Metabolomic Applications in Marine Mollusc Development and Aquaculture

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A thesis submitted to Auckland University of Technology in fulfilment of the requirements for the degree of Doctor of Philosophy

(PhD)

Faculty of Health and Environmental Sciences

School of Science

2016

Abstract

Metabolomics is a rapidly emerging discipline within functional genomics to better understand biochemical phenotypes across a range of biological systems. The approach has many demonstrated applications in aquatic biology, but has not yet been applied to study early lifestages of marine molluscs. This Thesis evaluates metabolomics as an approach to characterise early lifestage phenotypes of molluscs, and demonstrates unique applications in aquaculture, developmental biology, immunology, and toxicology.

GC/MS-based metabolomics was first tested for its capacity to classify good and bad quality mussel larvae (i.e., slow- vs fast-growing organisms). Based on the composition of metabolites, larval classes could clearly be discriminated and the data indicated differences energy metabolism, osmotic regulation, immune function and cell–cell communication.

Mussel larvae which had been subjected to handling stress and different culture conditions were also assessed. A decrease in succinate and an increase in alanine were observed after the water exchange, which indicated alterations in energy production and osmotic balance. However, these variations were subtle and it is unlikely that the water exchange practice had any lasting negative effects on larval physiology and performance. A culture condition classification model was also constructed which revealed that larvae from flowthrough vs static systems differed in terms of energy, protein and lipid metabolism. The data also suggests that growth performance is metabolically buffered through an adaptive physiological mechanism to provide similar developmental characteristics under these conditions.

Oyster larvae were assessed during a viral (OsHV-1 µVar) infection to characterise the host-virus interaction at a metabolic level. Responses included a coordinated disruption of the TCA cycle in accordance with mammalian macrophage stimulation via

activation of immunoresponsive gene 1 and production of itaconic acid, induction of a Warburg-like effect, and production of free fatty acids for virion assembly, among others. These results provide new insights into the pathogenic mechanisms of OsHV-1 infection in oyster larvae, which may be applied for selective breeding programmes aiming to enhance viral resistance.

Lastly, metabolomics was applied to investigate mechanisms of toxicity in mussel larvae exposed to copper contamination. At sublethal dose levels, metabolic trajectory analysis indicated that larvae were successfully employing various endogenous mechanisms involving biosynthesis of antioxidants and a restructuring of energy-related metabolism in an attempt to alleviate the toxic effects on cells and developing tissues.This was partly confirmed by a targeted analysis of oxidative stress biomarkers (e.g., enzymes). A lethal copper dose induced severe metabolic dysregulation after 3 hrs exposure which worsened with time, substantially delayed embryonic development, initiated the apoptotic pathway, provided many evidences for the occurrence of oxidative stress (validated via oxidative stress biomarkers), and resulted in cell/organism death shortly after 18 hrs exposure.

In summary, this Thesis provides strong support for the application of metabolomics to assess the health status of marine mollusc embryos and larvae.

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Attestation of Authorship

"I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning."

Signed: 

Date: 28.10.2016

Co-author contributions

The co-authored literature reviews in this Thesis have been broken down into five task components, each of which have been given a weighting (% time) to produce the completed output:

Concept & structure	= 5%
Article retrieval	= 10%
Article assessments	= 20%
Writing	= 60%
Reviewing & editing	= 5%

Chapter 2: *Literature Review 1 (Alfaro & Young 2016)*

Author	Contribution	Contribution	Total	Signature
T. Young	Concept & structure Article retrieval Article assessments Writing	50% 100% 100% 85%	83.5%	
A.C. Alfaro	Concept & structure Writing Review/edit	50% 15% 100%	16.5%	

Chapter 3: *Literature Review 2 (Young & Alfaro 2016)*

Author	Contribution	Contribution	Total	Signature
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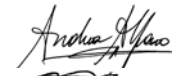
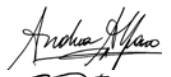
The co-authored experimental manuscripts in this Thesis have been broken down into five task components, each of which have been given a weighting (% time) to produce the completed output:

Experimental design	= 5%
Sample collection & analysis	= 20%
Data analysis & interpretation	= 20%
Writing	= 50%
Reviewing & editing	= 5%

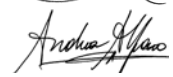
Chapter 4: *Case Study 1 (Young et al. 2015)*

Author	Contribution	Contribution	Total	Signature
T. Young	Experimental design Sample analysis Data analysis Data interpretation Writing	100% 100% 100% 100% 100%	95%	
A.C. Alfaro	Review/edit	50%	2.5%	
S.G. Villas-Bôas	Review/edit	50%	2.5%	

Chapter 5: *Case Study 2 (Young et al. 2016)*

Author	Contribution	Contribution	Total	Signature
T. Young	Experimental design Sample analysis Data analysis Data interpretation Writing	100% 100% 100% 100% 100%	95%	
A.C. Alfaro	Review/edit	50%	2.5%	
S.G. Villas-Bôas	Review/edit	50%	2.5%	

Chapter 6: *Case Study 3 (Young et al. 2017)*

Author	Contribution	Contribution	Total	Signature
T. Young	Experimental design Sample analysis Data analysis Data interpretation Writing	100% 95% 100% 100% 98%	92%	
A. Kesarcodi-Watson	Sample analysis Writing	5% 2%	3%	
A.C. Alfaro	Review/edit	50%	2.5%	
S.G. Villas-Bôas	Review/edit	50%	2.5%	

Chapter 7: *Case Study 4 (Young et al. in prep)*

Author	Contribution	Contribution	Total	Signature
T. Young	Experimental design Sample analysis Data analysis Data interpretation Writing	50% 50% 100% 100% 95%	80%	
S.G. Gale	Experimental design Sample analysis Writing	50% 50% 5%	15%	
A.C. Alfaro	Review/edit	50%	2.5%	
S.G. Villas-Bôas	Review/edit	50%	2.5%	

Acknowledgments

Thank you Andrea for being such a fantastic primary supervisor!

My PhD buddies – ugggggh, thaaaankyou!! Paul McBride and Jarrod Cussens for being there when I needed you and for being my flatties, Dr Rebecca Jarvis for always making me smile and feel happy, Carine Bourgois for the honesty and laughs. And lastly to my partner in crime, Dung Viet Le, what fun we had driving to the hatchery every morning, working through the nights, cheeky coldies after work on Friday's, hiking in the forest, and travelling the West Coast. Couldn't have done it without you mate!

To the Nelson crew. I am specifically thankful to my friends Nicola Hawes, Sarah Cumming, Dan McCall and Rodney Roberts from SPATnz for providing great chinwags, broodstock, technical expertise, physical assistance, and for truly enabling my research. I also am greatly indebted to Samantha Gale, Norman Ragg, Zöe Hilton, and Helen Mussely from Cawthron Institute for empowering and supporting me, for fostering the close relationship that we have between our research groups, providing expert knowledge and advice, working late with me in the cold, and most of all, for being such great mates. I would also like to thank my friends Aditya Kesarcodi-Watson, Hannah Mae, and Ellie Watts from Cawthron Institute for helping me to get the job done, I owe you one. Thank you Archim Jenkin for your kind hospitality, your inescapable bearhugs, and your friendship. Catherine, you are a star and a legend!

To Silas Villas-Bôas, Margarita Markovskaya, Francesca Casu, and Erica Zarate from the Metabolomics Laboratory at the University of Auckland – thank you so much for training me and providing guidance with the technical aspects of my research.

Finally, to some of the most important people in my life. Lynda Young, Patrick Tanner, Paula Luthardt, Smokee Young, and Jodi Hale – I could never have done it without you, at times it was a challenge for all of us for which I am sorry, I am eternally grateful for your support, and I love you all so very much! XOX

This research was supported by the Cawthron Cultured Shellfish Programme (NZ Ministry of Business, Innovation and Employment contracts CAWS0802, CAW1315), and a Faculty Research Grant to A.C. Alfaro. Logistical and technical support was provided by Spat Production and Technology Ltd., the Auckland University of Technology's School of Science, and the University of Auckland's Metabolomics Laboratory. A stipend was supported by an AUT Vice Chancellors Doctoral Scholarship.

Arial sunset photo of the Cathwron Aquaculture Park where all animal experiments were conducted for this Thesis. Sunny Nelson, Sth Island, New Zealand.

