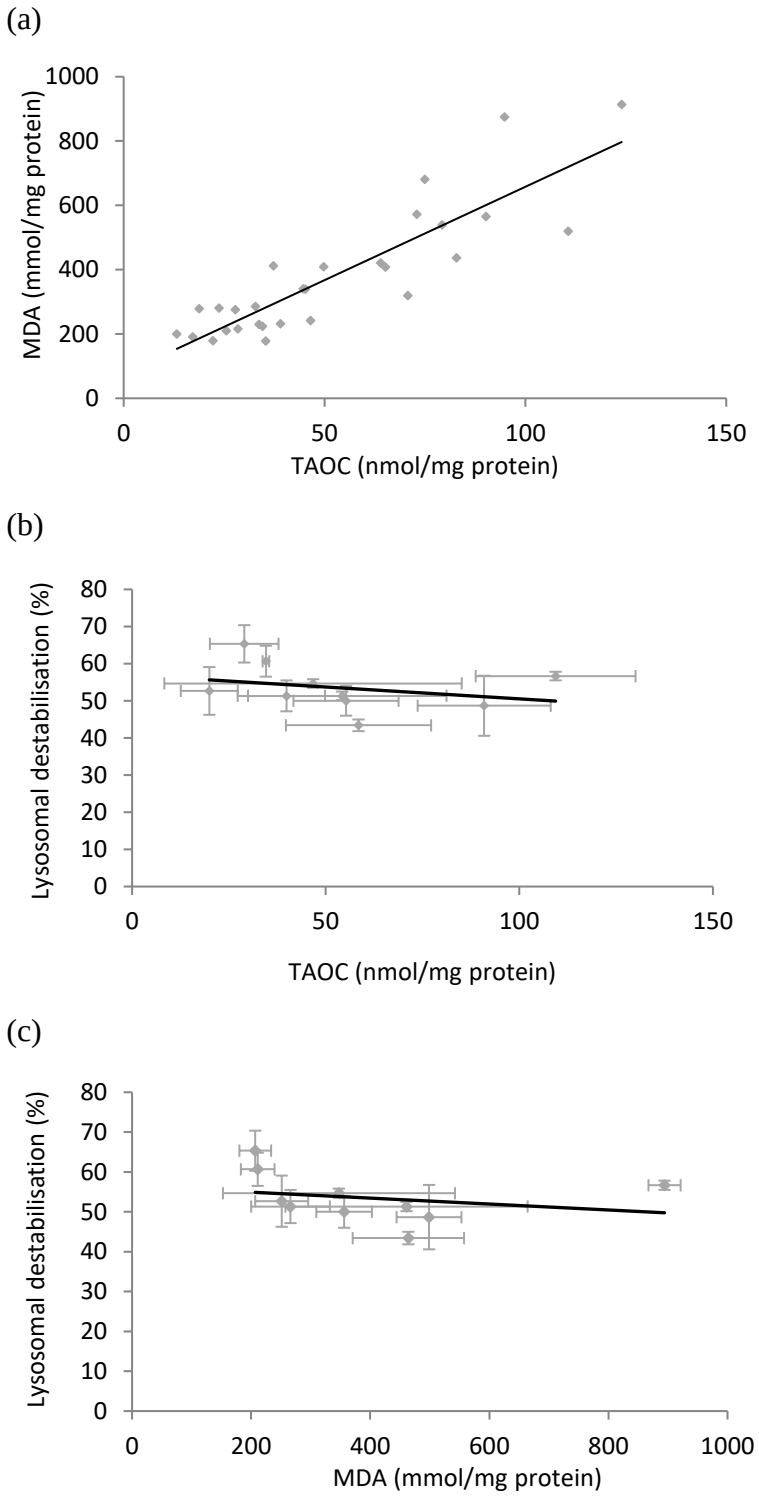


Figure 3-15. Relationships between biomarkers. (a) Total antioxidant capacity (TAOC) and malondialdhyde (MDA), (b) TAOC and Lysosomal membrane destabilisation (LD) and (c) MDA and LD. Data points in part (a) are individual organisms, data points in in part (b and (c) are means for replicates.

3.3.2.11.4 Relationship between cellular biomarkers and individual effects

Adult survival was positively correlated to TAOC and MDA but negatively correlated to LD (Table 3-8). Feeding rate was negatively correlated to TAOC (Table 3-8). All other relationships between cellular biomarkers and individual level effects were not significant.

Table 3-8. Correlation analysis (Spearman's) between cellular biomarkers (total antioxidant capacity (TAOC), malondialdehyde (MDA) and lysosomal membrane destabilisation (LD) and individual effects (adult survival, mean number of clutches per snail, mean number of eggs per snail, proportion of eggs hatched (egg hatching) and juvenile survival) in the F_3 generation of *Isidorella newcombi* sub-populations with differing Cu exposure histories, exposed to 75 µg L^{-1} Cu for 72 hours. Data used are for means individual replicates.

Variables		*p*	*rho*
TAOC	Adult survival	0.014	0.74
TAOC	Mean clutches	0.23	NA
TAOC	Mean Eggs	0.086	NA
TAOC	Egg hatching	0.44	NA
TAOC	Juvenile survival	0.64	NA
MDA	Adult survival	0.015	0.74
MDA	Mean clutches	0.18	NA
MDA	Mean Eggs	0.067	NA
MDA	Egg hatching	0.23	NA
MDA	Juvenile survival	0.61	NA
LD	Adult survival	0.005	-0.80
LD	Mean clutches	0.98	NA
LD	Mean Eggs	0.57	NA
LD	Egg hatching	0.88	NA
LD	Juvenile survival	0.47	NA

3.4 Discussion

The experimental design for many multi-generational studies involves the exposure of organism to a constant concentration of a contaminant over the entire life cycle (e.g. Bal *et al.* 2017). While this may be realistic for some contaminants that are released continually, it does not reflect the way Cu is often released into the environment. Cu introduced to aquatic systems is known to adsorb to organic matter and sediments with bioavailable Cu quickly returning to background concentrations (Stevens *et al.* 2014). As a result, in many cases where Cu contamination occurs, bioavailable concentrations are likely to be elevated for a short time and then a return to background levels. Here we address a knowledge gap associated with the intergenerational effect of pulse exposures over multiple generations. The exposure conditions used in this study were designed to mimic a pulse event rather than exposure to a constantly elevated concentration. In this study, a 3 day pulse exposure was used when *I. newcombi* were approximately 60 days of age. In other studies that have exposed freshwater snails to Cu over at least a generation, exposures to constant Cu concentrations were used (Das and Khangarot 2011; Peña and Pocsidio 2007; Rogevich *et al.* 2008). In other studies with other taxa, pulse exposures have been used within a single generation ((Chen *et al.* 2012; Diamond *et al.* 2005; Hoang *et al.* 2007) While pulse exposures to Cu have been used in the past on other taxa, the current study is the only on to date that uses a pulse exposure to investigate the response of freshwater snails to Cu over multiple generations. It is also unique in using a single relatively short exposure within each generation over multiple generations to investigate effects.

3.4.1 Parental to F2 generations

3.4.1.1 Adult Survival

The 100 µg L^{-1} Cu treatment was discontinued after the parental generation exposure as a total of 6 individuals from the combined replicates survived the exposure period. This is similar to the lethal concentration calculated for the freshwater snail *Melanoides tuberculata,* where Cu was found to be the most toxic metal when the snail was exposed to a range of metals including Cu, Cd, Zn, Pb, Ni, Fe, Al and Mn, with a 96-hour LC_{50} concentration of 140 µg L^{-1} Cu (Shuhaimi-Othman *et al.* 2012).

In the remainder of the treatments exposed to concentrations of up to 75 µg L^{-1} Cu, more than 50% of the snails survived in each treatment in each generation (**Error! Reference source not found.**). The lethal concentrations in this study generally agreed with those from other studies. Reported values for LC_{50} Cu concentrations for freshwater snails range from 13 to 79 µg L^{-1}

Cu (Besser *et al.* 2016; Brix *et al.* 2011; Das and Khangarot 2011; Ng *et al.* 2011; Watton and Hawkes 1984). There is some variability in the LC_{50} values reported in these studies which are likely to be associated with differing interspecific sensitivity, life stages of animals tested, water chemistry of media, temperature and exposure period. Watton and Hawkes (1984) calculated a 96 hr LC_{50} value of 77 µg L^{-1} Cu for adult *Potamopyrgus jenkinsi*. While LC_{50} concentrations were not calculated in the current study, in each of the generations more than 50 percent of the snails exposed to 75 µg L^{-1} Cu survived and only 7 percent of snails exposed to 100 µg L^{-1} Cu in the parental generation survived. This indicates that the LC_{50} would lie between 75 and 100 µg L^{-1} Cu, which is similar to the value calculated by Watton and Hawkes (1984) who also used adult snails and had a similar exposure period to the current study.

In the parental generation, *I. newcombi* exposed to 75 µg L^{-1} Cu had significantly lower survival then the other treatments. In the subsequent F_1 and F_2 generations the 75 µg L^{-1} Cu treatment had lower survival than the other treatments but these were not significantly different from the other treatments. A comparison of survival among the four generations exposed to 75 µg L^{-1} Cu, showed the F_3 generation had a significantly higher survival rate than the parental generation. The increased survival at the same concentration in a later generation indicates that at this exposure concentration the snails developed resistance over the experimental period. *Daphnia magna* exposed to 35 µg L^{-1} Cu showed a significant increase in tolerance to Cu in the fourth generation (Bossuyt and Janssen 2004). The marine copepod, *Tigriopus japonicas*, developed increased resistance to Cu after just one generation of exposure at 100 µg L^{-1} Cu (Kwok *et al.* 2009). *T. japonicas* also increased tolerance to Cu after 3 generations of exposure to 13.74 µg L^{-1} Cu (Sun *et al.* 2014). All of the above studies used longer exposure periods than the current study, however, they are further evidence that adaptive change can occur rapidly when populations are exposed to a contaminant that exerts strong selection pressure over multiple generations (Hoffmann and Hercus 2000). In the current study, the main purpose of the parental to F_2 generations was to establish populations of *I. newcombi* that had different exposure histories to Cu so a comparison of the responses of the snails with different exposure histories could be undertaken in the F_3 generation. The reduced survival in the 75 µg L^{-1} Cu treatments compared to the other treatments in these three generations was evidence of the high selection pressure being exerted by the exposure concentration in this treatment.

3.4.1.2 Food consumption

During the parental, F_1 and F_2 generation exposures, only the F_2 generation had significant differences in the amount of food consumed. There was a negative correlation between food consumption and exposure concentration in *I. newcombi* indicated that exposure to increased Cu concentrations affected snail feeding behaviour during the exposure period. There is prior evidence of exposure to high concentrations of Cu effecting feeding behaviour of freshwater snails. Feeding rates in the freshwater snail *Pomacea canaliculata* reduced when exposed to 67.5 µg L^{-1} Cu (Peña and Pocsidio 2007). *Lymnaea luteola* exposed to 56 µg L^{-1} Cu ceased feeding (Das and Khangarot 2011). While not specifically measured, there was a general behavioural response observed of reduced movement at the high exposures, with the snails in the 75 µg L^{-1} Cu treatment staying on the bottom of the aquariums, while the snails from the other treatments moved freely around the aquariums and fed (Figure 3-16). In the freshwater snail *Lymnaea luteola*, a reduction in locomotion has been reported in response to Cu exposure (Das and Khangarot 2011). The reduction in feeding may be associated with a broader behavioural response of reduced locomotion. The use of wet mass of lettuce as the measure limited the accuracy of the food consumption measurements, due to the effect of water on the lettuce weight, and some breakdown of the lettuce during the Cu exposures. Despite the lettuce mass being calculated against control lettuce left in media for 3 days rather than starting mass, this method still introduced variability into the data. Combined with the small sample sizes, this made the detection of statistically significant differences challenging.

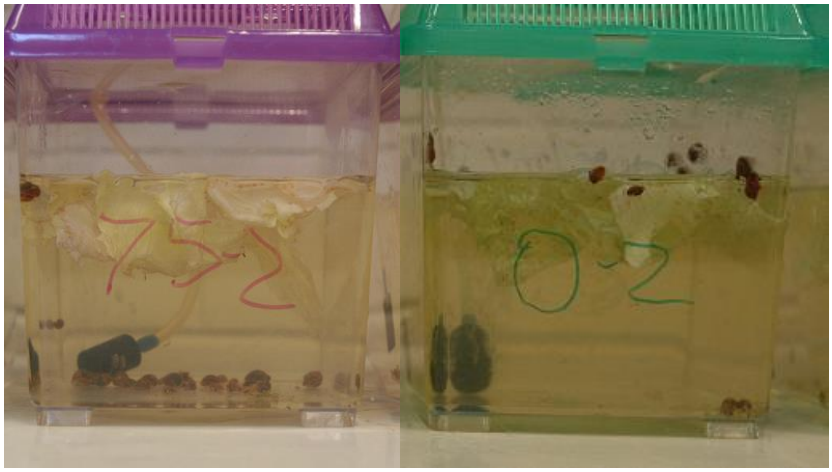


Figure 3-16. Images of exposure showing *Isidorella newcombi* exposed to 75 µg L^{-1} Cu on the left mainly remaining at the bottom of the aquarium, while in the control exposures the snails moved readily to the surface and fed on the lettuce.

3.4.1.3 Fecundity

Fecundity was assessed in surviving snails two weeks after the exposure period. In various studies exposing freshwater snails to Cu there has been a reduction in fecundity (Das and Khangarot 2011; Khangarot and Das 2010; Rogevich *et al.* 2008). These studies tested the direct effects of Cu on fecundity, whereas the current study tested the fecundity of surviving snails after exposure to assess trade-offs associated with Cu resistance development. There were no differences in fecundity when comparing the treatments within the generations Despite t the number of clutches in the 75 µg L^{-1} Cu treatment and the mean number of eggs per adult being lower in F_1, F_2 and F_3 generations than the parental generation, there were no significant differences among these generations in the number of eggs laid per adult. So, while minor evidence of biological trade-offs in the form of reduced number of clutches laid was present in the snails from the 75 µg L^{-1} Cu treatment this did not translate to a significant reduction in the number of eggs per snail and would be expected to have limited relevance at the population level. Significant biological trade-offs associated with resistance to metal have been reported in various species (e.g., Agra *et al.* 2011; Mireji *et al.* 2010; Shirley and Sibly 1999), however, in the current study, while there is some evidence of trade-offs occurring they are unlikely to have an effect at the population level.

3.4.1.4 Egg hatching success and juvenile survival

There were no significant differences in either egg hatching success or juvenile survival either among the treatments within a generation or among generations within any of the treatments. Direct effects of Cu exposure on freshwater gastropod eggs and juvenile survival have been reported (Khangarot and Das 2010). In the current study there is no evidence of the exposure of the adults to Cu affecting the hatching success of eggs or juvenile survival.

3.4.2 F_3 generation

In the F_3 generation all treatments were exposed to 75 µg L^{-1} Cu to allow a common baseline for the assessment of changes in tolerance and associated biological trade-offs resulting from previous exposure history. Analyses tested the differences between pre-exposure concentration that the snails were exposed to in previous generations rather than the exposure concentration itself. In the F_3 generation discussion, the term pre-exposure concentration will be used to describe the Cu concentration that snails were exposed to in the parental to F_2 generations.

3.4.2.1 Survival

In the F_3 generation, there was a positive correlation between the pre-exposure concentration and survival during the three-day exposure period. This suggests that populations of *I. newcombi* developed some resistance that was related to the pre-exposure concentration of previous generations to Cu. While this was the case the only significant difference in survival was between 0P-75E and the 75P-75E snails.

3.4.2.2 25 and 50 µg L^{-1} Cu pre-exposure treatments

The trend of increased survival in the F_3 generation in the 25 and 50 µg L^{-1} Cu pre-exposure treatments as indicated by the correlation analysis is likely to be related to increased phenotypic plasticity as a result of prior Cu exposure rather than directional selection. In the parental, F_1 and F_2 generations of these treatments, there were high survival rates that were not significantly different from the controls. Additionally, there were no significant differences in the reproductive output among these treatments in the parental to F_2 generations. Changes associated with directional selection would require tolerant individuals to make an increased contribution to the genetic make-up of subsequent generations. Given that there was no significant difference in mortality or change in reproductive output per adult this seems unlikely. While changes associated with phenotypic plasticity are often short term, some of the epigenetic changes associated with phenotypic plasticity can be passed across generations (Head 2014). An analysis of genetic differences among the treatments, combined with an investigation of gene expression and associated epigenetic changes would be the only way to show definitively that the changes in survival of these treatments were associated with intergenerational changes to phenotypic plasticity rather than directional selection, however, the weight of evidence suggests that increased phenotypic plasticity is the most likely cause.

3.4.2.3 75 µg L^{-1} Cu pre-exposure treatments

The basis of the increased survival of the snails from the 75P-75E treatment may be due to a number of factors. During the parental exposures, the 75 µg L^{-1} Cu exposed snails had significantly lower survival than the other treatments. In the F_1 and F_2 exposures, the trend of lower survival in the 75 µg L^{-1} Cu treatment continued, although there were no significant differences in these generations. Klerks *et al.* (2011) reported that when contamination affects survival or reproduction, natural selection will favour those individuals that are less sensitive to the contaminant. It is possible that the mortality of some of the snails in the 75 µg L^{-1} Cu treatment in the parental, F_1 and F_2 generations was associated with genetic variability, with

differential fitness resulting from variation in genotypes being the determining factor in their ability to survive the exposure. Under this scenario, directional selection could be expected in the 75 µg L^{-1} Cu pre-exposure treatment. In the 75 µg L^{-1} Cu pre-exposure treatment the effect of intergenerational changes in phenotypic plasticity must also be considered. As the increases in survival in the F_3 generation of the 25 and 50 µg L^{-1} Cu pre-exposure treatments appears to be associated with an increased phenotypic plasticity in these populations, it is possible that some or all of the increased survival in the 75 µg L^{-1} Cu pre-exposure treatment is also associated with increased phenotypic plasticity. It is likely, that in the 75 µg L^{-1} Cu pre-exposure treatment, both of these mechanisms have contributed to increased survival. It has previously been found that freshwater snails taken from differing field and laboratory populations with differing exposure histories to Cu had significantly different mortality when exposed to 65 µg L^{-1} Cu under controlled conditions (Côte *et al.* 2015). The current study further highlights the ability of freshwater gastropods with differing Cu pre-exposure histories to develop resistance and demonstrates that the development of resistance can occur in as little as 3 generations following 72 hours Cu exposure within each generation.

In the F_3 generation, the increased survival in the treatments that had pre-exposure histories to higher Cu concentrations continued in the 14 day recovery period directly after exposure when snails were maintained in uncontaminated water. This was indicated by a strong positive correlation between survival two weeks post-exposure and pre-exposure Cu concentration. This suggests that the snails that had a higher pre-exposure to Cu not only had a greater ability to survive the exposure period but had greater ability to recover after the exposure. In the freshwater snail *Pomacea paludosa* exposed to Cu in sediment, the highest mortality was associated with the treatment that bioaccumulated the highest Cu concentrations during the exposures and the increased in mortality in this group continued after snails were removed from the exposure into uncontaminated water (Hoang *et al.* 2011). The findings from the current study indicate that resistance developed through generations of pre-exposure to Cu lead to increases in survival during this recovery period as well as during the actual exposure.

3.4.2.4 Food consumption

In the F_3 generation, there was no significant difference in lettuce consumption among the treatments. The small amount of lettuce consumed in all treatments in this generation was similar to consumption by the 75 µg L^{-1} Cu treatments in earlier generations. This suggests that despite the increased ability of *I. newcombi* with a high pre-exposure history to Cu to survive,

their behavioural responses relating to important behavioural markers such as food consumption did not change with repeated exposure to this Cu concentration. This was further reinforced by an observed lack of locomotion in snails in all treatments during the F_3 exposures, with snails spending most of the time at the bottom of the tanks (Figure 3-16). The reduction in feeding may be related to metabolic depression when the snails are exposed to high Cu concentrations. In another gastropod, the limpet *Siphonaria capensis*, a reduced metabolic rate measured by cardiac activity was reported in response to concentrations higher than 50 µg L^{-1} Cu (Marshall *et al.* 2004).

3.4.2.5 Bioaccumulation of Cu

While there were significant differences in the Cu bioaccumulation in the F_3 generation, they did not follow a pre-exposure Cu concentration-dependent pattern. The 0P-75E and 25P-75E were the only treatments that were significantly different from each other. It was suggested by Morgan *et al.* (2007) that one mechanism by which tolerance to a chemical can be achieved is through reduced accumulation of that chemical. As the differences in the tissue Cu concentrations did not follow a pre-exposure concentration-dependent pattern or relate to differences seen in survival or LD it is assumed that differences in bioaccumulation were not an important factor in differences in tolerances among populations.

3.4.2.6 Fecundity, hatching success and juvenile survival as biological trade-offs

It has been reported that there are biological trade-offs associated with increased tolerance to contaminants that manifest as reduced growth, reproductive output and survival compared to non-tolerant individuals in uncontaminated environments (Shirley and Sibly 1999; Xie and Klerks 2004). In the current study, biological trade-offs were assessed through fecundity (clutches and eggs per adult) of the surviving snails 15 to 26 days after Cu exposures. The juvenile hatching success and survival to six days were also assessed. Despite the differences in survival among the treatments in the F_3 generation there were no differences in either the number of clutches or eggs per adult, the hatching success or juvenile survival in this generation. As the control treatment was exposed to the common concentration of 75 µg L^{-1} Cu in the F_3 generation, there were insufficient snails to for a control treatment. To compensate for the lack of a control in this generation the results for fecundity, hatching success and juvenile survival were also compared to the control results for these endpoints in the parental, F_1 and F_2 generations. No significant differences were found between the F_3 results for these

endpoints and the controls from earlier generations. This suggests that although the snails with high pre-exposure Cu concentration histories had developed resistance to Cu as evidenced by the increased survival rates, there were no accompanying biological trade-offs. While trade-offs associated with resistance have been documented there are also examples where this is not the case. In *L. stagnalis* from genetically different populations with varying pesticide exposure histories cultured in uncontaminated media, there was no significant reduction in egg production (Boue'tard *et al.* 2014). In malathion-resistant flour beetles, as opposed to a trade-off, it was reported that the resistant individuals had increased reproductive capacity (Arnaud *et al.* 2005). It has been noted that while biological trade-offs associated with resistance are often discussed in the literature, the presence of trade-offs and underlying mechanisms are not well understood and require further investigation (Medina *et al.* 2007).

