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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Chapter 4

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