Chapter 1 Scope

Introduction and Thesis framework

1.1 General introduction

"My mollusc! What's a mollusc?" – Terry Pratchett, Men at Arms: The Play

Mollusca is a large phylum of invertebrates with ~85,000 extant species currently recognised. They are also the largest marine phylum with ~50,000 described species, and comprising around 23% of all known marine animals (Appeltans et al. 2012). From an ecological perspective, marine molluscs serve many important roles (reviewed by NRC 2010; Dame 2011). They are crucially positioned within lower trophic levels of marine food webs, and they are key to providing substrate complexity of benthic environments to support entire marine communities, increase species diversity, and reduce erosion. Many molluscs are filter feeders which help to lower levels of water eutrophication, and influence the nutrient and organic coupling of benthic and pelagic systems through biodeposition. Molluscs also contribute to biogeochemical processes through shell formation, carbon sequestration and providing an important source of sedimentary carbonate content. Some groups (e.g., mussels, oysters) act as excellent indicators of ecosystem health, and some may also be useful to gauge the conservation needs of other taxa (Hellou & Law 2003). Aside from their ecological roles, marine molluscs also deliver important socioeconomic services.

Mollusc fisheries and aquaculture combined provide around 24 million tonnes of shellfish per year (FAO 2016), and directly or indirectly support the livelihoods of many millions of people globally (Valderrama et al. 2010). Most of the revenues generated are accounted for by the demand for seafood. However, farmed molluscs are also valuable for the production of consumer items (e.g., pearls, jewellery), and are instrumental in providing a wide range of specific biotoxins and other metabolites used

in drug development and medical research (e.g., medications to prevent pain, accelerate recovery from nerve injury) (Lewis et al. 2012). Compared with other aquaculture sectors, such as fish farming, molluscs are generally considered to be a more environmentally sustainable source of food and income. Being major primary consumers in shallow marine ecosystems, many groups (e.g., mussels, oysters, clams) do not require provision of feed input, they help to keep coastal waters clean, and are well-suited for integrated multi-trophic aquaculture (Reid et al. 2010). So long as careful choices are made (e.g., avoiding introductions of unwanted organisms and high density/intensive farming practices), bivalve mariculture in particular can even be viewed as a paradigmatic example of a sustainable economy safeguarding the environment (Parisi et al. 2012; NOAA 2013; Baker et al. 2015).

1.1.1 Mollusc aquaculture

Mollusc aquaculture has a long history, with ancient Romans being well-practiced in the cultivation of oysters over two thousand years ago. These techniques have been gradually refined and expanded over the centuries. Today, over 70 marine species are widely farmed around the world. With annual harvests of almost 16 million metric tonnes, molluscs account for over 60% of global animal mariculture production by weight (Figure 1.1), and the industry is valued at around $19 billion USD per annum (FAO 2016). This is dominated by the cultivation of bivalves. The bivalve sector has steadily been growing at a rate of approximately 5% each year for the past couple of decades, and is expected to increase substantially in the coming years due to expansion of current markets via globalisation, establishment of new production areas, intensification of advanced farming practices (e.g., hatcheries), applications of innovative research technologies (e.g., advanced molecular techniques), and cultivation of new species (Pawiro 2010; Alfaro et al. 2014a).

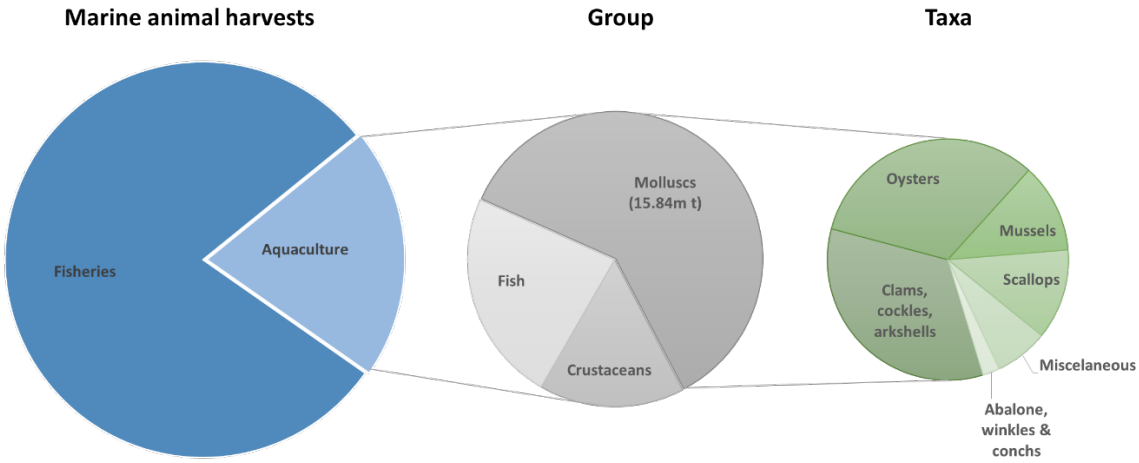


Figure 1.1. Breakdown of global marine aquaculture production in 2014 (including brackish water and excluding plants). Information was extracted from the FAO (2016) global capture fisheries and aquaculture production databases.

1.1.1.1 Aquaculture in New Zealand

The New Zealand aquaculture industry is working hard towards sustainably expanding various sectors to achieve a revenue goal of $1 billion NZD in annual sales by 2025; more than doubling its current value (MPI 2012). Aquaculture exports are dominated by Greenshell™ mussels (*Perna canaliculus*) which account for 81% of exports, followed by Chinook salmon (14%) and Pacific oysters (5%) (Allison & Destremau 2015). To achieve this goal, an increase in both production volume and product value will be required. The New Zealand aquaculture industry is thus aiming to further develop its marine mollusc sectors through establishing commercial scale mussel and oyster hatcheries, and advancing its selective breeding programmes. With these strategies well underway, their success will enable farms to be supplied with high quality stock which have strong marketable traits, and also produce family lines which are resilient to problematic diseases caused by ubiquitous marine pathogens.

1.1.1.1.1 Mussels

The New Zealand mussel sector currently relies entirely on wild-caught spat for stocking farms. However, successful spatfalls are extremely dependant on local weather conditions

and other natural environmental fluctuations (Alfaro & Jeffs 2003; Alfaro et al. 2010, 2011a). In addition, the 'quality' of wild-caught animals is highly variable, and the available sources of this spat can no longer supply demand (Smith et al. 2016). Thus, there is considerable interest and investment to develop the ability and capacity for full-lifecycle culturing techniques, and to implement a selective breeding programme to provide farms with high quality juveniles that have desirable traits (e.g., shell coloration, biochemical/nutritional composition, high post-deployment retention, growth and survival) (Allison & Destremau 2015).

To achieve this technological development, a Primary Growth Partnership (PGP) was established in 2012 between the New Zealand Ministry for Primary Industries (MPI) and the country's largest seafood company Sanford Ltd. The PGP programme resulted in the formation of a Sanford Ltd subsidiary company, Spat Production and Technology Ltd (SPATnz), based in Nelson, New Zealand. Equal initial co-investments totalling $26 million NZD was provided for the development of a large scale mussel hatchery to be operated by SPATnz. The first phase of the project ensued in construction of the hatchery which began operations in 2015. With 15+ years of prior broodstock conditioning, larval rearing, and selective breeding research (initially pioneered by the Cawthron Institute [Nelson, New Zealand] and with an on-going collaboration), SPATnz is now successfully producing large quantities of selectively bred mussel larvae/spat.

The hatchery is currently set to begin its Phase II expansion to increase production in 2017 with the capacity to supply industry with around 30,000 tonnes a year equivalent of adult mussels (Smith et al. 2016). However, although proof of concept has been successfully realised, consistency of larval quality is a persistent challenge with variability in larval growth and settlement rates, and the occasional occurrence of entire 'batch crashes' for which causation has not yet been established (Smith et al. 2016; personal communication, R. Roberts; SPATnz, 2015). Thus, to support expansion of the

sector it is crucial that the reason/s for cases of high mortalities (up to 100%) and poor larval quality are identified, and for new tools to be developed in order to better understand and monitor larval health.

1.1.1.1.2 Oysters

Oyster farms in New Zealand similarly rely on wild-caught spat for stock, although this is currently being supported by a small-scale commercial hatchery. A hatchery, a nursery, and a selective breeding programme have been under development by the Cawthron Institute for around 10 years. The nursery and spat growing operations were taken over by Aotea Fisheries Ltd in 2013, but Cawthron Institute maintains the hatchery component under contract, and continues to direct the breeding programme and perform associated research.