3.4.2.7 Total antioxidant capacity

Total antioxidant capacity (TAOC) was measured for the F_3 generation to investigate the effect of multiple generations of pre-exposure to Cu on the antioxidant response to Cu-induced stress. The TAOC of the F_3 generation snails increased as the pre-exposure Cu concentration increased. This implies that the snails that had a higher Cu pre-exposure history had a greater ability to increase antioxidant function and neutralise reactive oxygen species (ROS). It is known that exposure to excess Cu increases ROS production in organisms (Livingstone 2001). Reactive oxygen species can lead to cellular damage in the form of lipid peroxidation, structural and functional changes to proteins, and damage to nucleic acids (Manduzio *et al.* 2005). The increase in TAOC in the snails with a pre-exposure history to high Cu concentrations would result in an increased ability to neutralise excess ROS generated by Cu interference in ROS homeostasis. On this basis, the increased antioxidant capacity of snails that had higher pre-exposure Cu concentration histories could play a role in the increased survival rate in the F_3 generation.

3.4.2.8 Lipid peroxidation

Lipid peroxidation (LP) occurs when organisms have high levels of ROS which interact with and breakdown membrane lipids (Livingstone 2001), and is, therefore, a measure of oxidative damage. The levels of LP in *I. newcombi* in the F_3 generation increased as the pre-exposure Cu concentration history increased. In the snails with a multiple generation exposure history to high Cu concentrations it was expected that LP would decrease, especially given that the level of total antioxidant capacity increased in the snails that had a history high Cu concentration

exposure. While this was not expected, previous studies measuring LP in gastropods have also not fitted expected patterns. In the marine gastropods *Nerita melanotragus* and *Bembicium nanum* exposed to a single 96 hr Cu exposure, lipid peroxidation was lower in organisms exposed to high Cu concentrations than those exposed to low concentrations (Unpublished, Taylor, University of canberra). In the same study, two bivalves *Saccostrea glomerata* and *Mytilus edulis* were also assessed and had Cu exposure concentration-dependent increases in LP. This indicates that gastropods respond differently to bivalves with relation to oxidative stress responses.

In many studies investigating LP in gastropods, the response seems to be transitory. In studies that investigated exposures of less than 4 days, stress-induced changes in LP were present (Deschaseaux *et al.* 2011; Deschaseaux *et al.* 2010; Pannunzio and Storey 1998; Singh *et al.* 2008). Studies that investigated the LP response over a longer period have had mixed findings with some studies reporting there were no differences in LP (Reid and MacFarlane 2003; Sureda *et al.* 2009) and others finding that under long term exposures LP continued to be elevated (Cacciatore *et al.* 2015; Zhou *et al.* 2010). Klobučar *et al.* (1997) found that changes to LP in *Planorbius corneus* exposed to pentachlorophenol were present but not dose-dependent after 2 days of exposure, were present and dose-dependent after 8 days of exposure, and were not present after 13 days of exposure, illustrating the transient and inherent variability of the LP responses in gastropods. These studies have involved exposure to a wide range of stressors, as and there are insufficient studies that have investigated the effect of Cu or even metals more generally on LP in gastropods for a useful comparison. The only consistent finding across studies is that gastropods exposed to oxidative stress-inducing chemicals over a short period of up to four days display a response. The reported responses are not always dose-dependent and the relative response between treatments can vary over time as demonstrated by Klobučar *et al.* (1997). In addition, the response can return to background levels over longer exposure periods (Klobučar *et al.* 1997; Pannunzio and Storey 1998; Sureda *et al.* 2009). While LP in *I. newcombi* in the current study did not follow the expected exposure concentration-dependent pattern, given the reported transitory nature of LP responses in gastropods as well as the fact that reported responses have not always been dose-dependent the result is not without precedent. These findings, along with the reported results for LP in gastropods, highlights the need for time-course studies to better understand the regulation of the antioxidant system and associated oxidative damage when gastropods are exposed to oxidative stress-inducing chemicals.

3.4.2.9 Lysosomal membrane destabilisation

Exposure history had a significant effect on LD, with those individuals with no pre-exposure history having higher LD than those previously exposed. This is most evident in the 75P-75E snails which had the lowest LD. All treatments that had been pre-exposed to elevated concentrations of Cu also had significantly lower LD than 0P-75E. As LD has been recognised as an effective biomarker of contaminant-induced stress in molluscs (e.g., Broeg *et al.* 2005; Moore *et al.* 1982; Moore *et al.* 2007; Ringwood *et al.* 2003), this indicates that the snails from treatments that had been exposed to Cu in previous generations were under less stress than the snails that had no previous exposure to elevated Cu concentrations. As all treatments were exposed to a common Cu concentration in the F_3 generation, the differences in LD occur because of adaptive changes associated with the selection pressure exerted by exposures in the previous 3 generations. Changes in LD have been related to the accumulation of high concentrations of contaminants into the lysosome, the onset of programmed cell death and oxidative damage in the form of lipid peroxidation (Izagirre and Marigómez 2009; Johansson *et al.* 2010; Taylor and Maher 2010). While the snails from the 0P-75E treatment did accumulate the highest tissue Cu concentrations, it was only significantly higher than those from the 25P-75E treatment. Given that LD and tissue Cu concentrations followed a different pattern between treatments, it is unlikely the sequestration of Cu to the lysosomes was an important factor in the LD result. Previous studies in bivalve molluscs have linked LD to oxidative stress and the breakdown of lipid membranes through LP (e.g., Marasinghe Wadige *et al.* 2014; Taylor *et al.* 2016). In the current study there is no correlation between LD and either TAOC or LP. On this basis, there is no clear evidence that the increased LD was as a direct result of oxidative stress. The other reported cause of increased LD is the initiation of programmed cell death mechanisms. Kiss (2010) reported the occurrence of apoptotic programmed cell death occurring in molluscs as a result of metal exposure. While apoptotic processes were not measured directly in this study they are investigated in the accompanying transcriptomic study (Chapter 5). As such, the underlying reason for the increase in LD is uncertain, however, there is clear evidence that the snails with a lower pre-exposure history to Cu are experiencing increased levels of general stress as indicated by increased LD.

3.4.2.10 Relationship life history traits and cellular biomarkers

In the F_3 generation, adult survival was positively correlated with TAOC and LP and negatively correlated with LD. The differences in antioxidant capacity and LP in surviving organisms from each treatment may indicate reasons for the differential survival among treatments. The

correlation between TAOC and survival is most likely due to increased TAOC allowing the neutralisation of ROS in the snails, with the associated reduction in oxidative stress leading to reduced mortality. In the bivalve mollusc *Mytilus galloprovinciallis* exposed to organophosphates, impaired glutathione redox status has been associated with an reduction of survival (Peña-Llopis *et al.* 2002).

The positive correlation between LP and survival is counter intuitive. As LP is a measure of oxidative damage it would be expected that increased LP would lead to increased mortality. As has been discussed in Section 3.4.2.8 and also in the Section 2.4.7, the LP results in this study were not as expected. While the underlying mechanism for this response is outside the scope of this study, two potential mechanisms could be hypothesised. The first is associated with the differences in timing of the antioxidant mechanisms which may lead to differences in the onset of oxidative damage between the treatments that also had different TAOC. The second is that the snails with higher survival rates that had been exposed to Cu over multiple generations may have transferred resources to other biological functions that are more important to survival. While this study is not able to determine the exact reason, this response further demonstrates the need for integrated studies that investigate the mechanistic basis of biomarker responses. This is especially so for LP and TAOC that are widely used as biomarkers to interpret antioxidant responses and oxidative damage.

There was a negative correlation between adult survival and LD in the F_3 generation. In the treatments that had been exposed to high Cu concentrations in the parental, F_1 and F_2 generations there was increased survival and lower LD. As discussed, the increased LD is likely to be related to increases in apoptotic programmed cell death. If organisms are experiencing sufficient stress-related damage that an increase in apoptosis is required, it is not surprising that there was also reduced survival in the treatments. LD is known to be a reliable biomarker of general stress (Broeg *et al.* 2005; Moore *et al.* 2007) and the correlation between increased LD and reduced survival in the treatments provides further evidence that this biomarker is able to predict changes at higher levels of biological organisation.

3.4.3 Summary and conclusions

In the parental to F_2 generations some general responses to short pulses of Cu were seen including reduced survival and reduced feeding in snails exposed to high Cu concentrations. These findings indicated that the snails from the high Cu exposure were under Cu-induced stress that would be likely to apply a selection pressure.

There was evidence that the selection pressures exerted on the snails in the parental to F_2 generations led to adaptive changes, resulting in increased resistance to Cu. In the F_3 generation, when all treatments were exposed to a common Cu concentration, there was an increase in survival that was correlated with the pre-exposure Cu concentrations of the treatments. The snails from treatments that had been pre-exposed to Cu also displayed a reduction in stress at a sub-lethal level as indicated through lower LD. While there were differences in the bioaccumulation of Cu in the F_3 generation, they did not follow the same trend as increased survival, so it is unlikely that changes in bioaccumulation of Cu are leading to differences in tolerance. Changes in antioxidant response were also investigated as a possible mechanism for an increase in tolerance. While TAOC increased in the snails from the higher pre-exposure treatments, the oxidative damage marker LP followed the opposite trend. A review of oxidative stress responses in gastropods highlighted the need for further research in this area as the responses often do not follow expected patterns and are different from closely related taxa such as bivalve molluscs. The mechanisms that led to the increase in Cu resistance in the treatments that had been previously exposed to high Cu concentrations are not clear. Transcriptomic responses of snails from the resistant treatments have been investigated and provide insight into the mechanistic differences in the responses of the resistant snails at a molecular level.

The study also investigated the presence of biological trade-offs, in the form of reduced reproductive capacity, that are often reported to be associated with Cu-resistant populations. There was no clear evidence that there was any reduction in reproductive capacity in the treatments that had developed Cu resistance.

This study demonstrates that Cu resistance can develop over a short evolutionary time scale. While other studies have demonstrated the development of resistance over multiple generations exposed continuously to Cu, this study was unique in the use of a single short term exposure to Cu in each generation. In aquatic environments excess Cu introduced into the environment is often only bioavailable for a short period of time and this study demonstrates the potential for changes in resistance to Cu in populations exposed in this manner. While the high Cu concentrations used in this study are higher than are often found in the environment and above the water quality guideline concentrations they are relevant for assessing the potential for adaptive changes where there are occasional high concentration point source events or areas where Cu is being used as a pesticide.

DECLARATION OF CO-AUTHORED PUBLICATION CHAPTER

For use in theses which include publications. This declaration must be completed for each co-authored publication and to be placed at the start of the thesis chapter in which the publication appears.

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Experimental design, snail exposures, preparation of samples, laboratory analysis, statistical analysis, and preparation of manuscript	80

The following co-authors contributed to the work.

Name	**Nature of contribution**	**Contributor is also a student at UC Y/N**
Tariq Ezaz	Advice on genomic techniques and reviewing of manuscript	N
Anne Taylor	Advice on experimental design, snail culture and reviewing manuscript	N
Mark Stevens	Reviewing manuscript	N
Frank Krikowa	Chemical analysis	N
Simon Foster	Chemical analysis	N
William Maher	Advice on experimental design, reviewing manuscript	N

Candidate' Signature

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;

(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s)	**University of Canberra, Canberra, Australia**

[Please note that the location(s) must be institutional in nature, and should be indicated here as a department, centre or institute, with specific campus identification where relevant.]

Signature

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Chapter 4. The response of *Isidorella newcombi* to copper exposure: Using an integrated biological framework to interpret transcriptomic responses from RNA-seq analysis.

4.1 Abstract

This study describes the transcriptomic response of the Australian endemic freshwater gastropod *Isidorella newcombi* exposed to 80 ± 1 µg L^{-1} of copper for 3 days. Analysis of copper tissue concentration, lysosomal membrane destabilisation and RNA-seq were conducted. Copper tissue concentrations confirmed that copper was bioaccumulated by the snails. Increased lysosomal membrane destabilisation in the copper-exposed snails indicated that the snails were stressed as a result of the exposure. Both copper tissue concentrations and lysosomal destabilisation were significantly greater in snails exposed to copper. In order to interpret the RNA-seq data from an ecotoxicological perspective an integrated biological response model was developed that grouped transcriptomic responses into those associated with copper transport and storage, survival mechanisms and cell death. A conceptual model of expected transcriptomic changes resulting from the copper exposure was developed as a basis to assess transcriptomic responses. Transcriptomic changes were evident at all the three levels of the integrated biological response model. Despite lacking statistical significance, increased expression of the copper transporting ATPase provided an indication of increased internal transport of copper. Increased expression in genes associated with endocytosis are associated with increased transport of copper to the lysosome for storage in a detoxified form. Survival mechanisms included metabolic depression and processes associated with cellular repair and recycling. There was transcriptomic evidence of increased cell death by apoptosis in the copper-exposed organisms. Increased apoptosis is supported by the increase in lysosomal membrane destabilisation in the copper-exposed snails. Transcriptomic changes relating to apoptosis, phagocytosis, protein degradation and the lysosome were evident, and these processes can be linked to the degradation of post-apoptotic debris. The study identified contaminant-specific transcriptomic markers as well as markers of general stress. From an ecotoxicological perspective, the use of a framework to group transcriptomic responses into those associated with copper transport, survival and cell death assisted with the complex process of interpretation of RNA-seq data. The broad adoption of such a framework in ecotoxicology studies would assist in comparison between studies and the identification of reliable transcriptomic markers of contaminant exposure and response.

4.2 Introduction

The input of copper into aquatic systems has increased substantially in the last century due to mining as well as its use in manufacturing, pipes, automobile brakes and pesticides (Neira *et al.* 2014). Copper is essential for biological function, acting as a cofactor in enzymes involved in biochemical processes including energy generation, iron acquisition, oxygen transport, cellular metabolism, peptide hormone maturation, blood clotting and signal transduction (Kim, Nevitt, & Thiele, 2008). Despite being an essential element, at elevated concentrations copper disrupts protein structure and function, causes DNA damage, alters fecundity levels and causes oxidative stress (Das and Khangarot 2011; Gaetke and Chow 2003). The dual roles of copper as an essential element as well as a toxic contaminant when present at high concentrations means that biological responses to copper contamination are complex.

A species' response to contamination in the environment can range from molecular to population level. Traditionally, the effect of contamination in the environment has been assessed through the measurement of tissue concentrations and biochemical, physiological and behavioural changes in biota (Taylor and Maher 2010). Advances in genomic technologies have enabled the application of new methods in ecotoxicology such as sequencing of the transcriptome (RNA-seq).

RNA-seq offer several advantages over traditional methods including recognition of contaminant-specific responses, early detection of responses, detection of functional changes indicative of an organism's level of stress and the ability to detect novel responses. Firstly, one of the early responses of an organism to an environmental stress such as copper contamination is a change in the transcriptional regulation of genes and biological pathways as a means of maintaining homeostasis (Ankley *et al.* 2006). This provides the opportunity to detect early signals that act as warnings of problems at higher levels of biological organisation. Secondly, the unique transcriptional footprint resulting from exposure to specific contaminants or groups of contaminants presents opportunities to detect contaminant-specific responses (Yang *et al.* 2007). In an effort to maintain homeostasis, organisms exposed to chemical stress alter their transcriptome regulation as a means of regulating a range of biological processes including contaminant uptake, transport, storage and detoxification as well as mechanisms that respond to cellular challenge or damage. RNA-seq provides the opportunity to gain a mechanistic understanding of how biological functions are altered, providing insights into organism health

status ranging from optimal through to high levels of stress. The global nature of RNA-seq also means that detection of changes in the regulation of novel transcripts, genes and pathways provides opportunities to increase our understanding of responses to contamination (Wang *et al.* 2009). RNA-seq has broad potential for use in ecotoxicology, but to gain maximum benefit from this method there is a need to understand transcriptomic responses from an ecotoxicological perspective.

Some of the issues with developing RNA-seq for use in ecotoxicology include using non-model species, linking transcriptional changes to biological function and determining contaminant-specific transcriptomic profiles. Often no model species fulfil requirements for specific ecotoxicological purposes, leading to the use of non-model species. An investigation of RNA-seq using non-model species found that shortcomings are associated with the proportion of transcripts annotated and errors in annotation, especially when sequences are annotated to widely divergent species (Hornett and Wheat 2012). Despite this, it was found that RNA-seq produced high quality *de novo* assembled and annotated transcriptomes for quantitative analysis (Hornett and Wheat 2012). A second issue is the need to relate transcriptomic results to biological significance. Significant changes in transcriptomic regulation of some genes can be related to maintaining homeostasis within an organism's normal tolerance range, and are not biologically significant. Relating changes in gene expression to established response biomarkers allows specific transcriptomic responses to be indicative of stress levels. A third issue is related to the sheer quantity of information that is produced in a RNA-seq study. The global response profile produced by RNA-seq is advantageous as it provides a broad quantification of the species response to the contaminant but the large amount of data as well as the complexity of the global biological response makes interpretation difficult. Functional databases such as KEGG and Gene Ontology assist by grouping genes associated with specific biological functions, however, the detection of the most important responses from an ecotoxicological perspective remains challenging. A framework that categorises responses into contaminant transport and storage, survival mechanisms and cell death assists in the interpretation of RNA-seq data from an ecotoxicological perspective and allow greater consistency of interpretation and easier comparison between studies.

The planorbid gastropod *Isidorella newcombi* is an endemic Australian gastropod that is widely distributed outside of the tropical regions of Australia and is considered a pest in rice fields, where it is controlled through the application of copper sulphate (Stevens *et al.* 2014). This

study established the transcriptomic response of *I. newcombi* to copper exposure. Copper tissue concentrations were analysed to determine if *I. newcombi* accumulated copper over the exposure period. Lysosomal destabilisation was assessed in the organisms as a marker of general stress. The transcriptomes of copper-exposed and control groups were sequenced in order to determine the response to copper exposure at the molecular level. Transcriptional changes resulting from copper exposure were categorised as being related to copper transport and storage, survival mechanisms or programmed cell death. The biological changes were then combined in an integrated framework of biological response to copper exposure. Links between the internal transport of copper, the lysosome, programmed cell death, phagocytosis and degradation of cellular debris were evident in the RNA-seq data.

4.3 Methods

4.3.1 Experimental design

4.3.1.1 Snail and husbandry

Isidorella newcombi were sourced from a captive culture maintained at a water temperature of 22 ± 1°C, with a 12L/12D light cycle. Snails were kept in a 12 litre polystyrene aquarium tank and the water was aerated using an air stone. Water sourced from Vanity's Crossing (35° 20' 43"S, 148° 53' 23" E), an uncontaminated site in the Cotter River, was used for culture media (pH 6.65, conductivity 0.044 ms/cm, turbidity 1.4 NTU, hardness 6.6, salinity 0.02 ppt and DOC 1.46 ppm). Snails were fed lettuce (*Lactuca sativa*) washed in the river water in which the culture was maintained. After 1 week live snails were removed from the tank and the tank was refilled with water to cover the egg masses present. The egg masses were allowed to hatch producing a cohort of snails of the same age. The hatched snails were grown for 90 days and randomly separated into six treatment replicates of 12 snails each.

4.3.1.2 Copper exposure

The six replicates were transferred to 770 mL polypropylene containers (Chanrol (01C30), Blacktown, Australia) containing 600 mL of medium. Three replicates were kept as controls in uncontaminated river water with a copper concentration of 4 ± 1 µg L^{-1} Cu for 72 hours (control group) and three of the replicates had 75 µg/L Cu as $CuCl_2$ added to the river water (exposed group). The measured concentration in the media for the exposed group was 80 ± 1 µg L^{-1} Cu. With the exception of the addition of $CuCl_2$ the media was the same as for the stock culture. A 72 hour exposure period was chosen as it has been demonstrated that releases of copper into

the environment are only bioavailable for a relatively short period (Stevens *et al.* 2014). Six grams of lettuce was added to each replicate at the start of the exposure period. At the end of the exposure period three snails from each replicate were immediately frozen at -80°C for use in sequencing of the transcriptome (RNA-seq). Due to mortality in some replicates there were insufficient snails to allow for 3 to be used for metal tissue analysis and 3 to be used for lysosomal destabilisation. The number of snails used for each analysis is provided in the Figure captions.

4.3.2 Copper tissue concentration analysis

Snails used for metal analysis were depurated in clean river water for 24 hours. Organisms were dissected and soft tissue removed from the shell. Lyophilised tissue was digested in nitric acid (AristaR BDH, Australia) at 600 W for 2 min, 0 W for 2 min and 450 W for 45 min in a microwave oven (CEM MDS-2000, Tokyo) (Baldwin *et al.* 1994). Copper was measured using a Perkin-Elmer Elan DRC-e ICP-MS (Perkin-Elmer, Boston, USA) (Maher *et al.* 2001). Certified reference material (NIST 1566b) and blanks were digested and analysed with samples. The certified copper concentration for NIST1566b is 71.6 ± 1.6 µg g^{-1} and our recovered value was 68 ± 3 µg g^{-1} (n=9) was within the acceptable range.

4.3.3 Lysosomal membrane stability

Lysosomal stability was assessed using a method adapted from Ringwood *et al.* (2003). The digestive gland was minced on ice and rinsed with calcium and magnesium free saline buffer (CMFS) (pH 7.35-7.4). Minced tissues were shaken for 20 min at 100 rpm on a reciprocal shaker in 600 µL CMFS, and a further 20 min at 100 rpm after the addition of 400 µL of 1.0 mg/mL trypsin (1426-1G, Sigma USA). Samples were sheared with a glass pipette and filtered (40 µm) in a 5804R centrifuge (Eppendorf, Wien, Austria) (230 × g, 5 min, 15 °C). The pellet was resuspended in 1 mL CMFS and centrifuged again (230 × g, 5 min, 15 °C) prior to being resuspended again in a further 100 µL of CMFS. The harvested cells were then incubated in a neutral red (Sigma, USA) solution 0.16 mg/mL for 40 min. Cells were observed at 400× magnification, with a determination of the stability of the lysosomal membranes scored as stable or unstable. At least 50 cells were scored for each sample.