Pacific oysters are a relatively high-value product compared to mussels. However, with a revenue of around $20 million NZD in 2015, oysters are currently one of the smallest contributing sector to New Zealand's main aquaculture exports. Furthermore, overall production volume has declined by around 30% during the past decade due to the recent emergence of an extremely pathogenic marine herpesvirus, ostreid herpesvirus micro-variant (OsHV-1 µVar) (Barrat-Boyes 2012). The virus was first detected in France in 2008 and has since been responsible for killing massive volumes of oyster stock around the world, resulting in huge socio-economic consequences. The true costs associated with the virus is difficult to establish, but with global oyster aquaculture representing a multi-billion dollar sector and mass mortalities exceeding 80% of stock in many cases, negative repercussions are undoubtedly great.

Adult oysters do not demonstrate mortality upon OsHV-1 µVar exposures, and can manage viral infections by controlling its replication (Segarra et al. 2014). However, early lifestages are particularly vulnerable to the pathogen and substantial commercial losses arise when outbreaks occur during larval culture in hatcheries, and on farms with

juvenile stock (Mortensen et al. 2016). Because invertebrates generally lack an acquired immune response, controlling diseases is a considerable challenge. Many of the therapeutic strategies that can benefit diseased fish are ineffective for marine molluscs, and there are no effective vaccines available for OsHV-1 µVar (Dégremont et al. 2015a; DoA 2015). Thus, selectively breeding oyster families/strains for viral resistance have been identified as a key long term strategy to overcome the consequences linked with widespread infections (Camara & Symonds 2014).

A number of research organisations, industry partners and funding agencies in New Zealand and around the world (e.g., Australia, France, Ireland, United Kingdom) are investing heavily in research to collaboratively identify heritable traits associated with viral susceptibility and develop selectively bred families which are resistant to the virus across all lifestages (Prado-Alvarez et al. 2016). However, most of the research conducted thus far has focused on post-metamorphic lifestages (i.e., spat and juveniles <1 year old), and considerable knowledge gaps exist for earlier larval lifestages when oysters are most susceptible to viral infection.

1.1.2 Early lifestage molluscan research

With the many services that marine molluscs provide, it is perhaps unsurprising that their adult biology is well-researched through academic and commercial investigative interests that have spanned many centuries. However, most molluscs have complex biphasic life-cycles and much less is known about their early ontogenic development, including their fundamental requirements in many cases. The great diversity of molluscan taxa is mirrored by various life strategies and ecologies of the larval stage. Some species are direct developers without a planktonic phase, but most have a free-swimming stage that lasts from days to months prior to settlement and the beginnings of a sedentary lifestyle. The pelagic larval period enables them to disperse over long distances, colonise new territory, and move away from overcrowded or unsuitable habitats. Very little is known

about the fate of larvae during this time due to the difficulties in tracking such tiny organisms in the vast seascapes of the open ocean. However, we have learned by culturing particular species in the laboratory that their nutritional requirements, physical tolerances (e.g., pH, salinity, temperature, oxygen availability), susceptibilities to toxins and pathogens, and the environmental cues which regulate their behaviours and developmental timing is often highly species specific.

As previously mentioned, marine molluscs are good indicators of ecosystem health. Furthermore, their embryonic and larval forms have particular relevance in this area. These early lifestages are extremely sensitive to environmental influences and are used widely as standard sentinel organisms for marine pollution and ecotoxicology studies (Losso et al. 2007; Fabbri et al. 2014; Gamain et al. 2016), and, more recently, for investigating potential consequences of global climate change scenarios, such as ocean acidification (Waldbusser et al. 2015; Cole et al. 2016; Dineshram et al. 2016). Thus, molluscan embryos and larvae present a unique opportunity to monitor the effects of anthropogenic impacts, help guide environmental management decisions, and ultimately keep our marine ecosystems healthy.

Within aquaculture, early lifestage molluscan research is varied with efforts being focused on maternal provisioning and offspring quality (de Sousa et al. 2015; Myrina et al. 2015; Joaquim et al. 2016), nutritional requirements and dietary preferences (Aarab et al. 2013; Sánchez-Lazo & Martínez-Pita 2014; Gui et al. 2016; Southgate et al. 2016), understanding the genetic basis for developmental variation (Pace et al. 2006; Francis Pan et al. 2015), optimisation of culture conditions or rearing systems (Kamermans et al. 2013; Turini et al. 2014; van Hung et al. 2015; Gale et al. 2016), disease management and pathology (Kesarcodi-Watson et al. 2012a,b; Rojas et al. 2015; Sohn et al. 2016), improving rates of larval settlement and metamorphosis (Mesías-Gansbiller et al. 2013; Young et al. 2015a; Çelik et al. 2015; Protopopescu & Beal 2015), and development of

cryopreservation capabilities for selective breeding purposes (Paredes et al. 2013; Suquet et al. 2014; Rusk et al. 2017b). Many advances in these areas have been made over the past couple of decades, thus providing significant opportunities for full lifecycle culture of certain taxa. Yet, many questions still remain to be comprehensively investigated; even for species with long culture histories. Until recently, this was partially due to limited application of advanced molecular and biochemical tools/techniques, or their lack of availability. However, over the past decade, numerous breakthroughs have been made in analytical and computational technologies and state-of-the-art instrumentation is relatively easier to access, and analysis costs are typically much lower.

We can now separate very complex biological matrices (e.g., cells, tissues, whole organisms) and characterise their composition in high detail and in a high throughput fashion. Many of these analytical developments owe themselves to efforts and advances made during the Human Genome Project (Venter et al. 2001), and largely to the needs which have followed its completion in 2001 to better understand gene function. Now, 15 years into the 'post-genomic era', global untargeted analysis of all gene transcripts (mRNA), their protein products, and metabolites which are not encoded in the genome (but are functionally modified by proteins) is possible. Rather than measuring one or a few target genes, proteins or metabolites of interest in a single assay, hundreds to thousands of biomolecules can be analysed simultaneously. The large sets of data can then be mined to discover often unexpected and novel information. Different strategies are used to assess these three levels of biological organisation, and have evolved into their own disciplines coined transcriptomics, proteomics, and metabolomics (reviewed by Schneider & Orchard 2011); collectively known as 'omics'-based approaches. The advent of omics technologies has brought about a major change towards the systematic analysis of biological processes at many different scales, and has triggered a revolution in exploratory based research. Used alone or in conjunction with one another, these

untargeted analytical strategies have recently proved incredibly valuable for functional genomic investigations and numerous other applications in various animal models, including marine molluscs.

1.1.2.1 Application of 'omics'-based approaches

Omics-based approaches controversially represent a paradigm shift in the way science is being conducted towards non-hypothesis driven research. However, it is important to note that the 'scientific method' based on empiricism is by no means violated, and with the shift rather signalling a change from primarily hypothesis-driven to data-driven research, the concept that omics-based approaches are 'hypothesis-free' is not really true. Indeed, for any omics investigation, the underlying hypothesis is that genes, proteins and/or metabolites are responsible for the phenotypic trait being studied, and that the omics data will find them. It is hypothesis-free only in the sense that we do not have to specify in advance which genes/proteins/metabolites/pathways we think are responsible. Thus, whether omics is considered as a paradigm shift or not, the approach is undoubtedly a transformation in the *way* biologists 'do business'. Omics-based approaches have evolved to better-understand the complexity of biological systems and organisms' responses to environmental disturbances, and signifies an exciting new era in modern biology.

Omics-based analyses of early lifestage invertebrates is still in its infancy, but a few examples do exist which demonstrate applications of transcriptomics and proteomics in molluscan embryos and larvae. For example, global analysis of gene and/or protein expressions have revealed the complex mechanisms in pre-metamorphic stages which are involved in developmental timing (Huan et al. 2012, 2015), intraspecific growth variation (Meyer & Manahan 2010), larval quality and health (Bassim et al. 2015), metal toxicity (Navarro et al. 2011), and environmental stress responses (Dineshram et al. 2013, 2015; 2016). Each of these studies have applications which are relevant for enhancing full

lifecycle mollusc aquaculture capabilities, and together have set a precedent for omics-based research within the general field of larval biology. Furthermore, by very broadly scanning multiple biomolecules, results from these studies also show that omics-based approaches are incredibly useful for generating novel hypotheses which would have been difficult or highly time-consuming, if not impossible, to devise through targeted analytical strategies based on current *a priori* knowledge. However, metabolomics-based analyses have not thus far been applied to such early lifestage investigations which is perhaps surprising considering the unique benefits that metabolomics has proven to deliver in other disciplines, such as medicine and human health, microbiological research, plant physiology, and agricultural sciences, among others (reviewed by Nadella et al. 2012; Aldridge & Rhee 2014; Tenenboim & Brotman 2016; Wishart 2016).