4.3.4 RNA–seq analysis

4.3.4.1 RNA extraction and sample preparation

For RNA-seq analysis, whole snails were transferred immediately to a -80°C freezer at the end of the exposure period. The digestive gland was dissected and RNA was extracted using an E.Z.N.A.® mollusc RNA kit (Omega-Biotek 6875-01). Initial quality and quantity of each RNA sample were checked using a Nanodrop® 1000 spectrophotometer and three samples from each treatment replicate were pooled. For transportation pooled samples were transferred to RNA stable® tubes (Biomatrica) and shipped to BGI (Shenzhen, China) for sequencing. RNA QC was performed using a Bioanalyzer 2100 (Agilent technologies) and one sample that did not meet BGI QC criteria was excluded (exposed replicate 2). 100 bp paired end sequencing libraries were prepared following standard illumina chemistry and protocols and were sequenced using an Illumina HiSeq 4000 platform. After sequencing all reads with low quality (more than 20% bases with a quality less than 10), adapter polluted or with a high content of unknown base reads were removed from the raw reads leaving the clean reads.

4.3.4.2 de novo assembly and annotation

de novo assembly and annotation were performed at BGI following their established pipeline. Briefly, assembly of the clean reads was performed with Trinity (v2.0.6) using three independent software modules (Inchworm, Chrysalis and Butterfly). The TIGR gene indices clustering tool was used to cluster transcripts into unigenes (Pertea *et al.* 2003). Unigenes were searched using blast analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against nt, nr, COG, KEGG and Swissprot databases. The best hits from the alignment were used to predict unigene coding regions and direction. When results from different databases were conflicting the following order was used for annotation: nr>SwissProt>KEGG>COG. The Blast2GO program in conjunction with nr annotations was used to obtain unigene GO annotation. InterProScan5 was used for the InterPro annotation. Raw reads and unigene sequences have been archived and are available at: https://figshare.com/s/5b30ccc9704345f0e471.

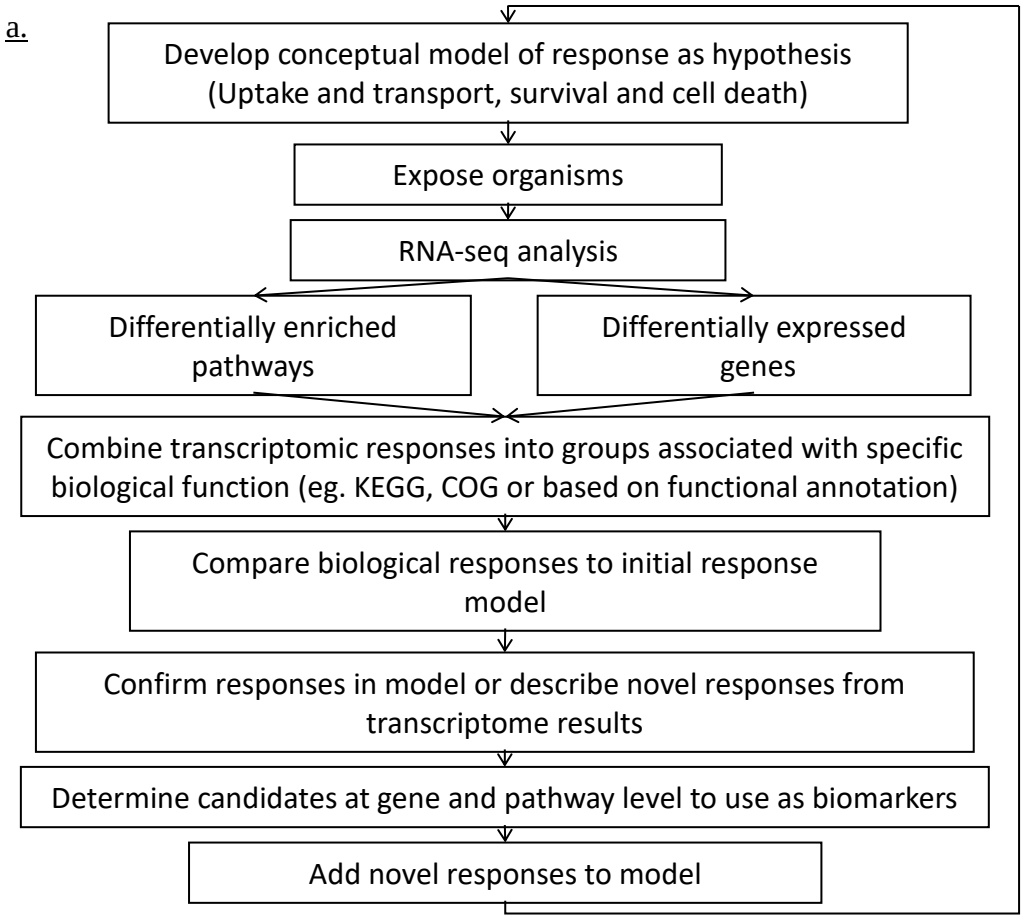
4.3.4.3 Analysis of differentially expressed genes

Differentially expressed genes (DEGs) were identified using the NOIseq program (Tarazona *et al.* 2011). For the comparison of DEGs the pooled samples made up of three samples from each of the replicates were used to compare the control to the copper-exposed organisms. Genes were considered differentially expressed when fold change ≥2 and probability ≥ 0.8.

4.3.4.4 Interpretation of transcriptomic response

We established a model outlining our method for the interpretation of RNA-seq data (Figure 4.1a). This included the development of a conceptual model of integrated biological response that outlined expected transcriptional changes associated with copper uptake and transport, survival mechanisms and programmed cell death (Figure 4.1b). Transcriptomic differences between the control and copper-exposed treatments were then interpreted based on this framework. Any novel responses detected during a study can be added to the model to improve the understanding of the response to the contaminant.

a.



b.

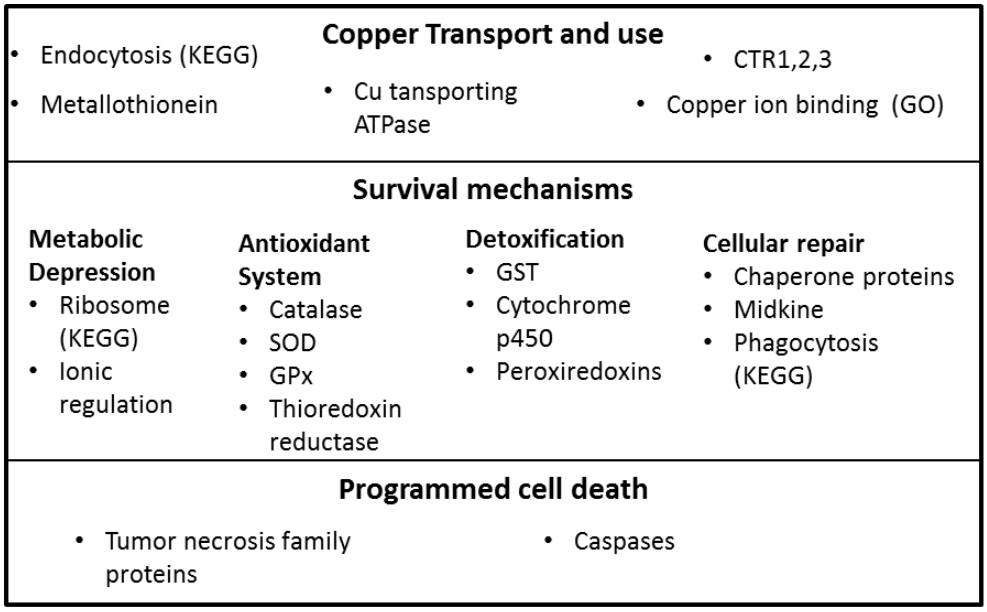


Figure 4-1. a) Flow diagram of method of interpretation of RNA-seq data. b) Conceptual model to use as a basis for interpretation of RNA-seq data with examples of genes from different responses.

4.3.5 Statistical analysis

Preliminary data analysis was conducted in Microsoft Excel 2010 with statistical tests being run in R version 3.1.2 (R Core Team 2014). For Cu tissue concentrations and lysosomal destabilisation, data from the three replicates were pooled for analysis. The data were tested for normality and equality of variance. If these assumptions were met a two sample students t-test with equal variances assumed was used, if assumptions were not met a Mann-Whitney U-test was used.

4.4 Results

4.4.1 Bioaccumulation of copper

The Cu exposed snails had significantly higher tissue copper concentrations than the control group (t = -11.7275, $d.f.$ = 10, $p < 0.0001$). The copper-exposed snails had a mean tissue copper concentration of 26 ± 3 µg g^{-1} Cu dry mass compared to those from the control treatment which had 10 ± 1 µg g^{-1} Cu dry mass (Figure 4.2a).

4.4.2 Lysosomal membrane destabilisation

Isidorella newcombi from the exposed treatment had a significantly higher percent of cells with lysosomal membrane destabilisation than the control group (W = 0, p = 0.003). The median percentage of cells with destabilised lysosomal membranes in the copper-exposed snails was 42 compared with 22 in the control group (Figure 4.2b).

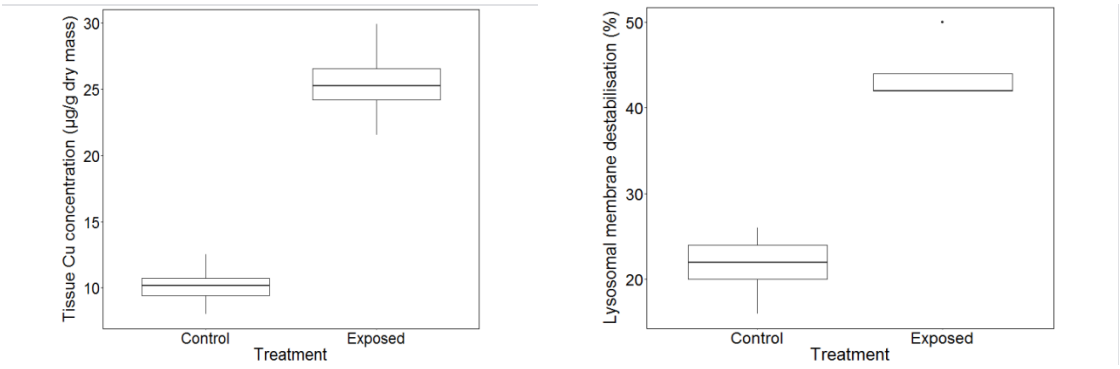


Figure 4-2. Bioaccumulation and response of *Isidorella newcombi* exposed to copper a) tissue copper concentration (control n=7, exposed n=5) and b) percent of cells with lysosomal membrane destabilisation (control n=9, exposed n=5). Horizontal lines indicate median values, boxes show 25th–75th percentiles, whiskers show the range from 0 to the100th percentile and individual points indicate outliers.

4.4.3 RNA sequencing and *de novo* transcriptome assembly

Clean reads (254.7 Mb) were generated and aligned to 112,387 contigs (Table 4.1). There was a total of 2630 differentially expressed contigs between the copper-exposed and control group (Figure 4.3). A Blast search (blast p; E-value $<10^{-5}$) of protein and nucleotide databases (Nr, Nt, Swiss-prot, KEGG, COG or GO databases) provided functional annotation for 59512 of the 112387 contigs (Table 4.2).

Table 4-1. Quality parameters and number of reads for RNA-seq analysis.

Total clean reads (Mb)	Q20 (%)	GC (%)	No. of contigs	Mean length (bp)
254.7	96.38	37.10	112387	548

Table 4-2. Summary of annotation results from *Isidorella newcombi*. 'All annotated' includes all contigs annotated to at least one of the databases.

	Number of contigs	Percentage (%)
All contigs	112387	100
All annotated	59,512	52.95
NR annotation	36,136	32.15
NT annotation	39,110	34.80
Swiss-Prot annotation	25,048	22.29
KEGG annotation	24,318	21.64
COG annotation	10,610	9.44
Interpro annotated	20,613	18.34
GO annotation	3,042	2.71

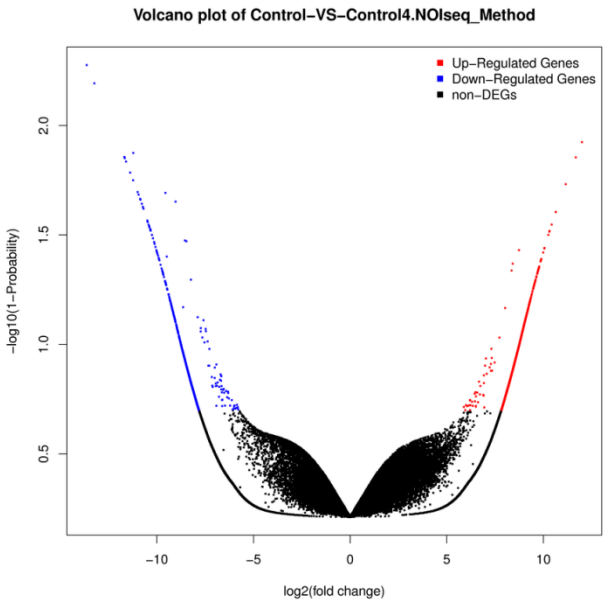


Figure 4-3. Volcano plot of gene expression in *Isidorella newcombi* exposed to copper. Log_2 fold change in expression are plotted against – log_{10} (1-probability). Each point represents a contig, with contigs shown in blue up-regulated and genes shown in red down-regulated (foldchange >2 and probability >0.8).

4.5 Differential gene expression

4.5.1 Pathways

Of the differentially expressed genes, 357 were associated with at least 1 KEGG pathway (Kanehisa *et al.* 2008). These genes were mapped to 251 different KEGG pathways. The pathways to be discussed in this paper are ribosome, apoptosis, FcγR mediated phagocytosis, lysosome and endocytosis (Table 4.3). The differentially expressed genes associated with these pathways and their levels of differential expression are listed in the appendix (Appendices 1, 2, 3, 4 and 5). In addition to the KEGG pathways, gene ontology analysis identified significant differential enrichment (corrected p = 0.00875) between the copper-exposed and control groups for the gene ontology term copper ion binding with three genes encoding for enzymes that use copper as a cofactor being down-regulated (Appendix 6).

Table 4-3. KEGG pathways chosen for investigation of the functional response of *Isidorella newcombi* to copper exposure.

KEGG pathway (No.)	Differentially expressed genes	KEGG classification Level 1	KEGG classification Level 2
Ribosome (3010)	66	Genetic information processing	Translation
Apoptosis (4210)	13	Cellular processes	Cell growth and death
FcγR-mediated phagocytosis (4666)	6	Organismal systems	Immune system
Lysosome (4142)	9	Cellular processes	Transport and catabolism
Endocytosis (4144)	8	Cellular processes	Transport and catabolism

4.5.2 Candidate genes

The differentially regulated genes were searched for specific genes known to be associated with metal ion handling and stress response. Among these, heat shock proteins, cytochrome P450, and metallothionein 1 were up-regulated (Table 4.4).

Table 4-4. Differentially regulated genes identified by searching the DEG for terms associated with stress response with degree of differential expression and annotation details.

Gene	Log 2 fold change	Annotation and *transcript identification*
HSP90 domain containing protein	8.47	XP_004347209.1|/1.26e-12 (Nr) *Unigene66008*
Heat shock protein 70	8.04	CDJ62290.1|/1.53e-26 (Nr) *Unigene75839*
Metallothionein 1	7.97	HQ166888.1|/3e-07 (Nt) *Unigene37519*
Cytochrome P450 2J2	8.82	XP_005479013.1|/4.36586e-13 (Nr) *Unigene38673*
Cytochrome P450 II f2	8.22	AEO50743.1|/2.08e-57 (Nr) *Unigene28566*
Cytochrome P450, family 4	7.89	dmo:Dmoj_GI19140/2e-10 (KEGG) *Unigene65721*

The copper transporting ATPase (CuATPase) was investigated as a potential candidate gene and while not significantly upregulated, five unigenes and one cluster list were returned for genes that were annotated as CuATPase (Appendix 7). One transcript on the cluster list was significantly up-regulated, however, this was one of 2 transcripts out of 8 on the cluster list that were not annotated as CuATPase. Of the 13 transcripts that made up the cluster list and the unigenes annotated to this CuATPase, 1 was significantly up-regulated, 9 were non-significantly up-regulated and 3 were non-significantly down-regulated in the copper-exposed treatment. The 3 down-regulated transcripts were 3 out of the 4 least expressed transcripts. The expression of the CuATPase annotated transcripts in the control treatment added up to 24.2 FPKM, whereas the expression in the exposed group added up to 354 FPKM. Even though no transcript annotated as CuATPase was significantly upregulated in the copper-exposed treatment, there is evidence to indicate that CuATPase expression was higher in the snails exposed to copper.

A search of the list of the 77 genes most often found in differential transcriptomic expression studies in bivalve mollusc toxicology (Miao *et al.* 2015) found that 57 of the genes listed were present as annotated in this study and of those 9 were differentially expressed (Appendix 8). The differentially expressed genes not included in pathways or other candidate gene categories previously discussed, include integrin, myosin light chain kinase, perlucin and serine proteinase inhibitor.

4.5.3 Genes with differential expression greater than 10 Log_2fold

There were 13 genes up-regulated and 34 genes down-regulated more than 10 log_2fold (Appendix 9). Of the 47 transcripts, 14 transcripts were annotated (Figure 4.4). The annotated transcripts with the highest and lowest level of differential expression were myosin light chain kinase and excitatory amino acid transporter 2 respectively.

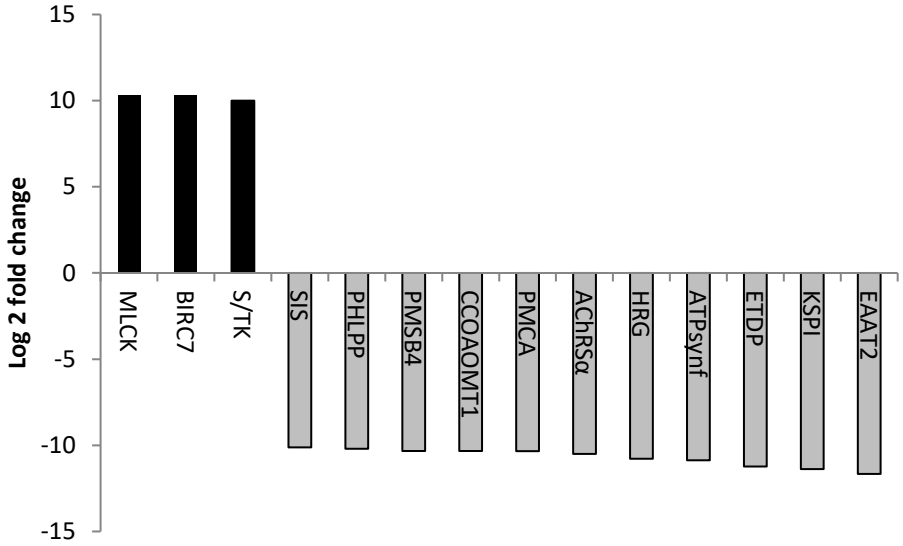


Figure 4-4. Change in gene expression between control and copper-exposed treatments of *I. newcombi*. Genes included are those differentially regulated greater than 10 log_2fold that were annotated. Annotation and transcript identification details are included (Appendix 5.9).

4.6 Discussion

4.6.1 Copper accumulation

The mean tissue copper concentration in *I. newcombi* was significantly greater in the copper-exposed snails than in the control snails after the three day exposure period. This indicates that *I. newcombi* is a net accumulator of copper and is able to accumulate copper rapidly. Dose-dependent uptake of copper has previously been shown in freshwater and terrestrial gastropods (Dallinger *et al.* 2005; Das and Khangarot 2011). A relatively short exposure period was chosen for this study as copper released into an aquatic environment quickly binds with dissolved organic matter and sediment particles causing bioavailable concentrations to rapidly return to original levels (Stevens *et al.* 2014). The short exposure period used in this study reflects exposure conditions likely to be associated with a copper spill or a pesticide application that would cause a relatively short increase in the bioavailable concentration of copper.

4.6.2 Lysosomal destabilisation

Lysosomal destabilisation was significantly greater in copper-exposed organisms. Lysosomal destabilisation is an established biomarker of general stress that has been widely measured in molluscs (Broeg *et al.* 2002; Edge *et al.* 2014; Moore *et al.* 2004; Taylor and Maher 2012b). Lysosomal destabilisation in gastropods has previously been shown to increase in a metal contaminated environment dominated by copper contamination (Ubrihien *et al.* 2017c). The range of lysosomal destabilisation in the control group is within the normal range indicative of

organisms not experiencing stress as described by Ringwood *et al.* (2003), while in the copper-exposed organisms all individuals had greater than 40% of cells with lysosome destabilisation indicative of stressed organisms. Lysosomal stability has been linked to contaminant-induced stress markers including DNA damage, lipid peroxidation, reduced scope for growth, reduced antioxidant capacity, apoptosis and reduced larval viability (Moore *et al.* 2006; Taylor and Maher 2012b). The increased lysosomal destabilisation in the copper-exposed organisms in this study provides a measure of organism condition as a baseline against which to assess differentially regulated genes that can be associated with stress responses.

4.6.3 The integrated response of *I. newcombi* to copper exposure

The tissue copper concentrations and lysosomal destabilisation results indicate that copper-exposed snails were taking up copper and that it was having adverse effects. To be useful in ecotoxicology, transcriptomic responses must be related to stress or specific contaminants. The integrated biological response framework proposed in Figure 1b was used to categorise transcriptomic responses associated with copper transport and usage, survival mechanisms and programmed cell death. This provides a logical framework for interpreting transcriptomic responses from an ecotoxicological perspective (Figure 4.5). Details of biological function and accompanying references for differentially expressed genes associated with pathways (Appendices 1, 2, 3, 4 and 5) as well as annotated genes differentially expressed greater than 10 $\log_2$fold (Appendices 9) are supplied.

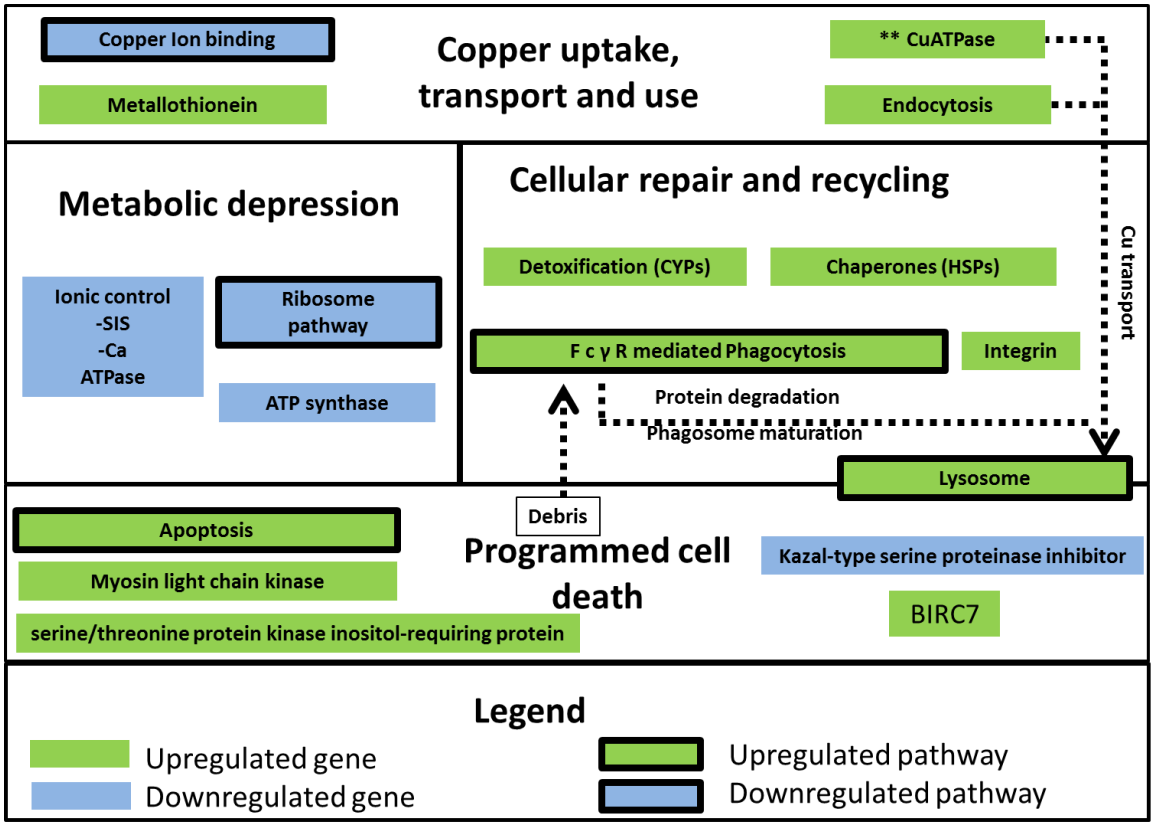


Figure 4-5. Integrated biological response model, based on transcriptomic responses of *Isidorella newcombi* exposed to copper. ** Indicates CuATPase was not significantly upregulated but included due to consistent up-regulation of transcripts annotated as this gene (See results section 4.5.2).

4.6.3.1 Copper uptake, transport and use

There was clear evidence of increased copper tissue concentrations in *I. newcombi* exposed to copper. Genes associated with copper-specific processes that were differentially regulated in the copper-exposed snails included genes that code for enzymes that use copper as a cofactor and facilitate the internal transport of copper. The expression of three genes; cytochrome *c* oxidase subunit II, cytochrome *c* oxidase copper chaperone and dopamine beta hydroxylase-protein associated with the gene ontology copper ion-binding function were down-regulated. This may indicate that under the high copper concentrations experienced the requirement for these genes is reduced or their efficiency is improved. There was evidence of the increased internal transport of copper in the exposed snails. Transcripts annotated as the copper-transporting ATPase which is responsible for much of the internal transport of copper across membranes of cells and organelles had consistently increased expression in the exposed

organisms, however, no individual transcript was significantly up-regulated. Several genes associated with the endocytosis KEGG pathway as well as the endothelial specific receptor tyrosine kinase were up-regulated. Endocytosis is the process used to transport excess copper to the lysosome for storage, as well as proteins for degradation and recycling (Goh and Sorkin 2013; Pryor and Luzio 2009; Varotto *et al.* 2013). Metallothionein 1 was also up-regulated and is associated with sequestration of metals and protection against metal toxicity (Carpenè *et al.* 2007). Overall there was evidence that genes coding for enzymes that use copper as a cofactor were down-regulated, but genes associated with the transport of copper generally were up-regulated in the copper-exposed organisms. These genes are potential candidates for markers of metal exposure and in some cases copper-specific responses.

4.6.3.2 Survival mechanisms

4.6.3.2.1 Metabolic depression

The down-regulation of genes associated with the ribosome pathway, ion pumping and the ATP synthase subunit f provide evidence of a reduction of metabolic rate as a response to stress in the copper-exposed organisms. The down-regulation of ribosome synthesis is associated with reduced metabolism in stressed organisms (Mayer and Grummt 2005). Ribosome biogenesis is both energy and resource demanding, and severe cellular stress can result in the shutdown of rRNA transcription (Golomb *et al.* 2014). The down-regulation of genes associated with the sodium influx stimulating peptide and calcium transporting ATPase which are associated with ionic homeostasis provide further evidence of a reduced metabolic state. Maintenance of ionic homeostasis is energetically expensive and has potential to be reduced in order to limit metabolic function in stressed organisms (Hand and Hardewig 1996). The down-regulation of the ATP synthase subunit f gene, involved in the production of ATP from ADP is likely to be associated with the reduced requirements for ATP in the copper-exposed snails due to reduced levels of activity associated with metabolic depression (Hand and Hardewig 1996). The reduced expression of genes associated with ribosome function, ion pumping and ATP generation provide candidate genes for transcriptomic markers of metabolic depression as a general stress response.

4.6.3.2.2 Cellular repair and recycling

In organisms stressed by contamination the processes of cellular repair and recycling are critical to limiting damage (Visick and Clarke 1995). The main components of the cellular repair and recycling effort evident in the copper-exposed organisms were lysosomal processes, phagocytosis, chaperone proteins and the phase 1 detoxification enzyme cytochrome P450. The

lysosome is an acidic organelle containing hydrolytic enzymes that plays a role in the digestion of a range of macromolecules including proteins, carbohydrates, nucleic acids and lipids, and is a regulator of cellular homeostasis (Appelqvist *et al.* 2013). Alpha-L-fucosidase, V-type ATPase, cathepsin F and cathepsin B, are likely to have been differentially regulated as they all perform various roles associated with acidification of the lysosome and degradation. The lysosome also plays an important role in copper storage and homeostasis (Polishchuk and Polishchuk 2016). The Fcγ-R mediated phagocytosis pathway was significantly enriched in the copper-exposed organisms. Within this pathway it was genes such as paxillin and cofilin that are associated with regulation of the actin cytoskeleton as part of the physical changes associated with phagocytosis, rather than the Fcγ receptor components that were differentially expressed. Phagocytosis is a multistep process that engulfs and degrades tissue debris resulting from apoptosis and is also crucial for tissue homeostasis and remodelling (Flannagan *et al.* 2012; Gitik *et al.* 2014). In the copper-exposed organisms it is likely that increases in phagocytosis is associated with engulfing and degrading cellular debris generated by apoptosis. The integrin α4 also had increased expression in the snails exposed to copper. This integrin tightly binds paxillin and has been linked to structural changes associated with phagocytosis (Liu *et al.* 2002), providing further evidence of increased phagocytosis in the copper-exposed organisms. Heat shock proteins (HSP) are a group of stress-inducible chaperone proteins involved in the folding and repair of damaged proteins (Leung *et al.* 2014). The increased expression of HSP70 and HSP90 provides evidence that repair is required for damaged proteins in the copper-exposed snails. Enzymes from the cytochrome P450 (CYP) family are responsible for the oxidative metabolism of a wide range of endogenous and exogenous substances (Rewitz *et al.* 2006). Transcripts from the CYP2 and CYP4 families of enzymes were found to be significantly up-regulated in response to copper exposure. The up-regulation of the CYP genes in the copper-exposed snails is likely to result from the breakdown of endogenous material associated with cellular damage and programmed cell death.

4.6.3.3 Programmed cell death

Apoptosis has a range of functions including the elimination of damaged cells (Elmore 2007). In the copper-exposed organisms, FAS receptor, FAS ligand (FASL), tumour necrosis factor apoptosis inducing ligand (TRAIL) and tumour necrosis factor apoptosis-inducing ligand receptor (TRAIL-R) are all part of the apoptosis KEGG pathway and were up-regulated. The FAS, FASL, TRAIL and TRAIL-R genes play a signalling role in this pathway and were amongst the most significantly up-regulated genes in the snails exposed to copper (Appendix

2), however, the genes associated with the functional process of cell death such as FAS-associated death domain and specific caspases were not differentially expressed. Within the apoptosis pathway, the inhibitor of apoptosis gene BIRC2/3 had reduced expression and BIRC7 had increased expression in the copper-exposed organisms. These genes are termed inhibitors of apoptosis, but BIRC7 shows both pro-apoptotic and anti-apoptotic activity (Saleem *et al.* 2013). The increased expression of myosin light chain kinase which has been shown to be involved in membrane blebbing associated with apoptotic processes is also additional evidence for increased apoptosis occurring in the snails exposed to copper (Mills *et al.* 1998). In addition to the genes relating directly to apoptosis, there is evidence of induction of genes that determine the fate of cells in stressed organisms. Kazal-type serine proteinase inhibitor (KSPI) was down-regulated. Reduction of KSPI has been shown to lead to autophagy and cell death as it allows greater proteolytic activity (Rimphanitchayakit and Tassanakajon 2010). The serine/threonine protein kinase inositol-requiring protein (IRE) was significantly upregulated in *I. newcombi* exposed to copper. Insitol requiring proteins and the protein kinases that regulate their function are associated with the choice between apoptosis and cell survival in stressed cells (Liu *et al.* 2015; Lu *et al.* 2013). The down-regulation of the PH domain and leucine rich repeat containing protein which is an apoptosis promoter does not support increased apoptosis occurring in the copper-exposed organisms (O'Neill *et al.* 2013). In addition, the increased expression of BIRC7 inhibitor of apoptosis protein indicates that there are anti-apoptotic as well as pro-apoptotic mechanisms occurring in the snails exposed to copper. The regulation of apoptosis and anti-apoptotic mechanisms in stressed organisms is complex and not fully understood (Portt *et al.* 2011). In the copper-exposed snails there were some indicators of anti-apoptotic processes, however, there is much more evidence for increased apoptosis occurring. Lysosomal membrane destabilisation accompanies apoptosis (Johansson *et al.* 2010) and the increase in lysosomal membrane destabilisation in the copper-exposed snails further supports increased apoptosis in this group.

4.6.3.4 Links between responses

The transcriptional changes in pathways and genes involved with apoptosis, the lysosome and phagocytosis indicates that linked processes are occurring (Figure 4.5). Increased apoptosis occurring in the copper-exposed snails would lead to an increase in cellular debris. As post apoptotic cells are engulfed by phagosomes this is likely to result in increased expression of genes related to phagocytosis. The changes in the regulation of genes associated with the lysosomal pathway, especially those associated with degradation of organic compounds, may

be partially explained by the phagosomes containing post-apoptotic matter maturing into phagolysosomes and degrading the cellular debris created by apoptosis.

4.6.3.5 Application of an integrated biological response model

Since RNA-seq was first used for ecotoxicology the interpretation of the data has been highly variable between studies. Models such as KEGG pathways and Gene Ontology that assist in understanding the biological function of differentially expressed genes, as well as adverse outcome pathways (Ankley *et al.* 2010) that link transcriptomic changes to higher levels of biological organisation have been used. Despite this, a framework for the interpretation of RNA-seq data from an ecotoxoicological perspective has not been developed. The discussion of every differentially expressed transcript in an RNA-seq study is impossible in a journal paper; in this study 2630 differentially expressed transcripts were identified. As a result, some subjectivity is required in choosing which differentially expressed genes to include in describing the response at a transcriptomic level. The categorisation of responses into a framework describing contaminant uptake and transport, survival mechanisms and programmed cell death provides a simple framework to assess global transcriptomic responses associated with contaminant exposure. This framework focusses the interpretation of transcriptomic response into contaminant-specific responses, mechanisms associated with cellular survival and changes associated with cell death as a response which are useful categories when identifying specific stressors and assessing the level of stress an organism is experiencing. Additionally, the use of a framework such as this may assist in the selection of candidate genes for other techniques such as qPCR. The integrated biological response model (Figure 4-5) along with the associated tables outlining gene expression and function provide an example of the application of this framework.

4.7 Conclusions

Copper tissue concentrations as well as lysosomal destabilisation results indicated that *I. newcombi* bioaccumulated copper and were stressed as a result of copper exposure. Transcriptomic responses to copper are evident in internal transport of copper, metabolic depression, cellular repair and recycling and programmed cell death. Genes associated with copper uptake and transport such as copper ion binding and endocytosis as well as metallothioneins offer high potential as contaminant-specific transcriptomic markers. Responses associated with changes in the expression of genes associated with the lysosome, apoptosis and phagocytosis offer high potential as transcriptomic markers of general stress. The use of an integrated biological response framework assists with the interpretation of complex RNA-seq data sets within the context of ecotoxicological investigations.

Chapter 5. Adaptive changes in the Cu response of *Isidorella newcombi* with a multi-generational history of Cu exposure: A mechanistic model based on changes in transcriptomic response

5.1 Introduction

Copper concentrations can become elevated in the environment through a range of human activities including mining, smelting, refining, manufacturing, the use of Cu-based pesticides and waste disposal (Wright and Welbourn 2002). In Australia, elevated Cu concentrations in the environment have been linked to mining, Cu pesticide use and industrial ports (Eriksen *et al.* 2001; Klessa *et al.* 1997; Merry *et al.* 1986; Stevens *et al.* 2014; Ubrihien *et al.* 2017c). Cu is a redox-active transition metal that is an essential element but exerts toxic effects at elevated concentrations (Gaetke and Chow 2003; Kim *et al.* 2008). The toxic effects associated with Cu exposure include interference with protein function due to non-specific binding to thiol groups, oxidative stress and alteration of normal metabolic activity (De Schamphelaere *et al.* 2007; Kakkar and Jaffery 2005; Livingstone 2001). The persistent nature of copper in the environment along with its repeated release in certain areas can result in multi-generational exposure of local populations to Cu.

The continuous or repeated exposure of a population to a contaminant can cause adaptations that result in increased tolerance to that contaminant (Whitehead *et al.* 2011). The response of organisms to adverse environmental conditions involves the use of specific and adaptive responses (Roelofs *et al.* 2010). It has been noted that organisms use plasticity to cope with environmental stressors that occur sporadically, but genetic adaptations occur to compensate for stressors that are experienced continuously (Whitehead *et al.* 2011). When exposed to Cu over multiple generations, aquatic species including *Lymnaea stagnalis, Daphnia magna* and *Tigriopus japonicas* have developed Cu tolerance (Bossuyt and Janssen 2004; Côte *et al.* 2015; Kwok *et al.* 2009).

The changes associated with increased tolerance to a contaminant are observed at the behavioural or metabolic level, however, they are driven by adjustments at the molecular level (Buckley *et al.* 2001). At a cellular level, response to contaminant-induced stress occurs through changes in; contaminant uptake and detoxification, regulation of metabolism, cell cycle progression, protein homeostasis, cytoskeletal organisation, vesicular trafficking and modification of enzyme activity (de Nadal *et al.* 2011; Morgan *et al.* 2007). Tolerance to contamination, therefore, is a result of an organism's ability to alter these essential processes

through changes in gene expression or protein function. The molecular response of an organism to environmental stress is controlled at a number of levels including transcription, regulation of translation, post-translational modification of proteins, protein degradation and allosteric regulation of proteins (Brinke and Buchinger 2017). The key to gaining a mechanistic understanding of changes associated with differential tolerance to chemicals is through understanding these changes and interactions at the molecular level (Schirmer *et al.* 2010). While there are a range of processes that control these functions, it has been suggested that the molecular mechanisms that support the adaptive change and phenotypic plasticity of an organism responding to contaminant-induced stress can be interpreted from the dynamics of its transcriptome (Leung *et al.* 2014).

An understanding of gene expression at the transcriptomic level provides insight into the changes in biological processes in response to environmental stressors. The transcriptome consists of all of the RNA molecules present in an organism at a particular point in time including mRNA. mRNA is the link between the genes which reside in the DNA and the proteins which determine biological function in organisms, and is indicative of the set of genes that are being actively expressed at a given time (Schirmer *et al.* 2010). The differential expression of genes at the transcriptomic level in populations with differing levels of tolerance to a contaminant can provide a mechanistic understanding of the differences at the molecular level that are associated with increased tolerance.

Isidorella newcombi exposed to elevated Cu concentrations over multiple generations were shown to develop Cu tolerance when compared to naïve snails (Sections 3.3.2.1 and 3.3.2.10). This provided an opportunity to investigate the differences in biological response to Cu exposure between populations with different levels of Cu tolerance. The aim of this study is to describe the mechanistic changes at the molecular level that provide the basis for differential Cu tolerance observed in *I. newcombi* with a multiple generation pre-exposure history to Cu. The transcriptomic gene expression in *Isidorella newcombi* with a multi-generational Cu exposure history and naïve snails are compared. The differentially expressed genes are then related to metabolic pathways and biological processes that differed between the pre-exposed and naïve snails to determine the mechanisms leading to increased tolerance in the pre-exposed snails. The differences between treatments are presented as an integrated biological model.

5.2 Methods

5.2.1 Experimental design

The snails used for RNA-seq analysis were taken from the F_3 generation of the intergenerational Cu exposure study (Figure 5-1). Experimental design and exposure conditions are described in section 3.2.2. The snails used were from the 0P-75E (naïve) and 75P-75E (pre-exposed) treatments (P represents the Cu exposure concentration in parental, F_1 and F_2 generations; E represents the Cu exposure concentration in the F_3 generation). Briefly, the snails used for the 75P-75E treatment had been exposed to 75 µg L^{-1} Cu for 3 days at 60 days of age in each of the parental, F_1 and F_2 generations. The snails from the 0P-75E had been maintained in uncontaminated water in the parental F_1 and F_2 generations. In the F_3 generation, the snails used for RNA-seq analysis were exposed for 3 days to 75 µg L^{-1} Cu at ~ 100 days of age. Each of the treatments had three replicates. The 0P-75E treatment that had no pre-exposure to elevated Cu concentrations will be referred to as naïve, and the 75P-75E treatment that was exposed to elevated Cu concentrations in the parental, F_1 and F_2 generations will be referred to as pre-exposed. From each treatment replicate RNA was extracted from three snails and pooled at the replicate level for RNA-seq analysis.

5.2.2 RNA extraction and sample preparation

RNA extraction and sample preparation methods were as described in section 4.3.4.1.

5.2.3 *De novo* assembly and annotation

de novo assembly and annotation methods were as described in section 4.3.4.2. Clean reads and contig sequences have been archived and are available at: https://figshare.com/s/01800f6f387725bb89f8

5.2.4 Analysis of differentially expressed genes

The method for analysis of differentially expressed genes is described in section 4.3.4.3. Differential expression is 75P-75E (pre-exposed) compared to 0P-75E (Naïve), and, as such, genes indicated as up-regulated have higher rates of transcription in the 75P-75E treatment than the 0P-75E treatment.

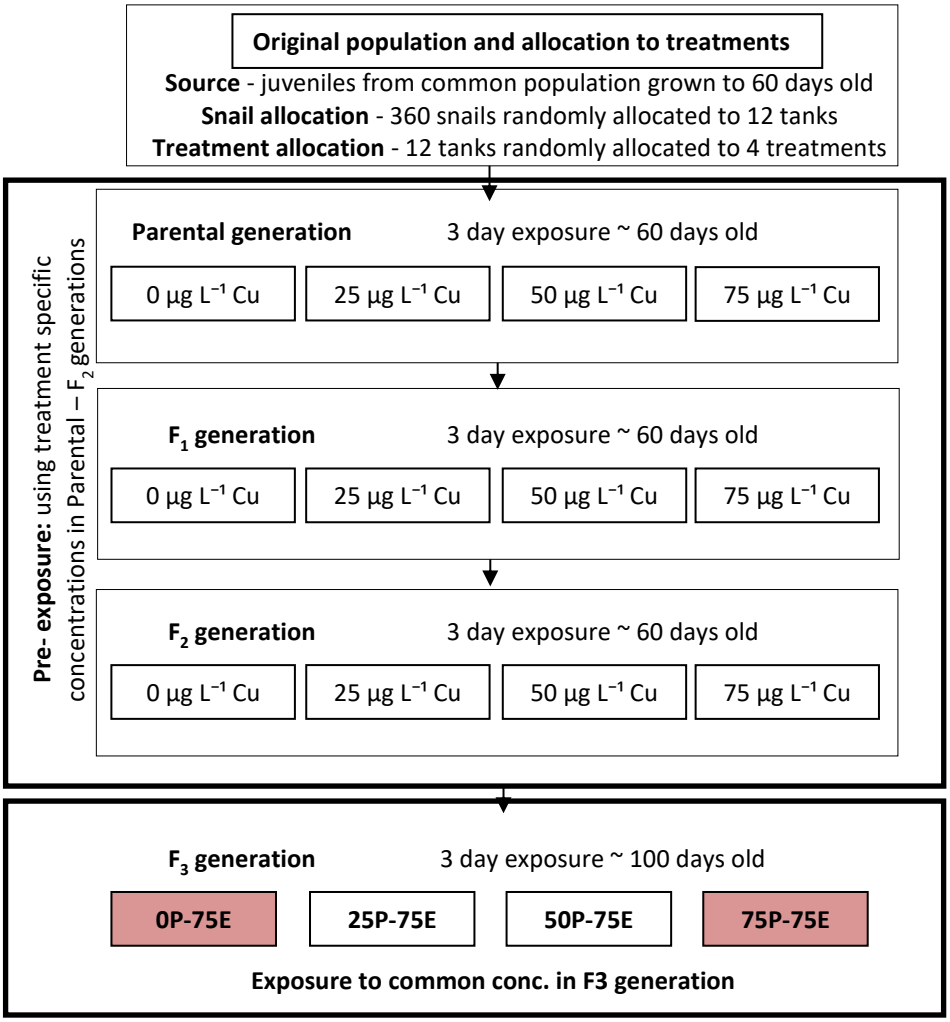


Figure 5-1. Experimental design for the multigenerational exposures of *Isidorella newcombi* to Cu. Treatment specific concentrations were used for the parental, F_1 and F_2 generations. In the F_3 generation all treatments were exposed to 75 µg L^{-1} Cu. Treatment labels for the in the F_3 generation, P = pre-exposure (µg L^{-1} Cu the treatment was exposed to in the parental, F_1 and F_2 generations), and E = exposure (µg L^{-1} Cu that the snails were exposed to in the F_3 generation). Treatments used for transcriptomic analysis in this chapter are coloured red.