1.1.3 Moving forward

To further develop the mollusc aquaculture sectors in New Zealand, and around the globe, full lifecycle hatchery culture is vital. However, reasons for variability in larval quality will need to be identified and remediated to achieve optimal production capacity, and strategies to combat or circumnavigate problems associated with disease and poor health will need to be devised. These issues can only be solved through gaining in-depth understanding of the physiological functioning of embryos and larvae. To achieve this, transcriptomic and proteomic research will certainly continue to contribute in the future, and foreseeably with much greater usages. But these approaches alone cannot provide the complete picture of any biological system since they do not fully capture metabolic processes responsible for energy conversion and repartitioning, and also are removed to some extent from certain environmental influences. Thus, to complete the picture, it is necessary to include detailed analyses of general metabolism, downstream of genes and proteins, through incorporation of metabolomic-based approaches into early lifestage molluscan research.

1.2 Thesis motivation

"The sea, once it casts its spell, holds one in its net of wonder forever"
– Jacques Yves Cousteau

I grew up in the Shetland Islands, a small isolated and rugged outcrop of rock bordered on three sides by the convergence of the North Sea, the Norwegian Sea, and the Atlantic Ocean; a place where it is impossible to be more than three kilometres from rolling swells, and equally impossible to stand up straight in the face of a 'light breeze'. I spent most of my free childhood days at the beach or along the clifftops, mostly wet and mostly very cold – but always happy. My friend's dads were fishermen or aquaculture farmers, mine worked at sea on the oil rigs. Skimming stones, whistling for seals, counting limpets, picking periwinkles, catching fish, feeding them in seapens, or scooping them from inside when no one was looking – those were the days! I have always had an affinity for the sea and the creatures which inhabit its shores and depths – apart from sharks, I am not very fond of sharks.

I have spent almost the last ten years of my life dedicated towards studying early ontogenic development of marine molluscs, albeit mostly in the warmer climes of subtropical New Zealand. During these years I have worked on various fundamental biology projects investigating pre- and post-metamorphic stages of many species, including the Mediterranean mussel (Sánchez-Lazo et al. 2012), the Chilean ribbed and blue mussels (Alfaro & Young 2010), the Pacific oyster (Suneja et al. 2014; Hilton et al. 2016; Young et al. 2017), the Japanese abalone (Young & Alfaro 2012), the New Zealand abalone (Alfaro et al. 2014b), the New Zealand pipi clam (Young et al. 2007), the New Zealand geoduck (Le et al. 2016a,b), the New Zealand scallop (de Jong 2013), the New Zealand ribbed mussel (Alfaro et al. 2011b), and the New Zealand green-lipped mussel

(Ganesan et al. 2008, 2012a,b; Young et al. 2008a,b,c 2009, 2011, 2015a,b, 2016; Gale et al. 2016; Ragg et al. 2016; Rusk et al. 2017a,b).

During my Masters thesis (2007–2009) I conducted research working closely with the founding team of what was to become SPATnz. The aim was to enhance mussel (*P. canaliculus*) settlement rates at the final stages of larval development immediately prior to metamorphosis. Excellent results were obtained in laboratory experiments using pharmacological agents to induce the behaviour by targeting particular receptors, and also providing substrates with different physicochemical properties (Young et al. 2009).

I then spent the next year or so working in collaboration with SPATnz and AUT at the Cawthron Institute's aquaculture facility to upscale laboratory results in a pilot hatchery using commercial practices, and to investigate the role of bacterial biofilms in promoting settlement and providing nutrition (Ganesan et al. 2012a,b; Young et al. unpublished data). It became apparent that the laboratory results were not as scalable as predicted when conducted over longer durations and under different culture conditions. The methods I had developed with my collaborators could significantly enhance larval settlement rates over the first few days of 'setting', but these rates could not be maintained due to subsequent post-settlement mortalities and/or poor larval retention on the settlement substrates.

Thus, with evidences of unpredictable variability in embryo and larval quality (i.e., differential developmental timing, growth rate, health, survival) prior to the settlement and metamorphic events, it became clear to me that a much better understanding of earlier ontogenic stages and larval condition should ideally be obtained due to likely carryover effects. This was really the original motivation behind my PhD Thesis which percolated with a strong vision for incorporating modern research techniques into early lifestage molluscan studies, and subsequently developed into a defined proposal and candidacy in 2012.

The newly-developing field of metabolomics was gaining its stronghold in the medical research arena, with a number of proven applications in human embryonic and stem cell research, and for a myriad of toxicology, pathology and general health studies. At that time, transcriptomic and proteomic approaches were also just starting to be applied to investigate early development of marine molluscs with great success, as previously outlined. Thus, I felt strongly that the addition of metabolomics (i.e., the 'final' omics approach in the genotype-to-phenotype cascade [see Chapter 2 Figure 2.1]) would significantly expand the scope of early lifestage molluscan research and serve a number of highly beneficial, as well as crucial, roles:

1. Unlike transcriptomics and proteomics, metabolomics is easily performed on non-model organisms with little or no genomic information (ideal for certain cultivated marine mollusc species).

2. Metabolomics is generally a low-cost analytical strategy compared to other omics approaches and data analysis is relatively quick (ideal for industry applications).

3. The metabolome is highly sensitive and fast-reacting to both genetic and environmental influences (providing a central link between genotype and phenotype).

4. Metabolomics can deliver additional information at a different level of biological organisation (to provide novel stand-alone findings).

5. Metabolomics can provide complimentary information on other levels of biological organisation (to lend support for hypotheses and to fully reconstruct complex molecular and metabolic networks when integrated, thus providing a good overview of the entire biological system).

1.3 Thesis aims

The primary aim of this Thesis is to pioneer the use of metabolomics-based approaches in early lifestage investigations of marine molluscs, and demonstrate that such approaches can deliver novel and valuable insights into their biology's with broad applications in aquaculture, immunology and toxicology. The secondary aim of this Thesis is to provide the general aquaculture research community with resources to incentivise and enable them to incorporate a metabolomics-based component into their existing or future research projects.

1.4 Thesis structure

This Thesis comprises of two literature reviews (Chapters 2 & 3), four experimental case studies (Chapters 4–7), and a final Thesis synthesis, discussion and conclusion (Chapter 8). The primary research chapters (Chapters 4–7) are classified into three general themes to experimentally demonstrate unique applications of metabolomics in mollusc aquaculture and early lifestage health and development:

- Mollusc larval quality: Chapters 4 & 5
- Mollusc host-pathogen interaction: Chapter 6
- Mollusc toxicology : Chapter 7 (Parts A & B)

Particular aspects of these case studies were developed in collaboration with our external research and industry partners (Cawthron Institute and SPATnz Ltd) to provide information with commercial relevance and value. The case studies were also designed to utilise different sampling regimes, organisms, lifestages, and data analysis approaches where appropriate to provide broad exemplars for different situations.

Chapter's 4–6 utilise metabolomics in isolation with each of these case studies being presented as they would appear for publication, with minor modifications. Chapter 7 combines metabolomics with a targeted analysis of selected biomarkers as a validatory

exercise, and is thus presented in two sections. The first section (Part A), primarily contextualises the body of work and reports on untargeted metabolomics-derived data and their interpretations. Taking this research further, some of these interpretations and hypotheses are subsequently tested in Part B through a targeted analysis of enzymes and other macromolecules to gain additional information at different levels of biological organisation.

1.5 Chapter contents and rationales

Chapter 2 – Literature Review 1: *Metabolomic applications in aquaculture research*

Overview: What is metabolomics, what can it tell us about cultured aquatic organisms, and how might it be used constructively in the future?

Very few aquaculture researchers have heard of the term 'metabolomics'. This is probably because the field is relatively new and has not yet been widely applied to gain knowledge with commercial potential – or at least it might not have been recognised. However, over 100 published studies have been conducted which incorporate a metabolomics component that are directly or indirectly relevant to the industry. Therefore, with the timely need for a first formal review of metabolomics as applied to aquatic biology and aquaculture, this chapter set out to provide such a review and showcase some unique applications of the approach. The rationale of providing this chapter content is to educate aquaculture scientists, and other interested parties, as to the benefits that metabolomics can deliver, to identify research gaps where metabolomics can advance knowledge in areas with commercial value (e.g., hatchery production of mollusc larvae), and to motivate interest and future inclusions of the approach into aquaculture research projects around the globe.