5.3 Results

5.3.1 RNA sequencing and *de novo* transcriptome assembly

Transcript sequencing generated 255.38 Mb clean reads and *de novo* assembly resulted in 105,901 contigs (Table 5-1). There was a total of 1,067 differentially expressed contigs between the 0P-75E and 75P-75E treatments (Figure 5-2). A BLAST search (blast p; E-value < 10^{-5}) of protein and nucleotide databases (Nr, Nt, Swiss-prot, KEGG, COG and GO) provided functional annotations of 49878 of the 105901 contigs (Table 5-2).

Table 5-1. Quality parameters and number of reads for RNA-seq analysis of *Isidorella newcombi* exposed to 75 µg l^{-1} Cu for 3 days in the F_3 generation.

Total clean reads (Mb)	Q20 (%)	GC (%)	No. of contigs	Mean length (bp)
255.38	96.19	37.11	105901	395

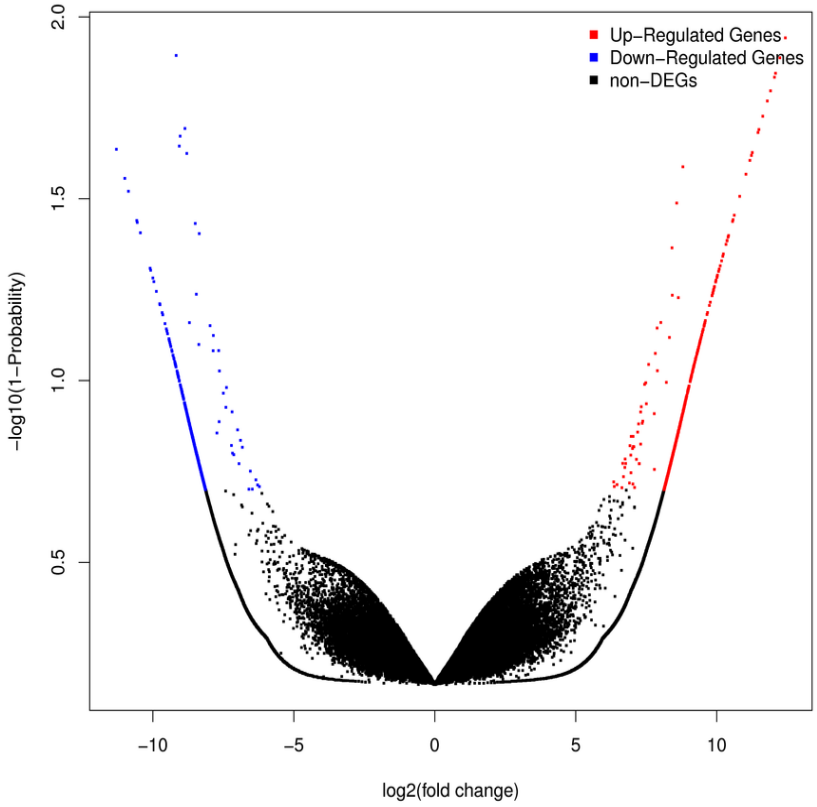


Figure 5-2. Volcano plot of gene expression in *Isidorella newcombi* with differing Cu exposure histories and exposed to 75 µg L^{-1} Cu for three days in the F_3 generation. Log_2 fold change in expression is plotted against $-\log_{10}(1 - \text{probability})$. Each point represents a contig, with contigs in blue down-regulated and contigs in red up-regulated (foldchange > 2 and probability > 0.8) in the snails with a pre-exposure history to Cu.

Table 5-2. Summary of annotation results from RNA-seq analysis of *Isidorella newcombi* with different Cu exposure histories exposed to 75 µg L^{-1} Cu for three days in the F_3 generation.

	Number of contigs	Percentage
All contigs	105,901	100
All annotated	49,878	47.09
Nr annotation	39,461	37.26
Nt annotation	36,014	34.01
Swiss-prot annotation	24,415	23.05
KEGG annotation	23,638	22.32
COG annotation	10,333	9.76
Interpro annotation	20,368	19.23
GO annotation	3,965	2.80

5.3.2 Differentially expressed genes and pathways

5.3.2.1 KEGG pathways

KEGG pathway analysis linked 79 differentially expressed genes to the functional processes described by 175 KEGG pathways (Kanehisa *et al.* 2008). These pathways were investigated and those chosen for discussion are; regulation of actin cytoskeleton, ribosome, RNA transport, phagosome, apoptosis and RNA polymerase (Table 5-3). These pathways were chosen based on their level of differential expression between the treatments and their relevance to response to Cu exposure.

Table 5-3. KEGG pathways chosen for investigation of the differences in response of *Isidorella newcombi* with different Cu exposure histories exposed to 75 µg L^{-1} Cu for three days in the F_3 generation.

KEGG pathway (no.)	Differentially expressed genes	KEGG classification level 1	Kegg classification level 2
Regulation of actin cytoskeleton (04810)	17	Cellular processes	Cell motility
Ribosome (03010)	10	Genetic information processing	Translation
RNA transport (03013)	19	Genetic information processing	Translation
Phagosome (04145)	13	Cellular processes	Transport and catabolism
Apoptosis (04210)	3	Cellular processes	Cell growth and death
RNA polymerase (3020)	2	Genetic information processing	Transcription

5.3.2.2 Individual differentially expressed genes

There were 1,003 differentially expressed genes (DEGs) that were not associated with one of the KEGG pathways that are being discussed. While all annotated genes were investigated, if genes were annotated as a vertebrate specific function, unspecified protein or the annotation could not be related to stress or metal exposure response, they were not included for discussion (Appendix 10). The results from the differentially expressed genes were separated based on functional categories including; metabolic activity, protection and repair, proteolytic function, immune function, transport and phagocytosis, and programmed cell death.

5.3.2.2.1 Metabolic activity

In relation to metabolic activity, the RNA polymerase (Table 5-4), RNA transport (Table 5-5) and ribosome (Table 5-6) KEGG pathways all had DEGs which indicated increased metabolic activity in the 75P-75E snails . In addition to these pathways there was differential expression of other genes associated with transcription and translation (Table 5-7), ionic transport (Table 5-8) and other metabolic functions (Table 5-9) that were related to differences in metabolism between treatments. The general pattern of expression indicated that 0P-75E snails had a lower rate of metabolic activity than the 75P-75E snails.

Table 5-4. Differentially expressed genes associated with the RNA polymerase KEGG pathway (KEGG 03020) in *Isidorella newcombi* with different Cu exposure histories exposed to 75 µg L^{-1} Cu for three days in the F_3 generation.

Gene (KEGG identification no.)	Log_2 fold change	*Transcript identification*
DNA-directed RNA polymerases I, II, and III subunit RPABC3 (K03016)	8.36	*Unigene241*
DNA-directed RNA polymerases I, II, and III subunit RPABC4 (K03009)	8.35	*CL4661.Contig2*

Table 5-5. Differentially expressed genes from the RNA transport pathway (KEGG 03013) in *Isidorella newcombi* with different Cu exposure histories exposed to 75 µg L^{-1} Cu for three days in the F_3 generation.

Gene (KEGG identification no.)	Log_2 fold change	*Transcript identification*
Elongation factor 1-alpha (K03231)	8.98	*Unigene 11901*
Translation initiation factor 2 subunit 3 (K03242)	8.98	*CL2169.Contig1*
Translation initiation factor 4A (K03257)	8.23	*Unigene21221*
Polyadenylate-binding protein (K13126)	8.50	*Unigene27676*
Serine/arginine repetitive matrix protein 1 (K13171)	8.71	*CL1786.Contig1*
THO complex subunit 2 (K012879)	-9.11	*Unigene34598*
Nucleoprotein TPR (K09291)	9.22	*Unigene12584*
Nucleoprotein TPR (K09291)	8.15	*Unigene3215*
Transcription initiation factor TFIID subunit 3 (K14650)	8.19	*Unigene70998*
Nucleoprotein TPR (K09291)	7.01	*CL554.Contig2*
Protein phosphatase 1 regulatory subunit 12A (K06270)	-8.34	*Unigen34070*
Apolipoprotein D and lipocalin family protein (K03098)	10.56	*Unigene70941*
Solute carrier family 39 (zinc transporter), member 7 (K14713)	8.39	*CL4015.contig11*
Peptidyl-tRNA hydrolase, PTH2 family (K04794)	8.39	*CL4015.Contig8*
Apolipoprotein D and lipocalin family protein (K03098)	7.20	*Unigene16218*
Ca^{2+} transporting ATPase, plasma membrane (K05850)	-10.44	*Unigene32360*
Nuclear pore complex protein Nup62 (K14306)	-9.76	*CL2123.Contig3*

Table 5-6. Differentially expressed genes from the ribosome pathway (KEGG 03010) in *Isidorella newcombi* with different Cu exposure histories exposed to 75 µg L^{-1} Cu for three days in the F_3 generation.

Gene (KEGG identification no.)	Log_2 fold change	*transcript identification*
Small subunit ribosomal protein S20e (K02969)	8.35	*Unigene1632*
Large subunit ribosomal protein L3e (K02925)	8.14	*Unigene57254*
Small subunit ribosomal protein S29e (K02980)	8.32	*Unigene12105*
Small subunit ribosomal protein S2e (K02981)	8.25	*Unigene71565*
Large subunit ribosomal protein LP1 (K02942)	8.18	*Unigene15019*
Large subunit ribosomal protein L21e (K02889)	9.88	*Unigene812*
Large subunit ribosomal protein L37e (K02922)	8.57	*CL12736.Contig1*
Small subunit ribosomal protein S3Ae (K02984)	8.18	*Unigene15156*
Small subunit ribosomal protein S28e (K02979)	8.99	*Unigene9200*
Large subunit ribosomal protein L27e (K02901)	8.40	*Unigene27359*

Table 5-7. Other differentially expressed genes related to transcription and translation in *Isidorella newcombi* with different Cu exposure histories exposed to 75 µg L^{-1} Cu for three days in the F_3 generation.

Gene	Log_2 fold change	Annotation and *transcript identification*
DNA-directed RNA polymerase II subunit 1 (RPB1)	8.41	XP_005113451.1 (Nr) *Unigene72918*
High mobility group AT-hook protein 2	8.70	mcf:102118074 (KEGG) *Unigene73199*
CCR4-NOT transcription complex subunit 11	8.36	XP_005102918.1 (Nr) *Unigene1632*
Pre-MRNA processing factor kinase 4 (PRP4)	9.58	bmor:101736377 (KEGG) *CL4606.Contig2*
Mediator of RNA polymerase II transcription subunit 16 (MED16)	-8.77	XP_005106027.1 (Nr) *Unigene7801*
Phenylalanyl-tRNA synthetase subunit beta	8.29	CDJ62264.1 (Nr) *Unigene72359*
Cytoplasmic tRNA 2-thiolation protein 2	7.15	Q55EX7 (Swissprot) *Unigene16605*
Midasin - like	-8.56	XP_005099547.1(Nr) *Unigene35237*
Dimethyladenosine transferase 1	-8.50	bfo:BRAFLDRAFT_125270 (KEGG) *CL3967.Contig1*

Table 5-8. Differentially expressed genes associated with ionic transport in *Isidorella newcombi* with different Cu exposure histories exposed to 75 µg L^{-1} Cu for three days in the F_3 generation.

Gene	Log_2 fold change	Annotation and *transcript identification*
Calcium binding protein 1 (CBP1)	6.34	AAV91525.1 (Nr) *Unigene5269*
Transient receptor potential cation channel subfamily V member 6 (TRPV6)	8.27	XP_005107388.1 (Nr) *Unigene72014*
Sodium influx stimulating peptide (SIS)	6.74	P42579.1 (Nr) *CL10743.Contig2*

Table 5-9. Other differentially expressed genes associated with general metabolic function in *Isidorella newcombi* exposed to Cu in the F_3 generation.

Gene	Log_2 fold change	Annotation and *transcript identification*
ATPase synthase F0 subunit 6	8.21	AFQ96942.1 (Nr) *Unigene24695*
Sugar transporter	8.45	XP_002785881.1 (Nr) *CL12840.Contig1*
Panthonate kinase 4	-9.21	XP_005102785.1 (Nr) *Unigene34814*

5.3.2.2.2 Protection and repair

Increased protection and repair mechanisms in the pre-exposed snails were indicated by a trend of up-regulation of transcripts that were annotated to biological processes associated with cellular protection and repair (Table 5-10).

Table 5-10. Differentially expressed genes associated with protection and repair in *Isidorella newcombi* with different Cu exposure histories exposed to 75 µg L^{-1} Cu for three days in the F_3 generation.

Gene	Log_2 fold change	Annotation and *transcript identification*
Intestinal mucin-2	6.96	acs:100563509 (KEGG) *Unigene2594*
Intestinal mucin-2	8.20	xtr:100493286 (KEGG) *Unigene43123*
Telomere associated protein RIF1 (RIF1)	8.25	XP_005103956.1 (Nr) *Unigene10688*
Leucine-zipper-like transcriptional regulator 1	7.79	EKC25786.1 (Nr) *Unigene20051*

5.3.2.2.3 Proteolytic function

Increased proteolytic function in the pre-exposed snails was evident by a general pattern of down-regulation of proteinase inhibitors and up-regulation of proteinases (Table 5-11).

Table 5-11. Differentially expressed genes associated with proteolytic function in *Isidorella newcombi* with different Cu exposure histories exposed to 75 µg L^{-1} Cu for three days in the F_3 generation.

Gene	Log_2 fold change	Annotation and *transcript identification*
Serine proteinase inhibitor dipetalogastin	-9.67	XP_975339.2 (Nr) *Unigene 44516*
Four domain proteinase inhibitor	-9.06	GAA47606.1 (Nr) *CL5047.contig1*
serine proteinase inhibitor antistasin	-6.89	XP_005092668.1 (Nr) *CL2868.Contig2*
kazal-type protease inhibitor 3	-8.86	ACL36282.1 (Nr) *CL5047.contig3*
serine protease 56	8.74	XP_004914443.1 (Nr) *Unigene29511*
transmembrane protease serine 9	8.90	ola:101157216 (KEGG) *Unigene27336*

5.3.2.2.4 Immune function

There was a general trend of increased regulation of genes associated with immune function. The majority of DEGs associated with immune function were up-regulated. The only exception to this were two transcripts annotated to interleukin 17 that were down-regulated (Table 5-12).

Table 5-12. Differentially expressed genes associated with immune function in *Isidorella newcombi* with different Cu exposure histories exposed to 75 µg L^{-1} Cu for three days in the F_3 generation.

Gene	Log 2 fold change	Annotation and *transcript identification*
Fibrinogen-related protein 14	8.19	ABO61860.1 (Nr) *Unigene21827*
GTPase IMAP family member 7	8.54	Q8NHV1 (Swissprot) *CL4394.Contig2*
Multi drug resistance protein	8.24	ABO36618.1 (Nr) *Unigene44186*
Interleukin 17C (IL17C)	-8.50	lgi:LOTGIDRAFT_152641 (KEGG) *Unigene33218*
interleukin 17D (IL17D)	-10.1	xma:102231184 (KEGG) *Unigene18488*

5.3.2.2.5 Vesicle mediated transport

There was evidence of difference in expression of genes related to vesicle transport. There was a general trend of increased expression of genes associated with the regulation of action cytoskeleton KEGG pathway with nine of thirteen differentially expressed transcripts that are associated with this pathway being up-regulated (

Table 5-13). The major area of changes in gene expression linked to changes in the actin cytoskeleton related to the phagosome KEGG pathway (Table 5-14). The general trend for this pathway was increased regulation, with eight of the twelve differentially expressed transcripts being up-regulated.

Table 5-13. Differentially expressed genes from the regulation of the actin cytoskeleton pathway (KEGG 04810) in *Isidorella newcombi* with different Cu exposure histories exposed to 75 µg L^{-1} Cu for three days in the F_3 generation.

Gene (KEGG identification no.)	Log_2 fold change	*transcript identification*
Wiskott-Aldrich syndrome protein (K05747)	10.01	*Unigene57921*
Actin beta/gamma 1 (K05692)	8.21	*Unigene5813*
Cofilin (K05765)	8.30	*Unigene23824*
Myosin-light-chain kinase (K00907)	8.84	*Unigene42607*
Myosin regulatory light chain 12 (K12757)	8.53	*Unigene57018*
Tyrosine-protein kinase Lyn (K05854)	-8.56	*Unigene26507*
Vinculin (K05700)	-8.29	*Unigene35524*
Ficolin (K10104)	9.07	*Unigene22151*
Tumor necrosis factor ligand superfamily member 10 (K04721)	8.32	*CL4582.contig2*
Killer cell lectin-like receptor subfamily D member 1 (K06516)	8.21	*CL12390.Contig2*
Contactin 1 (K06759)	8.29	*Unigene25707*
Collagen, type VI, alpha (K06238)	-8.63	*Unigene34382*
Collagen, type VI, alpha (K06238)	-8.58	*Unigene36043*

Table 5-14. Differentially expressed genes associated with the phagosome pathway (KEGG 04145) in *Isidorella newcombi* with different Cu exposure histories exposed to 75 µg L^{-1} Cu for three days in the F_3 generation.

Gene (KEGG identification no.)	Log_2 fold change	*transcript identification*
Actin beta/gamma 1 (K05692)	8.21	*Unigene5813*
V-type H+-transporting ATPase subunit a (K02154)	7.25	*CL2472.Contig4*
Killer cell lectin-like receptor subfamily D member 1 (K06516)	8.21	*CL12390.Contig2*
Integrin beta 3 (K06493)	11.04	*CL1172.Contig4*
Ficolin (K10104)	9.07	*Unigene22151*
Sialophorin (K06477)	-7.86	*CL1172.Contig3*
Sialophorin (K06477)	8.41	*CL1172.Contig2*
Disintegrin and metalloproteinase domain-containing protein 17 (K06059)	8.39	*CL88.contig2*
Contactin 1 (K06759)	8.29	*Unigene25707*
Integrin beta 1 (K05719)	-8.63	*Unigene34382*
Integrin beta 1 (K05719)	-8.58	*Unigene36043*
Mannose receptor, C type (K06560)	-8.21	*CL9068.Contig3*

5.3.2.2.6 Programmed cell death

The DEGs associated with programmed cell death indicated that there was a reduction of apoptosis occurring in the 75P-75E treatment compared to the 0P-75E treatment. While three genes associated with the apoptosis pathway were up-regulated in the pre-exposed snails, two of these genes were inhibitors of apoptosis (Table 5-15). Other genes linked to apoptosis were down-regulated, with the exception of multiple transcripts that were annotated to the PH domain and leucine-rich repeat-containing protein phosphatase (Table 5-16).

Table 5-15. Differentially expressed genes associated with the KEGG apoptosis pathway (KEGG 4210) in *Isidorella newcombi* with different Cu exposure histories exposed to 75 µg L^{-1} Cu for three days in the F_3 generation.

Gene (KEGG identification no.)	Log_2 fold change	*Transcript identification*
Tumour necrosis factor ligand supefamily 10 (TRAIL) (K04721)	8.32	*CL4582.Contig2*
Baculoviral IAP repeat-containing protein 7/8 (BIRC7/8) (K16061)	8.30	*Unigene12071*
E3 ubiquitin-protein ligase XIAP (XIAP) (K04725)	7.09	*CL2271.contig2*

Table 5-16. Other differentially expressed genes associated with apoptosis in *Isidorella newcombi* with different Cu exposure histories exposed to 75 µg L^{-1} Cu for three days in the F_3 generation.

Gene	Log_2 fold change	Annotation and *transcript identification*
tumour protein P53- inducible protein 11 (TP53I11)	-8.73	XP_005109031.1 (Nr) *Unigene46783*
NmrA-like family domain-containing protein	-8.23	Q8K2T1 (swissprot) *Unigene34367*
PH domain and leucine-rich repeat-containing protein phosphatase	8.37	dre:100331906 (KEGG) *CL2815.Contig1*
PH domain and leucine-rich repeat-containing protein phosphatase	8.88	dre:100331906 (KEGG) *Unigene8209*
PH domain and leucine-rich repeat-containing protein phosphatase	9.22	dre:100331906 (KEGG) *CL761.Contig1*
PH domain and leucine-rich repeat-containing protein phosphatase	9.35	dre:100331906 (KEGG) *CL1410.Contig3*

5.4 Discussion

The differentially expressed genes were grouped into 7 categories to assist in describing the major changes in biological responses between the pre-exposed and naïve snails. These are metabolic activity, protection and repair, proteolytic function, immune function, vesicle-mediated transport and programmed cell death (Figure 5-3). While there is clear weight of evidence for these responses in the gene expression from this study, it must be noted that there are still limitations with the use of RNA-seq in non-model organisms. When de novo assembly is used there are limitations associated with not all contigs being annotated (Hornett and Wheat 2012). As only 47.09 % of contigs were annotated in this study, the function of many of the differentially expressed genes remains unknown. As a result, some but not all the genes

associated with pathways and biological functions are known to be differentially regulated in the snails. Due to this the responses were grouped into the 7 categories discussed and these have multiple changes in gene regulation that provide evidence for the response that is described.