Chapter 3 – Literature Review 2: *Metabolomic strategies in aquaculture research*

Overview: How are metabolomics studies performed, what techniques are used, and how can researchers access the necessary facilities and expertise?

This chapter provides information on how metabolomics investigations are conducted by describing the different experimental, analytical and statistical strategies which are typically employed, and outlines some special considerations for samples derived from aquatic environments. For each of the strategies described, specific examples of primary research involving aquatic organisms are given. The rationale for this content is to act as a primer/tutorial for researchers who are new to the field to enable them to carry out metabolomic-based projects in aquaculture, or within any aquatic biology context, and encourage wider applications.

Chapter 4 – Case Study 1: *Assessment of larval quality*

Objective: To determine whether visually observable phenotypic traits in mussel larvae can be characterised and discriminated by metabolomic-based approaches.

To investigate this objective as a first test, metabolomics was applied to determine if mussel larvae of the same age and life experience but expressing different growth rates could be discriminated based on their metabolite profiles. Growth rate is a currently-used proxy for larval quality in shellfish hatcheries, with slow-growing organisms generally being considered inferior in quality (Helm et al. 2004). Slower growing larvae often reflect organisms with poor health due to disease and/or with structural abnormalities, and their presence can contribute towards decreasing the population health of the entire larval batch and reducing settlement success (Loosanoff & Davis 1963; Taris et al. 2006). Maintaining a tight bell curve on growth and development is also important to synchronise and hasten settlement and metamorphic competency which enhances production efficiency, and larvae with slow growth rates may produce slow growing spat

or juveniles which is undesirable for subsequent growout phases (Collet et al. 1999; Tanyaros & Tarangkoon 2016). The primary rationale of this study is simply to ascertain whether metabolomics can distinguish larval samples to the same effect as currently used techniques (i.e., microscopy, sieving/grading), and to perform the first appraisal of the sensitivity of the larval metabolome. This was an important step to reinforce the value of assessing further applications.

Chapter 5 – Case Study 2: *Assessments of handling stress and culture conditions*

Objective: To determine whether visually non-observable traits can be characterised and discriminated by metabolomic-based approaches.

The rationale of this case study was to better-understand the sensitivity and variability of the larval metabolome by establishing whether non-observable traits in larval samples could be distinguished via profiles of metabolites. With the successful outcome of the first case study, this second case study sought to establish if handling stress (i.e., sieving during a water exchange) and different rearing conditions (i.e., flowthrough vs. static culture) have influences on the mussel larval metabolome. Using larvae from a single cohort with different life experiences but expressing similar growth rates, this study also sought to verify the results of the first case study by re-analysing the larval quality metabolite biomarkers that had previously been identified.

Chapter 6 – Case Study 3: *Assessment of larval health during viral infection*

Objective: To determine if analysis of the larval metabolome can provide novel insights into mechanisms of disease and poor health caused by pathogens.

To investigate metabolomic applications in larval health and disease, the Pacific oyster (*Crassostrea gigas*) and its relationship with OsHV-1 µVar was selected as a model case example. Larval forms are most susceptible to OsHV-1 µVar infections, and

although it has been recognised that more of a research focus should be placed on early development stages (Dégremont et al. 2016), the host-virus interaction has not yet been investigated in larvae. This developmental stage has crucial importance for the production of commercial hatcheries, as well as explaining the abundance of natural spatfall. Selective breeding efforts have recently produced families which are resistant to OsHV-1 µVar (Dégremont et al. 2015b, 2016), but the underlying genetic and metabolic mechanisms responsible for resistance are wholly unknown. To further enhance selective breeding programmes and monitor their success, it is vital we understand precisely how larval host metabolism is modulated by the virus to proliferate, characterise which mechanisms are being activated by the host in efforts to counter the infection, and define the heritabilities of mechanistic components/traits for future genetic improvements. This particular early lifestage disease model represents the most important yet challenging obstacle that the global oyster aquaculture sector has ever faced (Castinel et al. 2015).

Chapter 7 – Case Study 4: *Assessment of embryo health during toxin exposure*

Objective: To determine if analysis of the embryonic metabolome can provide novel insights into mechanisms of toxicity and poor health caused by heavy metal exposure.

To test whether metabolomics can similarly be applied to assess poor mussel embryo and larval health in the final case study, a semi-commercial scale trial was developed to provide industry-relevant information. To experimentally induce poor health, consultation with our industry partner (SPATnz) and collaborating scientists at Cawthron Institute led to the decision to use copper toxicity as the experimental stressor. We chose to use copper in this study because it is commonly used as a reference toxicant for marine invertebrate embryotoxicity assays (Novelli et al. 2003; Fabbri et al. 2014; Libralato et al. 2013), it is very stable under a range of environmental conditions, its toxicological effects and biological fate are reasonably well-characterised in marine

molluscs (Fitzpatrick et al. 2008; Al-Subiai et al. 2011; Zhang et al. 2011; Giacomin 2014), dosing parameters are easily controlled, it is a highly relevant contaminant in New Zealand waters (Gadd & Cameron 2012), and there are some concerns regarding negative influences of potential trace metal pollution in the local seawater used during hatchery culture of mussel larvae (personal communication, R. Roberts; SPATnz, 2015). Selection of copper as the health stressor for this case study additionally provides a unique ecotoxicology context which broadens the scope of metabolomics-based applications in marine mollusc development for future researchers within the field of marine toxicology. This final case study also incorporated very early embryonic lifestages for the first time, involved a temporal aspect to the sampling design, and sought to validate interpretations gained from the metabolomics data through targeted analyses of a suite of oxidative stress biomarkers.

1.6 Research outputs arising from this thesis

1.6.1 Conference presentations

Young T., Alfaro A.C., Villas-Bôas S.G., Robertson J., Duxbury M., Higgins C., Brooks J., Pook C. **2013**. *Omics in Aquaculture: Research Trends and Future Directions*. Presented Oct 24th at the New Zealand Aquaculture Conference. Nelson, New Zealand.

Young T. Alfaro A.C., Villas-Bôas S.G., Robertson J. **2014**. *Muscling in on Mussels with Systems Science*. Presented Oct 23rd at the New Zealand Aquaculture Conference. Nelson, New Zealand.

Young T. Alfaro A.C., Villas-Boas S.G., Robertson J. **2014**. *Metabolomic Applications in Marine Invertebrate Development and Aquaculture*. Presented Aug 11th at the New Zealand Marine Sciences Society Conference. Nelson, New Zealand.

Young T. Alfaro A.C., Villas-Bôas S.G., Gale S. **2015**. *Shifting Paradigms towards Non-Hypothesis Driven Research in Marine Molecular Biology*. Presented July 6th at the New Zealand Marine Sciences Society Conference. Auckland, New Zealand.

Young T., Gale S.L., Burritt D., Sander S., Ragg N.L.C., Le D.V., Benedict B., Watts E., Taylor J., Alfaro A.C., Villas-Bôas S.G. **2015**. *Identification of Health Biomarkers in Marine Mussels: An Integrated, Multi-disciplinary Approach*. Presented Aug 25th at the International Society for Environmental Toxicology and Chemistry Conference. Nelson, New Zealand.

Adams S.L., Young T., Ragg N.L.C., Hilton Z. **2015**. *An Overview of Current Research to Enhance Shellfish Aquaculture in New Zealand*. Presented Sept 17th at the 6th International Symposium for Marine Biology and Biotechnology: The Omics in the Ocean. Pintung, Taiwan

Young T., Kesarcodi-Watson A., Alfaro A.C., Merien F., Nguyen T.V., Mae H., Le D.V., Villas-Bôas S.G. **2016**. *Disturbance of Larval Host Metabolism in Response to OsHV-1 Virus Exposes Novel Immunological Biomarkers*. Presented July 4th at the New Zealand Marine Sciences Society Conference. Wellington, New Zealand.

Young T., Gale S.L., Ragg N.L.C., Burritt D., Le D.V., Watts E., Taylor J., Benedict B., Alfaro A.C., Sander S., Villas-Bôas S.G. **2016**. *Metabolic Regulation of Immunotoxicity during Marine Invertebrate Embryogenesis*. Presented July 5th at the New Zealand Marine Sciences Society Conference. Wellington, New Zealand.