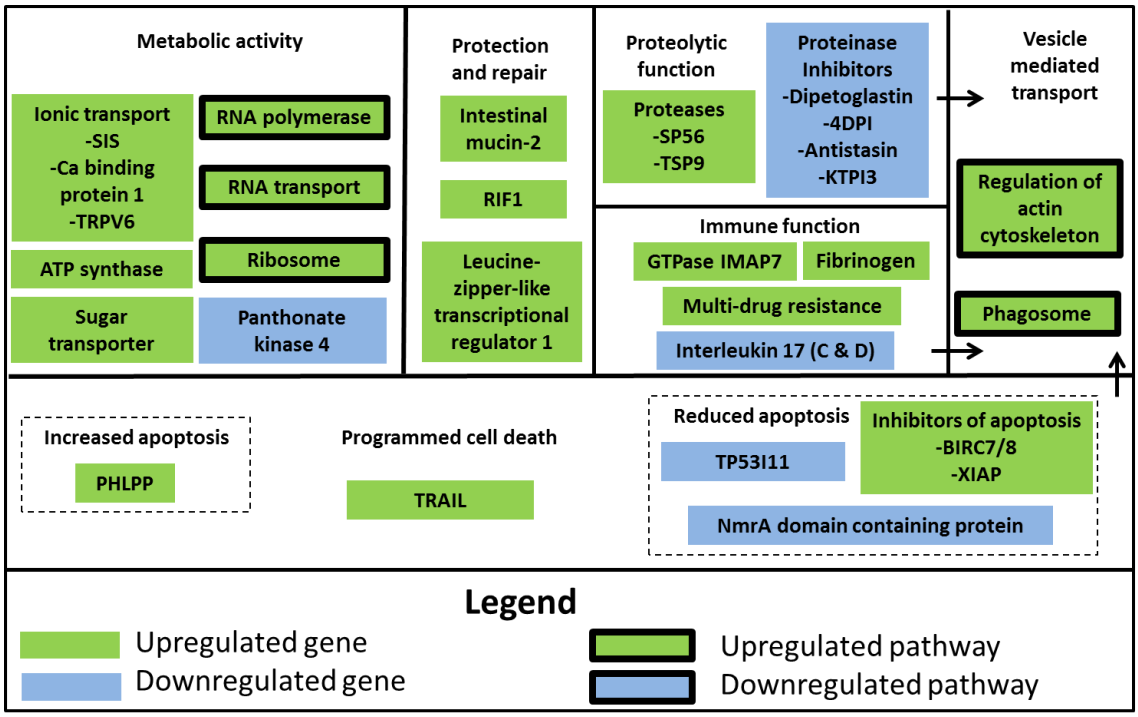


Figure 5-3. Integrated biological model indicating differences in gene expression to a 3 day exposure to 75 µg L^{-1} Cu exposure between *Isidorella newcombi* with multi-generational Cu exposure history and naïve snails. Differences in regulation are based on the relative transcriptomic expression between pre-exposed and naïve treatments. SIS = sodium influx stimulating peptide, TRPV6 = transient receptor potential channel subfamily V member 6, RIF1 = telomere associated protein RIF1, SP56 = serine protease 56, TSP9 = transmembrane protease serine 9, 4DPI = four domain proteinase inhibitor, KTPI3 = kazal type protease inhibitor 3, GTPase IMAP7, PHLPP = PH domain and leucine-rich repeat-containing protein, TP53I11 = tumour protein 53 – inducible protein 11, BIRC 7/8 = Baculoviral IAP repeat-containing protein 7/8, XIAP = E3 ubiquitin-protein ligase XIAP.

5.4.1 Metabolic activity

5.4.1.1 Transcription and translation

Transcription and RNA transport

There was evidence of increased expression of genes associated with transcription, transport of RNA molecules and translation in the pre-exposed snails. The RNA polymerase pathway had two differentially expressed genes, both of which were up-regulated (Table 5-4). In addition to genes specifically identified with the KEGG pathway analysis, several genes with functions relevant to transcription were also up-regulated. These included; DNA-directed RNA

polymerase II subunit 1 (RPB1), high mobility group AT-hook protein 2, pre-MRNA processing factor kinase 4 (PRP4) and CCR4-NOT transcription complex subunit 11(Table 5-7). RPB1 contains regulatory sites that are phosphorylated to regulate transcription initiation, elongation, termination and mRNA processing (Hahn 2004). High mobility AT–hook proteins allow the transcription of genes by both reducing the compactness of DNA to histones and as part of the enhanceosome that binds DNA, allowing transcription of certain genes (Ozturk *et al.* 2014). PRP4 is a serine/threonine kinase that is known for its role in pre-mRNA splicing and has been investigated in its role in alternate splicing associated with cell proliferation (Bates *et al.* 2017). The CCR4 Not complex is involved in the regulation of RNA metabolism at all steps from synthesis to decay (Collart 2016). A single transcript that was annotated to transcription activities was down-regulated in the pre-exposed population, the mediator of RNA polymerase II transcription subunit 16 (MED16) (Table 5-7). This gene is part of the mediator complex which is required for the DNA-binding transcription factors that control gene expression in response to environmental cues (Poss *et al.* 2013). MED16 has been specifically associated with the expression of heat shock proteins (HSPs) through the attachment of RNA polymerase II to HSP encoding genes (Kim and Gross 2013). HSPs are chaperone proteins that play a role in the repair of damaged proteins (Liu and Chen 2013). This may indicate that despite the general trend of increased expression of genes associated with transcription in snails with a multi-generational exposure history to Cu, the expression of genes such as MED16 that are associated with the transcription of genes linked to specific functions (ie. HSPs) were down-regulated. Despite the change in expression of this gene that has been linked to the initiation of HSP transcription, no differential expression of genes that specifically code for HSPs was detected. In addition to an increase in gene expression associated with transcription there was also evidence of increase in the transport of RNA. The RNA transport KEGG pathway had 17 differentially expressed genes, 13 of which were upregulated (Table 5-5). The results indicated that there was increased expression of genes associated with the transcription processes in the pre-exposed snails.

Translation

Ten significantly differentially expressed genes were identified in the KEGG ribosome pathway, all of which were upregulated in the pre-exposed snails (Table 5-6). In addition to genes specifically associated with the KEGG pathway there were two up-regulated genes; phenylalanyl-tRNA synthetase subunit beta and cytoplasmic tRNA 2-thiolation protein 2, and

two down-regulated genes; midasin and dimethyladenosine transferase 1, whose annotated function could be related to translation (Table 5-7). Phenylalanyl-tRNA synthetase subunit beta selectively couples amino acids to their cognate nucleic acid adaptor molecules during translation, facilitating polypeptide extension (Finarov *et al.* 2010). The cytoplasmic tRNA 2-thiolation protein 2 is involved in the 2-thiolation of tRNAs to ensure proper decoding of the mRNA by the ribosome (Dewez *et al.* 2008). The midasin protein is involved in the transport and maturation of the 60s ribosomal subunit (Romes *et al.* 2016). The dimethyladenosine transferase 1 is required for pre rRNA processing reactions that lead to the synthesis of 18S rRNA (Zorbas *et al.* 2015). While the last two transcripts mentioned are an exception, overall the transcriptomic results indicate that there was a general trend of up-regulation of genes associated with translation in the pre-exposed snails.

The changes in transcription, RNA transport and translation may be indicative of differing metabolic rates between in the snails from the two treatments. Reductions in transcription have been associated with stress-induced metabolic depression and, while the overall amount of mRNA in metabolically depressed organisms does not alter, there is a reduction in the rate of transcription in organisms with a reduced metabolic rate (Storey and Storey 2004). This is also true of translation, with ribosome biogenesis being energy and resource demanding, organisms experiencing severe stress have been reported to shut down rRNA transcription as a mechanism to reduce metabolic function (Golomb *et al.* 2014). For transcription, RNA transport and translation, the evidence indicates reduced activity in the naïve snails.

5.4.1.2 Ionic transport

Three genes that code for proteins involved in ionic regulation; calcium binding protein 1 (CBP1), transient receptor potential cation channel subfamily V member 6 (TRPV6) and sodium influx stimulating peptide (SIS) were up-regulated in the pre-exposed snails (Table 5-8). Calcium binding proteins play an important role in calcium-mediated cellular signal transduction. CBP1 regulates the gating of voltage-gated calcium ion channels (Oz *et al.* 2011). TRPV6 is a highly selective calcium ion transport channel that is essential for the maintenance of ionic homeostasis and metabolism (Stoerger and Flockerzi 2014). The SIS is associated with the homeostasis of sodium (Salzet and Verger-Bocquet 1996). The reduction in the energetically expensive process of ionic homeostasis has been reported to be a strategy used to limit metabolic function in stressed organisms (Hand and Hardewig 1996). The naïve snails

had lower expression of genes associated with ionic regulation, suggesting that these processes had been down-regulated to limit metabolic function.

5.4.1.3 Other metabolism-related changes

There were three other DEGs that were annotated to biologically important proteins that provide further insight into the differences in metabolic response between the snails that came from a population that had previously been exposed to copper and those that do not (Table 5-9). ATPase synthase F0 subunit 6 was up-regulated in the pre-exposed snails. The ATP synthase F0 is associated with rotation in ATP synthase C which generates cellular energy in the form of ATP (Sambongi *et al.* 1999). The sugar transporter was also up-regulated in the pre-exposed snails. In multicellular organisms sugars play essential roles in a broad range of functions, including use in carbon skeletons, as osmolytes, signalling, transient energy stores, and transport molecules, with sugar transporters functioning to mediate uptake or release from cells or cellular compartments (Chen *et al.* 2015). The regulation of these genes generally supports the trend of increased metabolism in the pre-exposed snails. Conversely, panthonate kinase 4 which plays an important role in the synthesis of coenzyme A was down-regulated in the pre-exposed snails (Rock *et al.* 2000). Coenzyme A has a broad range of biological functions including involvement in, or signalling for cell growth, proliferation, energy generation, balance of anabolic and catabolic reactions and autophagy (Pietrocola *et al.* 2015). Coenzyme A is also known to have different expression patterns between tissues (Pietrocola *et al.* 2015, and references therein). While coenzyme A is important in a wide range of biological functions, the reduced expression of the associated panthonate kinase 4 in pre-exposed snails does not follow the general trend of increased metabolic function in these snails. It is possible that the increased expression of the coenzyme A linked panthonate kinase 4 in the digestive gland of naïve snails is a result of a tissue specific function in the digestive gland. Due to the broad range of biological functions of coenzyme A, it is difficult to isolate a single process that is associated with the change in expression observed.

5.4.2 Protection and repair

The pre-exposed snails had increased expression of genes related to protection and repair mechanisms. Intestinal mucin-2, telomere associated protein RIF1 (RIF1) and the leucine-zipper-like transcriptional regulator 1, were up-regulated (Table 5-10). The increased expression of mucin related genes in response to exposure to elevated metal concentrations has been recognised in invertebrates (Rayms-Keller *et al.* 2000). Mucins are an important part of

the digestive gland epithelial layer that serves to protect the digestive epithelium (Rayms-Keller *et al.* 2000). RIF1 is involved in telomere length maintenance, DNA replication, DNA repair and the maintenance of general DNA integrity (Kumar and Cheok 2014). RIF1 is reported to be important in the repair of damaged DNA (Buonomo *et al.* 2009). The specific function of the leucine-zipper-like transcriptional regulator 1 remains uncertain, however, it comes from a protein family reported to interact with actin to regulate cellular structure as well as being involved in the down-stream regulation of detoxification and antioxidant proteins (Nacak *et al.* 2006). The expression of these genes indicates that the pre-exposed snails had an increased ability to regulate genes to protect against and repair Cu- induced damage.

5.4.3 Proteolytic function

There was evidence of increased proteolytic function in the pre-exposed snails through the reduced expression of proteinase inhibitors and the increased expression of proteinases (Tbale 5-11). Proteinases are enzymes that catalyse the hydrolysis of peptide bonds (Ramachandran *et al.* 2012). Allosteric regulation of proteinases can occur through the binding of proteinase inhibitors at allosteric sites of the protein, rendering the protein inactive (Hauske *et al.* 2008). Changes in the regulation of proteinase inhibitors will affect the level of activity of the proteinases in an organism. The proteinase inhibitors dipetaloglastin, the four domain proteinase inhibitor, serine proteinase inhibitor antistasin and kazal-type protease inhibitor 3 were all down-regulated. Dipetaloglastin is a serine proteinase inhibitor linked to the inhibition of thrombin, trypsin and plasmin (Mende *et al.* 1999). The four domain proteinase inhibitor and kazal-type protease inhibitor 3 are forms of kazal-type proteinase inhibitors (KSPIs). KSPIs have been linked to autophagy and cell death as they allow increased proteolytic activity (Rimphanitchayakit and Tassanakajon 2010). Antistasin is known to suppress the proteolytic activity of serine proteases (Yan *et al.* 2016). The down-regulation of these proteinase inhibitors would be indicative of reduced suppression of proteinases and, therefore, increased degradation of proteins in the pre-exposed snails. The serine protease 56 and transmembrane protease serine 9 were both up-regulated. While the specific function of these proteases is uncertain they provide further evidence of increased proteolytic activity in the pre-exposed snails.

5.4.4 Immune response

In invertebrates the immune system has been demonstrated to respond to a broad range of environmental challenges including metal contamination (Ellis *et al.* 2011). Three genes with

immune related function; fibrinogen-related protein 14, GTPase IMAP family member 7 and the multi-drug resistance protein were up-regulated in the pre-exposed snails (Table 5-12). Fibrogen related proteins are linked to the activation of the complement system which enhances immune function and phagocytic clearance of damaged cells in invertebrates (Hanington and Zhang 2011). GTPase IMAP proteins have been related to immune function and associated with changes in apoptosis and development (Schwefel *et al.* 2013). GTPase IMAP proteins have been reported to be differentially regulated in the marine bivalves *Mytilus galloprovincialis* fed with toxic dinoflagellates and *Crassostrea gigas* experiencing salinity stress (Gerdol *et al.* 2014; Zhao *et al.* 2012). Multi-drug resistance proteins are transport proteins that remove xenobiotics from the cytoplasm directly as well as after conjugation by phase II enzymes and play an import role in detoxification and cellular homeostasis, as well as in defence against oxidative stress (Luedeking *et al.* 2005). The only genes associated with similar functions that showed the opposite of this trend in the pre-exposed snails were associated with interleukin 17. Interleukin 17C and interleukin 17D were both down-regulated (Table 5-12). Interleukin 17 is a pro-inflammatory cytokine that is known to have important roles in the clearance of extracellular bacteria and is also associated with autoimmune and allergic conditions (Li *et al.* 2014). The reason for the down-regulation of interleukin 17 related genes is uncertain, but overall there was increased expression of genes associated with detoxification, immune function, cellular protection and cellular repair in the pre-exposed snails.

5.4.5 Regulation of the actin cytoskeleton and phagocytosis

The regulation of actin cytoskeleton KEGG pathway, had thirteen differentially expressed transcripts associated with twelve genes (Table 5-13). Many important biological processes including embryonic morphogenesis, immune surveillance, angiogenesis and tissue repair and regeneration require changes in the actin cytoskeleton (Lee and Dominguez 2010). The phagosome KEGG pathway had twelve differentially expressed genes (Table 5-14). There were six genes that were differentially expressed that were common to both the regulation of actin cytoskeleton pathway and the phagosome pathway. Phagocytosis is facilitated by changes in the actin cytoskeleton, and while changes to the actin cytoskeleton are required for many biological processes, the changes observed in phagocytosis are likely to be a major reason for the differential expression of the genes associated with the regulation of actin cytoskeleton pathway (May and Machesky 2001). The general up-regulation of genes associated with both of these pathways suggests in increase in regulation of actin cytoskeleton and phagosome

function in the pre-exposed snails. While phagocytosis is predominantly associated with pathogens in vertebrates, in invertebrates phagocytosis is used to respond to a wide range of toxicants including metals (Galloway and Depledge 2001).The process of phagocytosis is an important part of the mollusc immune-toxic defence system with haemocytes using phagocytosis as a means of removing contaminants, pathogens, apoptotic cells and tissue debris from the haemolymph (Dupuy and Caron 2008; Gitik *et al.* 2014; Ladhar-Chaabouni and Hamza-Chaffai 2016). In molluscs, the use of phagocytosis to sequester metals by molluscan digestive cells has been reported (Marigómez *et al.* 2002). In snails experiencing Cu-induced stress, it is likely that phagocytosis is used to sequester copper and remove cellular debris.

5.4.6 Apoptosis

In general there was evidence of reduced apoptotic function in the pre-exposed snails. In the apoptosis KEGG pathway, three genes, tumour necrosis factor ligand superfamily 10 (TRAIL), baculoviral IAP repeat containing protein 7/8 (BIRC7/8) and the E3 ubiquitin ligand XIAP were up-regulated (Table 5-15). BIRC7/8 and XIAP are both inhibitors of apoptosis (Finlay *et al.* 2017). TRAIL has been reported to bind to five different cell receptors and, depending on the specific receptor, can result in cell survival or apoptosis (Azahri and Kavurma 2013). In addition to the genes directly related to the apoptosis pathway there was further evidence of reduced apoptosis in the snails that had a multigenerational exposure history to Cu. The tumour protein P53- inducible protein 11 (TP53I11), and an NmrA-like family domain-containing protein were both down-regulated (Table 5-16). The expression of TP53I11 has been associated with the activation of tumour suppressor p53 and increased apoptosis (Liang *et al.* 2004). NmrA-like family domain-containing proteins have been reported to assist in redox balance by acting as an NAPDH sensor, with expression associated with the induction of apoptosis (Zhao *et al.* 2008). The down-regulated genes and changes to the genes annotated to the apoptosis KEGG pathway provide evidence of a relative reduction of apoptosis occurring in the pre-exposed snails. Conversely, the up-regulation of four transcripts annotated to the PH domain and leucine-rich repeat-containing protein phosphatases (PHLPP) and the down-regulation of protein phosphatase 1D (PP1D) provide evidence of increased apoptosis in the pre-exposed snails (Table 5-16). PHLPP is known to suppress cell survival both by inhibiting proliferative pathways and by promoting apoptotic pathways (O'Neill *et al.* 2013). PP1D has been associated with a feedback loop that leads to the proteolysis of tumour suppressor p53 and an associated reduction in apoptosis (Lu *et al.* 2007). The regulation of apoptotic mechanisms have been reported to be complex and not fully understood (Portt *et al.* 2011). Overall the

evidence suggests a reduction of apoptosis in the pre-exposed snails. In addition to the transcriptomic evidence, the increase in lysosomal membrane destabilisation in the naïve snails (Section 3.3.2.10) provides further support for increased apoptosis in naïve snails. It has been reported that increased lysosomal membrane destabilisation occurs as part of the apoptosis process (Johansson *et al.* 2010).

5.4.7 Summary of responses

Major differences between pre-exposed and naïve snail responses to 75 µg L^{-1} Cu were evident in the transcriptional regulation of genes associated with metabolic depression, protection and repair mechanisms, proteolytic function, immune function, phagocytosis, and apoptosis (Figure 5-3). In the pre-exposed snails there was evidence of higher metabolic activity compared to the naïve snails. Animals faced with severe environmental challenges can retreat into a reduced metabolic state until conditions are conducive to active life (Storey and Storey 2004). The process of metabolic depression involves the suppression of energetically expensive biological processes. Storey and Storey (2004) suggest that reduced expression of genes associated with ribosome function, ion pumping and ATP generation are transcriptomic markers of metabolic depression in stressed organisms. In the current study the analysis of the differentially expressed genes indicated that the pre-exposed organisms had relatively higher rates of ribosomal function, ionic transport and ATP generation. This indicates that pre-exposed snails had an increased ability to maintain metabolic rates when exposed to elevated concentrations of Cu.

The differential expression of genes associated with proteolytic function, phagocytosis, and other cellular protection and repair mechanisms indicate that different cellular survival and repair mechanisms are used by the pre-exposed and naïve snails. The increased expression of genes related to phagocytic processes in these snails indicates that they have an increased ability to remove toxicants and debris through phagocytosis. There was also evidence of increased proteolytic function in the pre-exposed snails. The repair of stress-induced damage or the degradation and replacement of heavily damaged macromolecules in stressed organisms can allow cellular integrity to be maintained (Flick and Kaiser 2012). Proteinases are responsible for a range of processes including the elimination of irreversibly damaged proteins (Wickner *et al.* 1999). The increased proteolytic function in the pre-exposed snails is likely to be associated with the degradation of damaged proteins. These processes, along with the other cellular protection and repair mechanisms, were generally up-regulated in the pre-exposed

snails compared to the naïve snails. The increased expression of inhibitor of apoptosis genes in the pre-exposed snails indicates apoptotic function is suppressed. This is likely to be associated with a reduction in severe Cu-induced damage requiring the extreme measure of programmed cell death. The mechanisms of cellular repair used by an organism vary depending on the level of stress and associated damage that cells are experiencing. In situations where stress is not severe, the repair of stress-induced damage or degradation and replacement of damaged biomolecules is sufficient to maintain cellular function (Flick and Kaiser 2012). When these measures are insufficient, more radical options such as apoptosis or macro-autophagy are required (Flick and Kaiser 2012). The transcriptomic responses of the pre-exposed snails show evidence of an increase in expression of genes associated with mechanisms to manage the Cu-induced stress through increases in protection and repair processes, the degradation of damaged biomolecules, increased immune function and, vesicle-mediated transport. The regulation of this broad range of protective mechanisms is likely to allow these snails to maintain metabolic function. Conversely, the naïve snails have a reduced metabolic state and are more reliant on radical mechanisms such as apoptosis to remove damaged cells. The increased ability of the pre-exposed snails to manage Cu-induced stress is also evident in reduced mortality (Section 3.3.2.1) and lysosomal membrane destabilisation compared to the naïve snails (Section 3.3.2.10).

This study shows that populations of *I. newcombi* with different exposure histories to Cu mount different responses to cope with the Cu-induced stress. Snails that have been exposed to Cu previously are more able to manage the Cu-induced stress through cellular protection and repair mechanisms, while snails that have not been exposed resort to killing of damaged cells. At an individual level this is also represented as a decrease in mortality and sublethal stress in organisms that come from a population that has been exposed to copper. While the concentration used in this study are well above the water quality guideline concentrations, they are relevant to areas where copper is used and processed industrially or it is used as a pesticide (ANZECC/ARMCANZ 2000).

Chapter 6. Conclusions

This project investigated the response of the endemic Australian freshwater gastropod *I. newcombi* to Cu exposure. The major research foci were investigating the potential of *I. newcombi* as a biomonitor and the development of resistance in *I. newcombi* over multiple generations of Cu exposure.

The focus on a multiple lines of evidence approach to environmental risk assessment requires the validation of appropriate biomonitor species (Simpson *et al.* 2013). In an Australian context there is a shortage of freshwater species that are validated for use as sentinel species in monitoring and assessment programs (Taylor and Maher 2016b). *I. newcombi* has many of the desirable attributes of a biomonitor in being sedentary, widely distributed, abundant and easy to identify (Phillips and Rainbow 1994). Freshwater gastropods generally have useful attributes for use in toxicity testing, being easy to culture, reproducing readily in captivity, having a relatively short embryonic development and having embryonic stages that are easy to identify (Ravera 1977). These attributes make *I. newcombi* an ideal species for development as a biomonitor. The development of a species as a biomonitor requires an understanding of the response to contaminant exposure through investigation of the exposure-bioaccumulation-response relationship, and the validation of appropriate biomarkers to detect response. The current knowledge on the response of *I. newcombi* to chemical exposure relates to the investigation of lethal concentrations of pesticides to control *I. newcombi* populations in rice growing areas where they are considered a pest (Stevens *et al.* 2014; 1996). The current method for controlling *I. newcombi* in rice fields is by application of $CuSO_4$. One aim of the current project was to investigate the response of *Isidorella newcombi* to sub-lethal Cu concentrations in order to determine the potential of this species as a biomonitor.

A second aim was to investigate the response of *I. newcombi* to multiple generations of Cu exposure. Populations exposed to contaminants over multiple generations can experience rapid evolutionary change as a result of selection pressures exerted by the contaminant (Hoffmann and Hercus 2000). The micro-evolutionary changes that occur in this situation can result in increased tolerance to the contaminant (Medina *et al.* 2007). When returned to a non-contaminated environment the tolerant organisms can have reduced fitness associated with biological trade-offs in the re-allocation of energy and resources, reduction in uptake of

essential elements and pleiotropic effects (Agra *et al.* 2011; Klerks *et al.* 2011; Shirley and Sibly 1999). From a risk assessment perspective an understanding of the development of tolerance to contaminants and associated biological trade-offs is necessary as traditionally used dose-response models rely on the assumption of a consistent response. In order to account for adaptation in risk assessment, there is a need to quantify the adaptive potential of populations (Côte *et al.* 2015). As *I. newcombi* has been controlled in rice fields for over 40 years using $CuSO_4$ it makes an ideal model for the investigation of resistance to Cu in freshwater species.

The response of *I. newcombi* was investigated using two major studies; a 28 day exposure to provide an indication of chronic response, and short term exposures to different concentrations over four generations to investigate multi-generational responses and the development of resistance. In both studies a range of responses that included subcellular biomarkers and life history traits were used. In the multi-generational study, transcriptomic response was also investigated in order to gain a mechanistic understanding of the response of *I. newcombi* to Cu at the molecular level and to detect differences in biological mechanisms used in response to Cu exposure in snails with differing levels of copper tolerance. A summary of the major findings of the project in relation to the objectives identified in the general introduction follows.

1. Determine *I. newcombi* Cu bioaccumulation.

In the 28 day exposure experiment there was a positive relationship between Cu exposure concentration and Cu tissue concentration. These results indicated that *I. newcombi* is a net accumulator of Cu. This is important in the assessment of a species as a biomonitor as it indicates that the tissue copper concentration of *I. newcombi* would provide a time-integrated assessment of the concentration of bioavailable Cu in the environment (Rainbow 1995).

2. Investigate mortality rates of *I. newcombi* exposed to Cu a wide range of Cu concentrations for an exposure period of up to 28 days.

Over a 28 day exposure period all snails exposed to concentration 60 µg L^{-1} Cu and above died. The rate of mortality increased with increasing Cu concentration with 100% mortality in snails exposed to 120 µg L^{-1} Cu dead by day three, while the last of the snails exposed to 60 µg L^{-1} Cu survived until the final day of the experiment. While the lethal Cu concentrations reported for freshwater gastropods vary, the results from this study are similar to those found in other adult freshwater snails (Besser *et al.* 2016).

In the intergenerational study, where three day exposures were used, all replicates in all generations exposed to 75 µg L^{-1} Cu had less than fifty percent mortality. In the 100 µg L^{-1} Cu treatment the high mortality in the parental generation caused this treatment to be discontinued. This indicates a 3 day LC_{50} concentration of between 75 and 100 µg L^{-1} Cu in adult snails, which is also similar to reported values in other freshwater snails (Watton and Hawkes 1984).

3. Investigate the effect of Cu exposure on *I. newcombi* life history traits including; fecundity, embryo viability and juvenile survival.

Fecundity was assessed by the number of egg clutches and eggs laid by the snails. Despite variability in the data from the 28 day exposure study there were three specific trends in egg laying. First, the snails that were exposed to high concentrations of 80 µg L^{-1} Cu and above laid no eggs. Second, the treatments that were exposed to concentrations of 20 – 60 µg L^{-1} Cu laid eggs in the first three weeks, but oviposition ceased completely in the fourth week of the study. Snails exposed at concentrations of up to 15 µg L^{-1} Cu continued oviposition throughout the exposure period. From these results it suggests that snails exposed to low concentrations do not experience stress that leads to loss of fecundity. At intermediate exposure concentrations, snails experience moderate stress and reproductive function can be maintained for a period but is eventually shut down which is likely due to the need to transfer biological resources to other functions in order to manage Cu-induced stress. In snails that are exposed to high concentrations, reproductive function is shut down immediately. Delayed, concentration-dependent reductions in oviposition have been previously recorded in freshwater snails (Das and Khangarot 2011). In the current study, there was also a trend of reduced embryo viability at exposure concentrations above 20 µg L^{-1} Cu. The reduction of hatching rates at similar concentrations has been recorded in other freshwater snails (Rogevich *et al.* 2008).

4. Investigate oxidative and general stress responses of *I. newcombi* to sub-lethal concentrations of Cu with the following biomarkers.
    a) Antioxidant capacity – total antioxidant capacity (TAOC).
    b) Oxidative damage – lipid peroxidation (LP).
    c) General stress - lysosomal membrane destabilisation (LD).

In the 28 day exposure study there were differences in the TAOC in snails exposed to different Cu concentrations, however, the changes did not occur in a Cu concentration-dependent manner. This may be due to changes in specific enzymatic and non-enzymatic components of the system varying independently at different concentrations and timeframes (Atli and Grosell 2016). In the multigenerational study in snails with differing multigenerational pre-exposure histories all exposed to 75 µg L^{-1} Cu, the TAOC increased with increasing pre-exposure history.

Oxidative damage as measured by LP was not significantly different in the snails exposed to different Cu concentrations for 28 days. In the multi-generational study LP increased with increasing pre-exposure history. It has been suggested that when organisms experience increasing oxidative stress there is an increased demand on the antioxidant system and that past a threshold level, the antioxidant system will become overwhelmed, resulting in an decrease in TAOC and an increase in LP (Taylor and Maher 2010). This was not the pattern observed, especially in the snails from the multigenerational study. In studies using lipid peroxidation as a biomarker in gastropods, responses have not always been dose-dependent; they have varied over time, and often return to background levels after extended periods of exposure (Klobučar *et al.* 1997; Pannunzio and Storey 1998; Sureda *et al.* 2009; Ubrihien *et al.* 2017c). These results highlight the need for a greater understanding of oxidative stress responses in gastropods.

In the 28 day exposure, snails exposed to 10 µg L^{-1} Cu and above had increased rates of LD. In the multi-generational study there was a negative relationship between pre-exposure history and LD, suggesting snails with a multi-generational history of exposure to elevated Cu concentrations were experiencing a reduced level of sub-lethal stress. These results indicate that LD is a reliable biomarker marker of Cu-induced stress in *I. newcombi.* LD has previously been shown to be a reliable indicator of metal-induced stress in a range of mollusc species (Moore *et al.* 1982; Ringwood *et al.* 2003; Taylor and Maher 2010). Changes in LD have been related to the accumulation of high concentrations of contaminants into the lysosome, the onset of programmed cell death and oxidative damage in the form of LP (Izagirre and Marigómez

2009; Johansson *et al.* 2010; Taylor and Maher 2010). As LP was not related to LD, and in the multigenerational study the Cu accumulation did not follow the same pattern as LD, it is likely that increased programmed cell death was a major contributor to increased LD in Cu-stressed snails. The increase in the expression of genes associated with apoptosis in snails exposed to Cu in the transcriptomic study provided further evidence for increases in programmed cell death in the Cu-stressed snails.

5. Establish Cu exposure-bioaccumulation-response relationships for the sub-lethal responses outlined in aims 3 and 4 above.

In the 28 day exposure chapter there was a positive relationship between exposure Cu concentration and Cu tissue concentration. The responses observed in life history traits and biomarkers often did not follow a linear pattern. The changes in fecundity and LD occured at a threshold concentration rather than following a linear relationship (points 3 and 4 above). These changes are the most relevant for the evaluation of *I. newcombi* as a potential biomonitor. The increased LD in snails at 10 µg L^{-1} Cu is just below the concentration where oviposition ceased after three weeks of exposure (15 µg L^{-1} Cu). This indicates that significant increases in LD occur at an exposure concentration and associated level of Cu- induced stress just below that which causes cessation of oviposition. This suggests that increases in LD are a warning sign that organisms are approaching a level of stress that would affect reproductive capacity and as such could be used as warning signals for changes at higher levels of biological organisation.

6. Develop an integrated biological model of the mechanistic response of *I. newcombi* to Cu based on changes in transcriptomic regulation.

A model was developed for the interpretation of RNA-seq data in ecotoxicology that categorises the responses in three groups of ecotoxicological relevance. These are the uptake, transport and use of the contaminant, organism survival mechanisms, and programmed cell death. The model was applied to transcriptomic data for *I. newcombi* exposed to Cu and differential expression of genes in the three categories were identified and combined in an integrated biological response model (Figure 4-5). The transcriptomic changes identified in the area of copper uptake, transport and use were; the down-regulation of genes associated with Cu ion binding, and the up-regulation of Cu transport genes including metallothionein, Cu transporting ATPase and endocytosis. At the cellular survival level changes associated with

metabolic depression and cellular repair and recycling were identified. At the programmed cell death level, the differences in gene expression indicated an increase in apoptotic processes. This model provides a description of the mechanistic response of *I. newcombi* to Cu exposure at the molecular level. The use of such a framework assists with the complex task of interpretation of RNA-seq data in the area of ecotoxicology, and the consistent use of an approach such as this will allow effective comparisons among studies investigating different contaminants and species.

7. Investigate adaptation in *I. newcombi* over multiple generations of Cu exposure.

The multigenerational study investigated the development of resistance in snails over four generations. This experimental design used more generations then other lab based studies investigating the development of resistance in freshwater snails. This study also used a single three day Cu exposure in each generation. While it is noted that Cu can be released into the environment in many ways, often it occurs in pulse events and due to its chemical properties bioavailable concentrations return quickly to background levels (Stevens *et al.* 2014). Studies investigating responses to Cu over multiple generations have generally used exposure to a consistent Cu concentration. In this way this study is novel and adds to our understanding of how organisms respond over multiple generations when exposed in this environmentally realistic manner.

When the F_3 generation was exposed to a common Cu concentration, multigenerational pre-exposure concentration was positively correlated with survival and negatively correlated with LD. This suggests that during the study the snails had developed increased tolerance in a pre-exposure concentration-dependent manner. The changes in tolerance in the 25 and 50 µg L^{-1} Cu pre-exposure snails are likely to be associated with an increase in the range of phenotypic plasticity through acclimation, as there were no differences in survival and reproductive output of these snails compared to controls in the parental to F_2 generations. Changes in tolerance in the 75 µ g L^{-1} Cu pre-exposed snails may also be associated with directional selection, as they had reduced survival compared to the other treatments in the parental to F_2 generations. The presence of reduced fitness associated with biological trade-offs was also tested. Despite a reduced number of clutches laid by the high pre-exposure treatment, there was no significant difference in the total number of eggs laid, so this is unlikely to have any significant effect at the population level.

8. Investigate transcriptomic changes associated with the development of Cu resistance in *I. newcombi*.

The purpose of investigating the differences in transcriptomic response in Cu-tolerant snails was to describe the changes at the molecular level that provide an increased ability to manage cellular homeostasis and survive at stress-inducing levels of Cu exposure. A comparison of the transcriptomic response to Cu exposure between snails with a multigenerational pre-exposure history to Cu that had developed tolerance and snails that had not previously been exposed to Cu was undertaken. The major differences in the transcriptomic response of snails that had a multi-generational exposure history to elevated Cu concentrations (pre-exposed) compared to snails that had not been exposed to elevated concentrations of Cu (naïve) were in metabolic activity, cellular defences, vesicle mediated transport and apoptosis. The pre-exposed snails showed an increased ability to maintain metabolic function compared to naïve snails. This was evident through changes in the expression of genes relating to transcription, translation and ionic transport. The changes in cellular defence mechanisms were evident through increases in proteolytic function, immune function and specific genes associated with intestinal mucin and DNA repair. This suite of responses combined under the term of cellular defences indicated that the pre-exposed snails had an increased ability to limit Cu-induced damage, degrade damaged biomolecules and maintain immune function. There was also increased expression of genes associated with phagocytosis in the pre-exposed snails. In invertebrates the roles of phagocytosis include the clearance of a range of potentially damaging materials including toxicants, pathogens and debris resulting from cellular damage. Finally, there was evidence of a reduced occurrence of apoptosis in the pre-exposed snails. This was particularly evident in the increased expression of apoptosis inhibiting genes in the pre-exposed snails. These responses indicated that the tolerant pre-exposed snails have an increased ability to induce defence mechanisms which allow them to minimise cellular damage, degrade damaged biomolecules more effectively and to transport excess copper and cellular debris to avoid further cellular damage. These mechanisms allow the pre-exposed snails to maintain a higher level of metabolic activity. Alternatively, in the naïve snails there is evidence of a higher rate of apoptosis which is accompanied by a reduced metabolic rate in order to limit cellular damage from contaminant exposure.

Areas identified for further study

The need for further study on the antioxidant response of freshwater gastropods and the response of freshwater snails to contaminants at the molecular level was identified. As discussed (objective 4) the results for the TAOC and LP did not follow expected patterns. Aquatic organisms adjust their antioxidant system in response to environmental variability to maintain a stable redox state (Costantini *et al.* 2010). Roelofs *et al.* (2010) point out that in response to stress there is an immediate response stage where the stressor interacts with cellular targets, an intermediate stress response stage where stress responses are activated to cope with damage and an accommodation stage where function of the cell is established in the presence of the stressor. Reactive oxygen species balance is maintained by a range of enzymatic and non-enzymatic antioxidants (Atli and Grosell 2016). Concentration and time-dependent changes in specific enzymatic and non-enzymatic components of the antioxidant system can make antioxidant response difficult to interpret. As antioxidant measures are used widely to investigate responses to contamination in ecotoxicology there is a need for a clear understanding of contaminant-induced antioxidant response and associated damage in gastropods. Time-course studies in gastropods that include assessment of overall TAOC, as well as the function of individual enzymatic and non-enzymatic components of the system are required to fully understand the antioxidant response in gastropods.

In this project the mechanistic response at the molecular level was investigated through changes in the transcriptome. While the transcriptomic response allows an understanding of the genes that are being actively expressed at transcriptome level, it is also important to recognise the other molecular controls are important in determining response to stress. The molecular response of an organism to environmental stress is controlled at a number of levels including transcription, regulation of translation, post-translational modification of proteins, protein degradation and allosteric regulation of proteins (Brinke and Buchinger 2017). While time and financial constraints limited the methods to transcriptomics, the broader investigation of mechanistic response at the molecular level would provide greater insight into function. This is particularly true for stress response in a non-model species such as *I. newcombi*, where the annotation to highly divergent species to determine the function of transcripts introduces uncertainty. The integration of proteomic and metabolomic methods along with the assessment of function for key proteins would assist in further understanding mechanistic response at the molecular level.

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Appendices

Appendix 1. List of genes associated with the KEGG ribosomal pathway with the amount of differential expression between control and exposed treatments and link for NCBI gene ID used for annotation with associated e-value.

KEGG ID	Gene	Log 2 fold change	Annotation and *transcript ID*
K02969	S20e	-8.01	ola:101166754/2e-43 *Unigene54005*
K02925	L3e	-9.05	aec:105155008/5e-178 *Unigene54441*
K02893	L23Ae	-9.17	fch:102058668/4e-49 *Unigene30864*
K02958	S15e	-9.46	cqu:CpipJ_CPIJ001180/2e-56 *CL7031.Contig1*
K02985	S3e	-9.28	crg:105335873/3e-101 *Unigene53993*
K02949	S11e	-8.64	pxy:105386822/2e-57 *Unigene56589*
K02894	L23e	-8.84	cqu:CpipJ_CPIJ011325/9e-66 *CL3174.Contig2*
K02898	L26e	-8.74	hmg:100215772/3e-43 *Unigene53782*
K02987	S4e	-8.26	pale:102891995/1e-116 *Unigene25461*
K02868	L11e	-9.07	ola:101171178/1e-77 *Unigene56870*
K02980	S29e	-9.26	pxy:105382390/9e-21 *Unigene13865*
K02957	S15Ae	-9.39	xtr:100125111/5e-59 *Unigene56799*
K02940	L9e	-8.28	spu:591874/3e-64 *CL9850.Contig2*

K02885	L19e	-8.29	hmg:100206718/2e-73 *Unigene53750*
K02981	S2e	-9.03	cmk:103186845/1e-115 *Unigene53899*
K02937	L7e	-8.60	ola:100049522/2e-86 *CL2827.Contig2*
K02915	L34e	-8.68	cfo:105259213/2e-34 *CL4904.Contig1*
K02875	L14e	-8.69	xma:102233444/3e-35 *CL6571.Contig2*
K02964	S18e	-8.34	crg:105347800/8e-66 *Unigene54489*
K02955	S14e	-9.28	mze:101487857/4e-68 *CL11793.Contig2*
K02883	L18e	-8.23	soc:105198518/1e-68 *CL10023.Contig2*
K02960	S16e	-8.63	crg:105344628/7e-61 *Unigene54330*
K02989	S5e	-8.53	tru:101061501/5e-89 *CL12846.Contig1*
K02908	L30e	-8.28	bom:102269226/6e-06 *Unigene26875*
K02936	L7Ae	-9.08	bmor:692662/4e-47 *Unigene19331*
K02943	LP1,LP2	-9.48	pbi:103057761/2e-32 *Unigene 54392*
K02865	L10Ae	-8.74	nvi:100115429/2e-89 *CL1882.Contig2*
K02870	L12e	-8.49	nvi:100117287/1e-65 *Unigene56403*
K02953	S13e	-8.52	pbi:103049505/7e-67 *Unigene56594*
K02866	L10e	-8.61	soc:105196408/2e-89

			Unigene53869
K02877	L15e	-9.91	hmg:100204269/1e-91
			CL5524.Contig2
K02889	L21e	-8.18	hmg:100203078/4e-53
			Unigene55643
K02896	L24e	-8.61	hmg:100201805/5e-43
			Unigene25133
K02910	L31e	-9.24	ame:413875/7e-39
			CL4205.Contig2
K02917	L35Ae	-8.92	hmg:100214200/2e-28
			Unigene55816
K02922	L37e	-8.86	bmy:Bm1_15745/4e-36
			Unigene53896
K02924	L39e	-8.54	cmy:102941490/7e-15
			Unigene27082
K02927	L40e	-9.24	tsp:Tsp_03772/6e-65
			Unigene53778
K02929	L44e	-8.83	hmg:100198396/3e-49
			CL7692.Contig2
K02984	S3Ae	-9.06	mgp:100547675/1e-96
			Unigene53747
K02991	S6e	-8.89	chx:102184058/9e-87
			Unigene54211
K02995	S8e	-9.21	amj:102559034/4e-83
			Unigene56193
K02962	S17e	-9.05	aqu:100636454/7e-49
			Unigene54624
K02966	S19e	-8.34	cfo:105248957/7e-41
			Unigene55811
K02974	S24e	-9.35	crg:105340909/2e-48
			Unigene54673
K02975	S25e	-10.07	mcf:101864921/4e-36
			Unigene54069

K02976	S26e	-9.40	spu:576654/1e-44 *Unigene56223*
K02977	S27Ae	-8.34	crg:105342397/5e-77 *Unigene53757*
K02979	S28e	-9.05	crg:105318569/3e-25 *Unigene54897*
K02983	S30e	-8.76	ecb:100033998/1e-39 *CL13267.Contig2*
K02934	L6e	-8.59	ngi:103745561/4e-62 *CL7417.Contig1*
K02882	L18Ae	-8.54	phi:102111748/6e-61 *CL10617.Contig1*
K02891	L22e	-8.10	fab:101817951/1e-36 *Unigene54478*
K02901	L27e	-9.36	hmg:101240604/1e-45 *Unigene56311*
K02903	L28e	-8.58	crg:105332917/2e-21 *Unigene55571*
K02905	L29e	-8.07	smm:Smp_066940/1e-18 *Unigene54511*
K02920	L36e	-8.83	ola:101158513/2e-24 *Unigene56463*
K02923	L38e	-8.91	xtr:448672/4e-20 *Unigene53670*
K02993	S7e	-8.72	xla:397774/1e-65 *CL1223.Contig1*
K02947	S10e	-8.80	crg:105341412/7e-48 *Unigene54423*
K02951	S12e	-8.75	crg:105348494/1e-47 *CL8515.Contig2*
K02971	S21e	-8.74	pss:102451908/1e-23 *Unigene55878*
K02971	S21e	-8.25	hmg:100206175/1e-15

Unigene12253

Appendix 2. List of genes associated with the apoptosis KEGG pathway showing the amount of differential expression between control and exposed treatments and link for NCBI gene ID used for annotation with associated e-value.