Gale S.L., Young T., Burritt D., Ragg N.L.C., Le D.V., Sander S., Benedict B., Watts E., Mae H., Hawes N., Berry J., Currie K.I., Alfaro A.C., Villas-Bôas S.G., King N., Hilton Z. **2016**. *Piloting Biochemical Tools to Assess Bivalve Early Life Health in Response to Ecologically Relevant Coastal Stressors*. Presented July 7th at the New Zealand Marine Sciences Society Conference. Wellington, New Zealand.

Alfaro A.C., Young T., Nguyen T.V. **2016**. *Novel Diagnostics to Boost Aquaculture Production*. Presented Sept 27th at the New Zealand Aquaculture Conference. Nelson, New Zealand.

Young T., Kesarcodi-Watson A., Alfaro A.C., Merien F., Nguyen T.V., Mae H., Le D.V., Villas-Bôas S.G. **2016**. *OsHV-1 Hijacks Host Metabolism in Oyster Larvae*. Presented Sept 27th at the New Zealand Aquaculture Conference. Nelson, New Zealand.

Hilton Z., Young T., Gale S.L., Dunphy Burritt D., Ragg N.L.C., King N., Adams S., Mae H., Alfaro A.C. **2016**. *Understanding Mechanisms of Herpes Virus Resilience in Pacific Oysters*. Presented Sept 27th at the New Zealand Aquaculture Conference. Nelson, New Zealand.

1.6.2 Industry portal publications

Alfaro A.C., Young T. **2015**. Metabolomics: An innovative and powerful tool that will revolutionize aquaculture. *World Aquaculture*, 46(2): 21–22.

Alfaro A.C., Young T. **2015**. Metabolomics approaches to improve mussel larval production. *Global Aquaculture Advocate*, 18(6): 78–79.

1.6.3 Peer-reviewed journal publications

Chapter 2

Alfaro A.C., Young T. **2016**. Showcasing metabolomics in aquaculture: A review. *Reviews in Aquaculture*, DOI: 10.1111/raq.12152

Chapter 3

Young T., Alfaro A.C. **2016**. Metabolomic strategies for aquaculture: A primer. *Reviews in Aquaculture*, DOI: 10.1111/raq.12146

Chapter 4

Young T., Alfaro A.C., Villas-Bôas S.G. **2015**. Identification of candidate biomarkers for quality assessment of hatchery-reared mussel larvae via GC/MS-based metabolomics. *New Zealand Journal of Marine and Freshwater Research*, 49(1): 87–95.

Chapter 5

Young T., Alfaro A.C., Villas-Bôas S.G. **2016**. Metabolic profiling of mussel larvae: Effect of handling and culture conditions. *Aquaculture International*, 24(3): 843–856.

Chapter 6

Young T., Kesarcodi-Watson A., Alfaro A.C., Merien F., Nguyen T.V., Mae H., Le D.V., Villas-Bôas S.G. **2017**. Differential expression of novel metabolic and immunological biomarkers in oysters challenged with a virulent strain of OsHV-1. *Developmental & Comparative Immunology*, 73: 229–245.

Chapter 7

Young T., Gale S.L., Alfaro A.C., Ragg N.L.C., Le D.V., Sander S.G., Burritt D.J., Benedict B., Villas-Bôas S.G. Assessing health of early life stage marine molluscs: Application of a multiresolution, multidisciplinary biomarker toolkit. *In prep*.

Young T., Gale S.L., Alfaro A.C., Ragg N.L.C., Le D.V., Sander S.G., Burritt D.J., Benedict B., Villas-Bôas S.G. Metabolic regulation of copper toxicity during marine mussel embryogenesis. *In prep*.

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Al-Subiai S.N., Moody A.J., Mustafa S.A., Jha A.N. 2011. A multiple biomarker approach to investigate the effects of copper on the marine bivalve mollusc, *Mytilus edulis*. *Ecotoxicology and Environmental Safety*, 74(7): 1913–1920.

Aldridge B.B., Rhee K.Y. 2014. Microbial metabolomics: Innovation, application, insight. *Current Opinion in Microbiology*, 19: 90–96.

Alfaro A.C., Jeffs A.G. 2003. Variability in mussel settlement on suspended ropes placed at Ahipara Bay, northland, New Zealand. *Aquaculture*, 216(1–4): 115–126.

Alfaro A.C, Young T. 2010. *Comparative Study of Settlement Responses to Neuroactive Compounds among Mussel Species*. Presented at the 9th International Larval Biology Symposium – LARVAL 2010, Wellington, New Zealand.

Alfaro A.C., McArdle B., Jeffs A.G. 2010. Temporal patterns of arrival of beachcast green-lipped mussel (*Perna canaliculus*) spat harvested for aquaculture in New Zealand and its relationship with hydrodynamic and meteorological conditions. *Aquaculture*, 302: 208–218.

Alfaro A.C., Jeffs A.G., Gardner J.P.A., Bollard Breen B.A., Wilkin J. 2011a. Green-lipped mussels in GLM9. *New Zealand Fisheries Assessment Report 2011/48*. Ministry of Fisheries, Wellington, New Zealand.

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Chapter 2 Literature review 1

Metabolomic applications in aquaculture

Note: This chapter has been published with the following citation:

Alfaro A.C., Young T. 2016. Showcasing metabolomics in aquaculture: A review. *Reviews in Aquaculture*, DOI: 10.1111/raq.12152

Abstract

Aquaculture production is currently challenged to meet the growing demands for seafood protein throughout the world. To achieve this growth in an efficient, safe and sustainable manner, novel tools and applications will need to be incorporated at each step of the production line. A variety of 'omics' (e.g., transcriptomics, proteomics, metabolomics) applications have already begun to emerge in aquaculture research with extreme success. A promising new 'omics' approach is metabolomics, which aims to use metabolite profiles to identify biomarkers indicative of physiological responses of living samples (e.g., whole organism, tissues, cells) to environmental or culture conditions. One of the benefits of this approach is that it uses a broad scan of biological conditions to identify often unexpected problem or risk areas to focus management attention. In this contribution, relevant research examples have been selected to showcase the applications of metabolomics in aquaculture in four major areas: hatchery production, nutrition and diet, disease and immunology, and food safety and quality. The novelty of this approach is highlighted by the fact that the majority of published papers in this field have been cited, and these are all recent contributions.

2.1 Global aquaculture

We live in a world of increasing environmental impacts and diminishing food resources. In addition, our growing population places more and more demands on food supplies, including animal-based protein. Undoubtedly, seafood supplies and demands have increased exponentially in recent years, and are expected to continue to grow in the years to come. This growth is mainly focused on aquaculture, since global wild-caught fish resources have declined due to overexploitation, and are unlikely to recover in the near future. Thus, over 50% of fish and seaweed food supplies currently come from aquaculture, amounting to about 90 million tonnes and worth US$144 billion in 2012 (FAO 2014). It is estimated that production will need to almost double by 2050 if we are to meet the growing demands (Waite et al. 2014). There have been many suggested scenarios as to how this growth could be achieved, but it is clear that future production will need to improve in efficiency, safety, sustainability and environmental performance. Regardless, technological innovation will be required to enhance reproduction and conditioning, larval rearing, disease diagnostics and immunology, nutrition and feed formulation, cultivation systems performance, and food safety and quality. Recent biotechnological advances have produced improvements in all of these areas, and new tools and approaches proliferate in the literature daily. One of the most promising biotechnological areas is that of ‘omics’ approaches. With rapidly expanding capabilities and diversity of analytical platforms and computational analysis, ‘omics’ applications are likely to revolutionise aquaculture research and development in the near future.

2.2 ‘Omics’ approaches

During the last century, biological research has benefited from the knowledge generated around the genome and a myriad of applications. A strong emphasis was placed on the idea that genes direct all processes within a cell, and thus the organism, is based on the

assumption that proteins, via mRNA, and then metabolites are synthesised in a hierarchical manner when genes are activated. However, this idea has now been superseded by the knowledge that many metabolic processes are controlled by complex feedback mechanisms and regulatory networks involving post-translational protein modifications and metabolite fluxes, which are de-coupled from gene expression (Suarez & Moyes 2012; Chubukov et al. 2013). At the heart of this new thinking are a range of newly created fields under the 'omics' banner, which promise to revolutionise biotechnological research across all life-base investigations. 'Omics' is a term which refers to the collective technologies used to explore the structures, functions, relationships and dynamics of various biomolecules within the cells of organisms. Recent advances in technology now enable us to use non-targeted 'omics'-based approaches to identify the global set of gene products (transcripts, proteins and metabolites) within a biological sample, rather than single products at a time. The exploratory nature of these approaches means that novel hypotheses are often generated rather than validated. At the same time, one of the advantages of 'omics' is that usually unexpected information is revealed, leading to high innovation and discovery in a very efficient manner.