Kegg ID	Gene name	Log 2 fold change	Annotation and *transcript ID.*	Function and reference
K04389	tumor necrosis factor ligand superfamily member 6	7.31	mgp:100551315/3e-08 *CL919.Contig4*	FAS receptor: Binds to FAS ligand to induce apoptosis (Waring and Mullbacher 1999)
K04721	tumor necrosis factor ligand superfamily member 10	8.36	crg:105344743/2e-08 *CL13460.Contig2*	FAS ligand: Transmembrane protein inducing apoptosis (Waring and Mullbacher 1999)
K04722	tumor necrosis factor receptor superfamily member 10	8.56	pps:100972073/7e-08 *Unigene22515*	TRAIL: Programmed cell death signalling (Azahri and Kavurma 2013)
K00922	phosphatidylinositol-4,5-bisphosphate 3-kinase	8.23	crg:105346594/9e-18 *Unigene43419*	PI3K: Inhibitor of apoptosis (Duronio 2008)
K16061	baculoviral IAP repeat-containing protein 7	10.32	crg:105323879/5e-49 *CL12139.Contig1*	BIRC7: Pro/anti-apoptotic processes (Saleem *et al.* 2013)
K16060	Baculoviral IAP repeat containing protein 2/3	-7.97	Crg:105335294/8e-22 *Unigene17495*	BIRC2/3: Inhibitor of apoptosis (Saleem *et al.* 2013)

Appendix 3. List of genes related to KEGG F-C gamma R mediated phagocytosis pathway showing the amount of differential expression between control and exposed treatments and link for NCBI gene ID used for annotation with associated e-value.

Kegg ID	Gene name	Log 2 fold change	Annotation and *transcript ID.*	Function and reference
K04371	mitogen-activated protein kinase 1/3	-7.89	asn:102375250/6e-14 *Unigene6787*	PAK1: Cell adhesion, migration, proliferation, apoptosis, mitosis and vesicle mediated transport(Talukder *et al.* 2005)
K05765	Cofilin	7.85	crg:105340112/7e-10 *Unigene66312*	Cytoskeleton function during phagocytosis (Gitik *et al.* 2014)
K12831	PDCD7	8.99	hst:105181958/6e-08 *Unigene31281*	Component of the minor U12-type spliceosome (Tian *et al.* 2014)
K04409	p21-activated kinase 1	8.53	cin:100178153/2e-28 *Unigene37457*	PAK1: signalling for cytoskeleton changes associated with phagocytosis (Itakura *et al.* 2013)
K00922	phosphatidylinositol-4,5-bisphosphate 3-kinase	8.23	crg:105346594/9e-18 *Unigene43419*	PI(4,5)P2: Membrane biogenesis, lipid homeostasis, vesicular trafficking, cytoskeleton organisation and signalling pathways (Delage *et al.* 2013).
K05760	Paxillin	8.45	mze:101481805/2e-06 *Unigene38777*	Cytoskeleton function during phagocytosis (Gitik *et al.* 2014)

Appendix 4. List of genes associated with the KEGG lysosome pathway showing the amount of differential expression between control and exposed treatments and link for NCBI gene ID used for annotation with associated e-value.

Kegg ID	Gene name	Log 2 fold change	Annotation and *transcript ID.*	Function and reference
K01206	alpha-L-fucosidase	8.55	crg:105332438/2e-27 *Unigene38685*	Glycoprotein degradation (Berteau *et al.* 2004)
K01539	endothelial-specific receptor tyrosine kinase	8.26	amj:102559179/2e-10 *Unigene37656*	Endocytosis (Goh and Sorkin 2013)
K02154	V-type H+-transporting ATPase subunit a	7.97	lcm:102365079/1e-26 *Unigene37352*	Proton pump to acidify intracellular compartments (Mauvezin *et al.* 2015)
K01373	cathepsin F	7.91	cge:100756958/5e-18 *Unigene30665*	Proteolytic enzyme (Griffitt *et al.* 2006)
K01363	cathepsin B	-8.76	cmk:103171629/4e-09 *CL5727.Contig2*	Proteolytic enzyme (Griffitt *et al.* 2006)

Appendix 5. List of genes associated with the KEGG Endocytosis pathway showing the amount of differential expression between the control and exposed treatments and link for NCBI gene ID used for annotation with associated e-value.

Kegg ID	Gene name	Log 2 fold change	Annotation and *transcript ID.*	Function and reference
K13649	Soluble scavenger receptor cysteine rich domain containing protein	7.90	crg:105326079/5e-7 *Unigene37884*	SSC5D: Macrophagic scavenging (Gonçalves *et al.* 2009)
K11824	AP-2 complex subunit mu	7.90	crg:105345381/5.8e-12 *Unigene63729*	Clathrin and cargo binding during endocytosis (Kelly *et al.* 2014)
K07547	Paxillin	8.45	Mze:101481805/2e-6 *Unigene38777*	Actin cytoskeleton regulation (Pribic *et al.* 2011)
K12494	Cyothesin-3	8.27	dre:100537448/4e-19 *Unigene37356 and Unigene33639*	Signalling for endocytosis regulation (Hofmann *et al.* 2007)
K12494	Insitol-polyphosphate multikinase	-8.04	lcm:102351435/4e-19 *Unigene53753*	Regulator of endocytosis (Xu *et al.* 2013)
K07903	Ras-related protein Rab10	8.27	Crg:105347459/4e-32 *CL5664.contig4*	Rab10: Cargo recycling mediator (Glodowski *et al.* 2007)

Appendix 6. List of genes associated with the gene ontology copper ion binding function showing the amount of differential expression between control and exposed treatments and link for NCBI Nr accession number used for annotation with associated e-value.

GO ID	Gene name	Log 2 fold change	Annotation and *transcript ID*	Function and reference
5507	Cytochrome C oxidase subunit II (mitochondrion)	-7.92	AGI78544.1|/5.94886e-31 *Unigene6750*	Cellular respiration (Ostermeier *et al.* 1996)
4500	cytochrome c oxidase copper chaperone-like	-8.16	XP_005099404.1|/4.89808e-21 *CL497.Contig1*	Cellular respiration (Ostermeier *et al.* 1996)
5758	dopamine beta hydroxylase-like protein	-8.08	NP_001191657.1|/6.06967e-47 *Unigene40804*	Catalyst for dopamine to norepinephrine reaction (Arredondo and Núñez 2005)

Appendix 7. Expression of transcripts that were related to copper transporting ATPase.

Unigene	Control FPKM	Exposed FPKM	log2FoldChange	Probability	Reg	Annotation and *transcript ID*
Unigene20011	0.8	15.69	4.3	0.7241976	*	Cu-transporting ATPase (NR) *Unigene20011*
Unigene37464	0.01	2.095	7.7	0.785143672	*	Cu-transporting ATPase (NR) *Unigene37464*
Unigene44503	0.12	7.85	6.0	0.781307542	*	Cu-transporting ATPase (NR) *Unigene44503*
CL716.Contig1	6.1	117.1	4.3	0.740231443	*	Cu-transporting ATPase (NR) *CL716.Contig1*
CL716.Contig2	1.42	1.33	-0.1	0.397118535	*	Cu-transporting ATPase (NR) *CL716.Contig2*
CL716.Contig3	0.01	32.76	11.7	0.986017601	Up	Not annotated *CL716.Contig3*
CL716.Contig4	2.28	12	2.4	0.672618767	*	Cu-transporting ATPase (NR) *CL716.Contig4*
CL716.Contig5	0.92	22.87	4.6	0.736397673	*	Not annotated *CL716.Contig5*
CL716.Contig6	5.47	14.36	1.4	0.601436719	*	Cu-transporting ATPase (NR) *CL716.Contig6*
CL716.Contig7	5.98	125.8	4.4	0.743017063	*	Cu-transporting ATPase (NR) *CL716.Contig7*
CL716.Contig8	0.62	0.01	-6.0	0.552338977	*	Cu-transporting ATPase (NR) *CL716.Contig8*
Unigene28640	0.5	0.01	-5.7	0.514086269	*	Cu-transporting ATPase (NT) *Unigene28640*

Unigene37464	0.01	2.095	7.7	0.785143672	*	Cu-transporting ATPase (NR) *Unigene37464*

Appendix 8. Transcripts annotated from the present study that align with the list of genes that are regularly present differential gene expression studies using bivalves (Miao *et al.* 2015)

Gene	**Present**	**DE**	**Expression**
Tubulin	Y	N	NA
Cytochrome C oxidase	Y	N	NA
NADH dehydrogenase subunit	Y	N	NA
Cytochrome b	Y	N	NA
Ferritin	Y	N	NA
Myc homolog	N		
Actin	Y	N	NA
Cathepsin L	Y	N	NA
EGF-1	N		
Collagen	Y	N	NA
GM2 ganglioside activator precursor	N		
Heat shock Protein 70	Y	Y	8.04
C-type lectin	Y	N	NA
Dopamine β hydroxylase	Y	Y	-8.24
Glutathione S-transferase	Y	N	NA
Scavenger receptor cysteine rich protein	N		
Galectin	Y	N	NA
Heat shock protein 90	Y	Y	8.47
Integrin	Y	Y	8.73
Trypsin	Y	N	NA
VDG3	N		
Ankyrin repeat protein	Y	N	NA
Chitinase	Y	N	NA
Cytochrome p450	Y	Y	8.82
EGF2	N		
Gene	**Present**	**DE**	**Expression**
Metallothionein	Y	N	NA
Receptor of activated kinase C	N		
Troponin	Y	N	NA

Arginine kinase	Y	N	NA
Calmodulin	Y	N	NA
Cathepsin D	N		
Defensin	N		
Glutathione peroxidase	Y	N	NA
Heterogeneous nuclear ribonucleoprotein	Y	N	NA
Intermediate filament protein	Y	N	NA
Lysozyme	Y	N	NA
Intermediate filament protein	Y	N	NA
Myosinase	N		
Proteasome β subunit	N		
Superoxide dismutase	Y	N	NA
Translationaly controlled tumour protein	Y	N	NA
Ubiquitin/polyubiquitin	Y	N	NA
Vitelline membrane outer layer 1 homolog	Y		
Myosin	Y	Y	various
Aldehyde dehydrogenase	Y	N	NA
ATP synthase β subunit	Y	N	NA
Bromodomain-containing protein	Y	N	NA
C1q domain containing protein	Y	N	NA
Ficolin	Y	N	NA
Heat shock protein p26	N		
Macrophage expressed protein	Y	N	NA
Serine/threonine proteinase	N		
Sialic acid acetylesterase	N		
TCP-1 chaperonin family	N		
Universal stress protein	Y	N	NA
Gene	**Present**	**DE**	**Expression**
Cellulase	Y	N	NA
Cyclin dependent kinase	N		
DNA polymerase	Y	N	NA
Fibrillin	Y	N	NA

Heat shock protein 27 kDa	N		
Inhibitor of apoptosis protein	N		
Juvenile hormone diol kinase	N		
Keratinocyte-associated protein	Y	N	NA
Macrophage galactose n-acetylgalactosamine Specific lectin	N		
Paramysoin	Y	N	NA
Tropomyosin	Y	N	NA
NADH-ubiquinone oxidoreductase	Y	N	NA
Perlucin	Y	Y	-7.93
Proliferating cell nuclear antigen	Y	N	NA
Pyruvate carboxylase	Y	N	NA
Rab GTPase-activating protein	Y	N	NA
Serine proteinase inhibitor	Y	Y	-7.48
Serpine 1 m-RNA binding protein	N		
Tumour necrosis factor receptor/ligand	Y	Y	7.93 to 8.58
Voltage dependent anion channel	N		
Zinc finger protein	Y	N	NA
Filamin	Y	N	NA

Appendix 9. Transcript and annotation details for all genes with a difference in regulation of greater than 10 log2fold. Differentially regulated genes that are already discussed as part of a pathway are not included.

Gene name	Log 2 fold change	Annotation and *transcript ID*	Function and reference (for annotated transcripts)
Not annotated	11.68	*CL716.contig3*	
Not annotated	11.16	*Unigene36647*	
Not annotated	10.64	*Unigene36633*	
Not annotated	10.43	*CL8898.Contig2*	
myosin light chain kinase	10.32	mze:101479888/5e-06 (KEGG) *Unigene42607*	MLCK: paracellular transport – linked to apoptosis (Mills *et al.* 1998)
Behavioural IAP repeat containing protein 7	10.32	crg:105323879/5e-49 (KEGG) *CL12139.Contig1*	BIRC7: Pro/anti-apoptotic processes (Saleem *et al.* 2013)
Not annotated	10.26	*Unigene10616*	
Not annotated	10.06	*CL5707.Contig2*	
Not annotated	10.05	*Unigene66563*	
Serine/threonine kinase inositol requiring protein	10.00	Q55DJ8/5e-06 (Swissprot) *CL10597.Contig1*	Cellular fate in stressed cells (Liu *et al.* 2015)
Not annotated	-10.00	*Unigene6691*	
Not annotated	-10.02	*CL2648.Contig4*	
Sodium-influx-stimulating peptide	-10.12	P42579/ 4.28e-09 (Nr) *CL10743.Contig2*	SIS: Sodium pump activation (Smit *et al.* 1993)

Not annotated	-10.15	*CL7255.Contig2*	
PH domain and leucine-rich repeat-containing protein phosphatase	-10.20	dre:100331906/2e-09 (KEGG) *CL6897.Contig1*	Apoptosis promoter (O'Neill *et al.* 2013)
Not annotated	-10.26	*CL187.Contig2*	
Not annotated	-10.32	*CL622.Contig3*	
proteasome subunit beta type-4	-10.33	XP_005103192 (Nr) *CL1154.Contig1*	Protein degradation (Gu and Enenkel 2014)
caffeoyl-CoA O-methyltransferase 1	-10.33	XP_005105713 (Nr) *CL3925.Contig1*	
plasma membrane calcium-transporting ATPase 1	-10.34	ola:101155655 (KEGG) *Unigene32360*	Calcium homeostasis (Brix *et al.* 2011)
Not annotated	-10.34	*Unigene29808*	
Not annotated	-10.35	*CL5738.Contig2*	
Not annotated	-10.38	*CL6020.Contig2*	
Not annotated	-10.40	*CL9924.Contig1*	
Not annotated	-10.43	*CL10665.Contig1*	
Not annotated	-10.47	*Unigene21027*	
Not annotated	-10.49	*CL3769.Contig1*	

Acetylcholine receptor subunit alpha	-10.50	EZA51692.1|/3.81e-46 (Nr) *CL217.Contig4*	Function of ion-conducting channel across the plasma membrane (Smit *et al.* 2001)
Not annotated	-10.70	*CL13270.Contig1*	
Not annotated	-10.73	*CL3813.Contig4*	
Histidine-rich glycoprotein	-10.78	P04929/1e-09(Swissprot) *CL2123.Contig2*	Regulation of biological functions including angiogenesis, immune complex clearance and coagulation (Jones *et al.* 2005)
Not annotated	-10.85	*CL3184.Contig1*	
ATP synthase subunit f	-10.87	XP_005098038.1|/2.98e-43(Nr) *CL12291.Contig1*	ATP synthesis (Jonckheere *et al.* 2012)
Not annotated	-10.95	*CL10352.Contig1*	
Not annotated	-11.00	*Unigene11039*	
Not annotated	-11.22	*CL9268.Contig1*	
Ectonucleoside triphosphate diphosphohydrolase 3	-11.23	crg:105337867/6e-66(KEGG) *CL5826.Contig3*	Hydrolysis of nucleotides outside of the cell wall (Belcher *et al.* 2006)
Kazal-type serine proteinase inhibitor	-11.38	PF07648/1.0E-11(Interpro) *CL9809.Contig3*	KSPI: Proteinase inhibitor (Rimphanitchayakit and Tassanakajon 2010)
Not annotated	-11.60	*Unigene9123*	
Excitatory amino acid transporter 2	-11.66	EKC23472.1|/1.57078e-07 (Nr) *CL7470.Contig2*	EAAT2: Glutamate transporter (Kim *et al.* 2011)

Not annotated	-11.68	*Unigene46969*	
Not annotated	-13.24	*CL8865.Contig4*	
Not annotated	-13.63	*CL278.Contig1*	

Appendix 10. Differentially expressed genes in *Isidorella newcombi* with different Cu exposure histories exposed to 75 µg L^{-1} Cu for three days in the F_3 generation that were annotated and not discussed.

Annotation	Log_2 fold change	Annotation and *Transcript identififcation*
Cyclic nucleotide gated channel beta 1	-9.21	gfr:102034228 (KEGG) *Unigene75023*
Genetic suppressor element 1	-8.83	Q1LWL6 (Swissprot) *Unigene34376*
HRAS-like suppressor 3 – like	-8.54	XP_005088861.1 (Nr) *Unigene33279*
Transducing-like enhancer protein 3 – like	-8.52	XP_005108955.1 (Nr) *Unigene756*
Phosphorylase b kinase gammacatalytic chain, liver/testis isoform like	-8.42	XP_006820127.1 (Nr) *Unigene33555*
Lipoxygenase homology domain-containing protein 1 – like	-8.41	XP_005101561.1 (Nr) *Unigene31229*
Slowpoke binding protein – like	-8.41	XP_005110734.1 (Nr) *CL9443.Contig1*
Probable serine/threonine-protein kinase cdc 7	-8.39	Q54DK3 (Swissprot) *Unigene34579*
Phospholipid scramblase 1-like	-8.38	XP_005095743.1 (Nr) *Unigene34967*
Ras and EF-hand domain-containing protein homolog	-8.38	spu:580444 (KEGG) *Unigene2408*
Solute carrier family 23 member 2-like - predicted	-8.36	XP_005089904.1 (Nr) *Unigene33481*
WD repeat-containing protein 7-like isoform X7 - predicted:	-8.34	XP_005092752.1 (Nr) *Unigene35798*
RNA binding protein 25	-8.33	bmor:101737821 (KEGG) *Unigene44344*
Regulatory-associated protein of mTOR-like - predicted	-8.26	XP_005090093.1 (Nr) *Unigene45107*
HRAS-like suppressor 3-like isoform - predicted	-8.16	XP_005088861.1 (Nr) *Unigene18946*
Rhomboid related protein	-8.13	lgi:LOTGIDRAFT_230028 (KEGG) *Unigene45100*
Mytimacin 6	-7.85	AHG59339.1 (Nr) *CL10471.contig1*

Tenascin-like - predicted	-7.73	XP_006173665.1 (Nr) *Unigene27330*
Caprin-2	-7.39	EKC37565.1 (Nr) *CL3505.contig1*
Expansin-yoaJ	-7.21	EKC31365.1 (Nr) *CL752.contig11*
Angiotension-converting enzyme – like - predicted	-6.35	XP_005096999.1 (Nr) *CL5692.Contig2*
Receptor-type tyrosine-protein phosphatase S	-6.29	spu:586604 (KEGG) *CL1044.Contig3*
Bromodomain-containing protein	6.64	Q55BK3 (Swissprot) *CL6973.Contig3*
Trichohyalin-like isoform X2	6.96	XP_005100053.1 (Nr) *Unigene4540*
Chitin binding type-2 domain profile	7.03	PS50940 (Interpro) *CL7925.Contig1*
DEC3	7.48	BAD16599.1 (Nr) *CL11164.Contig2*
EGF-like domain profile	8.02	PS50026 (Interpro) *CL10670.contig2*
Dynein heavy chain 5, axonemal-like isoform - predicted	8.14	XP_005091512.1 (Nr) *Unigene13779*
Microtubule-associated protein futsch-like - predicted	8.14	XP_005107862.1 (Nr) *Unigene14956*
Dolichyl-diphosphooligosaccharide—protein glycotransferase subunit STT3B-like isoform – predicted	8.18	XP_005095974.1 (Nr) *Unigene7651*
Interphotoreceptor matrix proteoglycan 2	8.20	EPQ16404.1 (Nr) *Unigene12646*
Intraflagellar transport protein 52	8.21	hro:HELRODRAFT_78455 (KEGG) *Unigene123901*
Type-1 angiotensin II receptor B-like	8.21	XP_005091360.1 (Nr) *Ungene11887*
Ubiquitin protein ligase MIB2	8.24	EKC26252.1 (Nr) *Unigene43446*
Putative methyltransferase DDB_GO268948-like isoform - predicted	8.26	XP_005109192.1 (Nr) *Unigene19981*
Ankyrin-1-like - predicted	8.28	XP_005091728.1 (Nr) *Unigene31411*

Putative protein phloem protein 2- like A3-like - predicted	8.30	XP_005097216.1 (Nr) *Unigene42838*
Patched domain-containing protein 3-like - predicted	8.34	XP_005099994.1 (Nr) *Unigene42627*
Glutamic acid-rich protein	8.37	P13816 (Swissprot) *Unigene28354*
Glutamine synthetase	8.49	EKC27196.1 (Nr) *Unigene43966*
Putative GPI-anchored protein PB15E9.01c-like – predicted	8.50	XP_005091610.1 (Nr) *CL5378.contig1*
Merozoite surface protein 3	8.51	AGR50640.1 (Nr) *Unigene21170*
Histidine-rich glycoprotein	8.58	P04929 (Swissprot) *Unigene59993*
Dual specificity protein kinase splA-like	8.59	XP_005097619.1 (Nr) *Unigene20789*
Putative protein TPRXL-like - predicted	8.60	XP_005113114.1 (Nr) *CL7880.Contig1*
Sorting nexin-29-like	8.63	lcm:102353553 (KEGG) *CL12958.Contig6*
NXPE family member 3-like - predicted	8.69	XP_005093953.1 (Nr) *CL4187.Contig1*
T-cell receptor beta chain ANA 11	8.75	XP_001892072.1 (Nr) *Unigene43852*
Solute carrier family 39 (zinc transporter), member 6	8.77	oas:100302554 (KEGG) *Unigene72592*
Carbonic anhydrase 7	8.85	KFM74432.1 (Nr) *Unigene29746*
RNA binding motif protein 25	8.96	tgu:100221046 (KEGG) *Unigene7988*
Chordin	9.02	dmo:Dmoj_GI21514 (KEGG) *Unigene61929*
Peptidase inhibitor 16-like - predicted	9.08	XP_005097635.1 (Nr) *CL823.Contig2*
Serine protease inhibitor 2	9.12	ACV66786.1 (Nr) *CL6049.Contig1*
Arrestin domain-containing protein 3-like - predicted	9.16	XP_005105543.1 (Nr) *CL555.Contig3*
Golgi-associated plant pathogenesis-related protein 1	9.31	EKC25609.1 (Nr) *CL9707.Contig2*

Dynein heavy chain	9.33	XP_001862168.1 (Nr) *Unigene73976*
General transcription factor II-I repeat domain-containing protein 2-like - predicted	9.34	XP_004077286.1 (Nr) *CL2652.Contig1*
Spondin-1 OS	9.54	Q9W770 (Swissprot) *CL232.Contig1*