New 'omics' fields are continually being generated, but the bulk of research from genotype to phenotype falls within genomics, transcriptomics, proteomics and metabolomics (Figure 2.1). Genomics aims to sequence, assemble and analyse the structure and function of a given genome. The DNA sequence provides information about what could potentially happen in the organism if they were to express particular genes. Transcriptomics focuses on sequencing the RNA transcripts that are produced by the genome and identifies which genes are actually being expressed under specific biological circumstances. This knowledge provides information about what appears to be happening in the organism at a given time. Proteomics is the study of proteins as a way to elucidate the structure, function and regulation of biological systems. This includes proteins which

are constructed by the direct instruction from genes, but also the functions of proteins which occur due to post-translational protein modifications. Since enzymes are involved in the operation and regulation of metabolic pathways in every living organism, their expressions reflect accurate responses to cellular and environmental conditions in real time. This information provides understanding about what makes cellular processes happen. Metabolomics (the study of metabolites) is often reported to represent a better picture of an organism's phenotype, since metabolites are very sensitive to environmental changes and provide information about what is actually happening on a metabolic and physiological level.

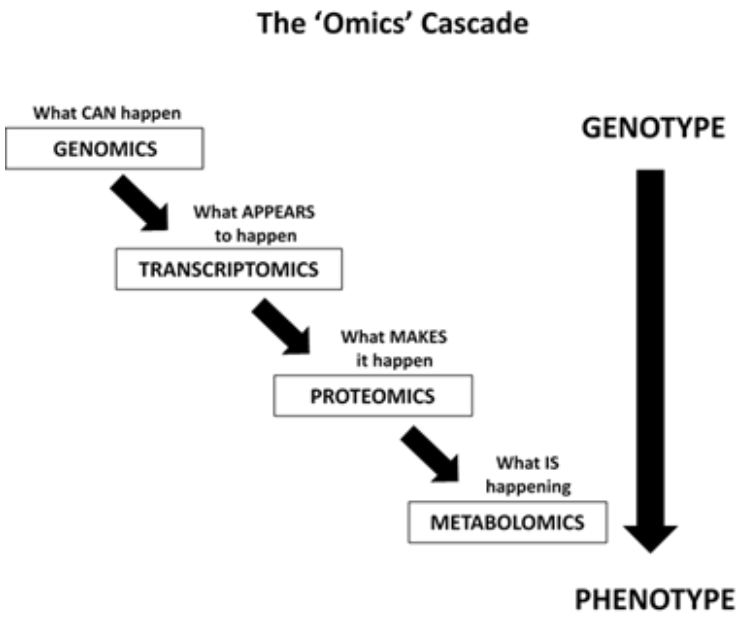

Figure 2.1. Diagram of 'omics' cascade defining genomics, transcriptomics, proteomics and metabolomics, and depicting their position along the genotype to phenotype continuum.

The rapid expansion in capabilities and applications within these fields owes a great deal to recent advances in analytical platforms (e.g., Nuclear Magnetic Resonance, Mass Spectroscopy) and bioinformatics, which allow us to obtain and mine huge amounts of data previously unattainable (reviewed in Chapter 3 [Young & Alfaro 2016]). These changes are reflected in the widening of scope of biological studies from simple

biochemical analyses of individual genes, proteins or metabolites to measurements of complex component mixtures within biological systems. This type of research is possible with the use of new sophisticated multi-platform technology that allows high-throughput analysis. The constant generation of new algorithms for software and search engines ensures maximum data production. These data can then be compared to gene and protein sequences and chemical fingerprints of metabolites stored in the public domain and proprietary databanks that continue to grow exponentially.

There is a wealth of information related to 'omics' research in the literature, especially for genomics and transcriptomics. However, newer 'omics' fields, such as metabolomics are represented by fewer publications, especially with regards to aquaculture applications. Indeed, there are comprehensive reviews for genomics (Quinn et al. 2012; Huete-Pérez & Quezada 2013), transcriptomics (Saroglia & Liu 2012; Qian et al. 2014) and proteomics (Rodrigues et al. 2012; Zhou et al. 2012; Carrera et al. 2013; Peng 2013) in aquaculture and seafood-related research. However, there is no such review for metabolomics as yet. To date, there are over 150 papers published that use metabolomics to address a range of issues with direct or indirect relevance to aquaculture (Figure 2.2).

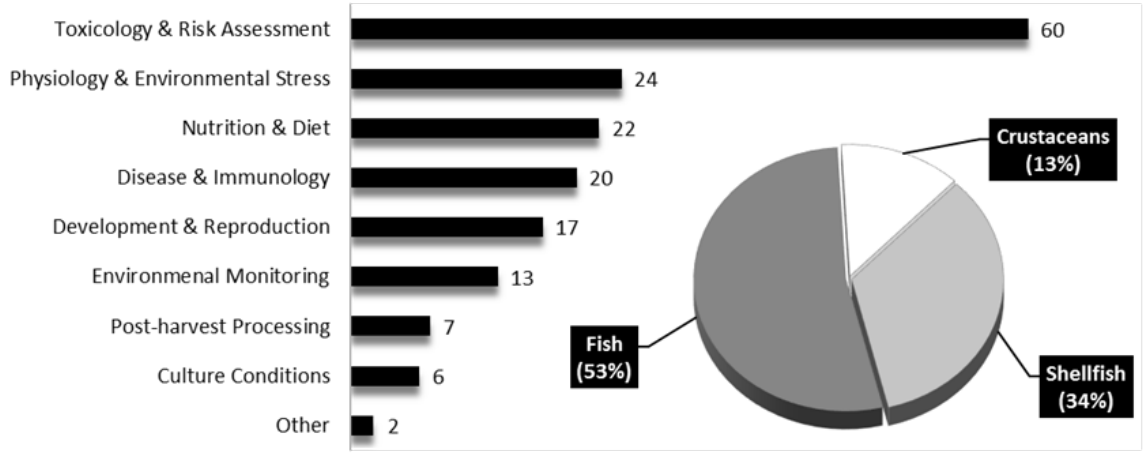

Figure 2.2. Trends in metabolomics-based research with applications to the aquaculture sector. The bar chart displays the number of publications from 2000-2014 within various categories. The pie chart displays the proportion of those publications, which involve relevant aquaculture organisms.

The majority of these papers are in the area of toxicology and risk assessment, although many other areas of aquaculture production are represented. Not surprising, over 50% of all papers have focused on fish, followed by shellfish and crustaceans. It is envisaged that publications in this field will continue to increase in the coming years, especially as more streamline workflows are generated. As a starting point, Chapter 3 comprises a companion review paper (Young & Alfaro 2016) that deals with the mechanics of how metabolomics projects can be carried out. This primer presents easy to follow metabolomics strategies, including sample collection and preparation, choice of analytical platforms, and statistics and bioinformatics for data analysis and interpretation.

Thus, the aim of this contribution is to highlight the potential applications of metabolomics to aquaculture by showcasing relevant papers within four general areas of aquaculture production: hatchery production, nutrition and diet, disease and immunology, and post-harvest product quality. It is important to note that the goal of the current chapter is not to discuss all publications in these areas, but rather papers have been carefully selected which clearly illustrate the metabolomics potential. At the end of this chapter, a list of future directions and implications for metabolomics in aquaculture are provided.

2.3 What is metabolomics?

Metabolomics is the study of chemical processes involving metabolites. Metabolite profiles can be obtained and investigated to find chemical signatures that reflect specific cell activities, such as a metabolic response to an environmental stimulus. This field does not use DNA, RNA or proteins, but only metabolites that are produced as a result of genetic coding specific to that cell or organism or as a response to environmental perturbation. In simple terms, it is possible to say that metabolomics is a ‘physiological snapshot’ of a living cell or tissue. This approach can then be used to identify physiological differences between cells, tissues, organs or organisms that have been

exposed to different environmental conditions (or culturing conditions). Thus, analytical techniques are used to search within the metabolomes (the total inventory of metabolites) of biological samples. Then, statistical examination of the metabolite profiles is performed to look for differences among samples. From such differences we can identify metabolite signatures that serve as biomarkers for that specific condition and identify the activities of particular metabolic pathways (e.g., glycolysis, TCA cycle, fatty acid biosynthesis, amino acid metabolism). Within experimental trials, metabolomics can be used to identify effects of water temperature, oxygen levels, dietary manipulations, and pathogen or toxin exposures, among others.

2.4 Advantages of metabolomics

Although other 'omics' approaches have their own unique benefits, there are a number of advantages to conducting metabolomics-based research. Compared to proteomic and transcriptomic analyses, metabolomics generally involves less sample preparation, has much shorter turnaround times from sample collection to data interpretation, and a typical experiment can be conducted for a fraction of the costs. Metabolomics can be performed using non-invasive body fluids/solid, such as plasma and faeces, which may be of particular value for studies involving fish. In addition, a number of analytical techniques (e.g., NMR and infrared spectroscopy) can be used without destruction of samples. This is extremely useful when biological material is limited and/or when multiple analyses are to be conducted on a single sample with the aim of data integration. Another distinguishing characteristic beneficial for aquaculture researchers is that the majority of metabolites are not species-specific, unlike genes and proteins. Consequently, the analytical assay does not need to be redeveloped for every animal model. Furthermore, metabolomics can easily be applied to the study of non-model organisms without prior knowledge of their genome. This makes the approach particularly well-suited to research

involving new and emerging aquaculture species, but also organisms with long culture histories.

2.5 Metabolomic research

Two main avenues exist for researchers who wish to conduct metabolomics investigations or add a metabolomics component to an existing research project. There are a number of commercial metabolomics laboratories throughout the world which offer streamlined services. Core facilities at various universities and centres house a unique combination of infrastructure and expertise to carry out a range of advanced metabolomics studies. These organisations can provide excellent support from consultation on experimental design to data analysis and interpretation of results. Inevitably, significant costs are usually associated with such commercial services. Alternatively, access to metabolomics facilities can be gained through academic institutions for substantially reduced charges based on collaborative agreements. For scientist wanting to conduct metabolomics research for the first time, it is important to note that running a successful metabolomics project requires an adequate experience in chemistry, statistics, bioinformatics and the advice from a metabolomics expert on hand. For researchers with sufficient chemistry knowledge and access to appropriate equipment and facilities, extraction and initial identification of metabolites may be relatively easy. However, there are some specific constraints in sample collection/preparation and experimental design that need to be considered. In addition, the bioinformatics required for data analysis and interpretation are significantly complex and may require the involvement of a bioinformatics expert. Regardless of the approach, new metabolomics projects should incorporate the appropriate expertise from the start. Furthermore, scientists are urged to give appropriate consideration to the expected results and implications of findings, since this approach is exploratory by nature.

The scope of this contribution is not to provide details of how metabolomics projects are undertaken or to describe the analytical and statistical strategies employed; these details are provided in Chapter 3. In this review, focus is limited to the application of metabolomics in aquaculture research, with specific examples in the areas of hatchery production, nutrition and diet, disease and immunology, and post-harvest product quality control.

2.6 Metabolomics applications in aquaculture research

2.6.1 Hatchery production

Successful larviculture of fish and marine invertebrates presents one of the foremost challenges in the development of full life-cycle rearing practices. Advances in hatchery technology are often incremental and directed by technical managers. Unfortunately, technical developments are poorly captured in the primary scientific literature and often not shared due to concerns over losing commercial advantage (Allan & Burnell 2013). While significant improvements in hatchery production technology have been made over the past two decades, considerable opportunities exist for optimising the quality of broodstock, feeding regimes and culture systems (Hamre et al. 2013). For many species, production yields are greatly hampered by high (>90 %) mortality rates during larval rearing (Salze et al. 2011; Purcell et al. 2012; Sørensen et al. 2014). In some cases, this appears to be due to inadequate culture practices for new species and a lack of knowledge regarding their fundamental requirements. In other cases, low success can be attributed to known environmental, genetic, nutritional or disease-related factors. However, unexpected batch crashes are routinely observed in some hatcheries and causation is often not identified. Metabolomics could be employed within various frameworks to help solve some of these issues by providing mechanistic and functional biochemical information, and to support development of remedial strategies for poor hatchery performance.

There are few reports on the application of metabolomics-based approaches to investigate the larval biology of aquatic organisms. However, recent inroads have been made with research on developmental biology using Zebrafish (*Danio rerio*) as a model organism (Papan & Chen 2009). The Zebrafish larval metabolome is highly dynamic through embryogenesis and can be used to successfully predict development stage (Hayashi et al. 2011), identify provisional requirements for energy acquisition and growth (Huang et al. 2013; Raterink et al. 2013), reveal coordinated flux in gene and protein expressions (Soanes et al. 2011), and characterise mechanisms of drug action and toxicity (Akhtar et al. 2016; Brox et al. 2016; Chai et al. 2016; Huang et al. 2016; Wang et al. 2016; Xu et al. 2016; Yan et al. 2016). While the zebrafish is not a commercially cultured species, such research provides an exemplary application and highlights the potential for studying the early life-stages of other organisms. Thus far, the only studies with specific applications of metabolomics in hatchery production of invertebrate larvae are those stemming from this thesis and involve investigation of mussel larval quality (Chapter 4 [Young et al. 2015]), the effects of handling stress and culture conditions during mussel larval rearing (Chapter 5 [Young et al. 2016]), characterisation of host-virus interactions in oyster larvae infected with ostreid herpesvirus (Chapter 6 [Young et al. 2017]), and analysis of mussel embryonic and larval health in response to trace metal contamination in seawater (Chapter 7 [in review]).

Our limited knowledge of exogenous and endogenous regulation of early development in different marine invertebrate and fish species restricts rapid advancements in larviculture practices. It is crucial that we mature our understanding of the factors that influence developmental timing, energy acquisition and allocation, nutritional requirements and preferences, and immunological and toxicological response mechanisms. With the imminent likelihood of an expansion in the diversity of farmed marine species, it becomes especially urgent that we develop solutions to address the

problem of larval nutrition and disease. Use of metabolomics could also be applied to other areas of hatchery production to help close the loop on full life-cycle culture for many species. For example, routine production of high-quality gametes for successful fertilisation and on-growing could be achieved through better broodstock management and understanding of maternal provisioning, paternal effects and factors associated with high fecundity. While not yet realised within the aquaculture industry, metabolomics has benefitted other areas of developmental biology to investigate reproductive disorders (Courant et al. 2013), identify biomarkers for assessment of sperm fertility (Kumar et al. 2015), to assess oocyte quality and predict embryo viability (Bertoldo et al. 2013; Cortezzi et al. 2013), and to identify the coordination of metabolic traits during selective breeding for stress resistance and longevity (Malmendal et al. 2013). Metabolomics will undoubtedly be a useful tool in the progression of future hatchery technologies.

2.6.2 Nutrition and diet

Nutritional research in aquaculture aims to improve the health of cultured species through diet. Providing the appropriate nutrition quality and quantity is likely to have broad ramifications into other cultivation aspects, such as individual performance improvement, disease prevention, enhancement of broodstock and gamete quality, development of sustainable and high-quality feed alternatives and mitigation of environmental impacts, among others. Optimal nutritional requirements for many new and emerging aquaculture species are unknown. Thus, there is an urgent need to determine peak dietary conditions for these organisms to support sector expansion and diversification. Even for many well-established species, the complex interactions between nutrition, health and environment is poorly understood. Furthermore, efforts to maximise production yields are inhibited by limited knowledge of larval dietary preferences and nutritional requirements for a number of species. Thus, considerable scope exists to boost full life-cycle culture productivities.

Metabolomics is uniquely suited to assess metabolic responses to nutritional deficiencies or excesses, and can provide in-depth mechanistic insights to assist development of optimised feeding regimes.

Currently, nutritional metabolomics research in aquaculture is an emerging field. Thus far, metabolomics-based approaches have proved useful for assessing: positive and negative effects of food deprivation in mussels, trout and salmon (Tuffnail et al. 2009; Kullgren et al. 2010; Baumgarner & Cooper 2012; Thunathong et al. 2012; Cipriano et al. 2015), interactions between diet, environment and disease in abalone and seabream (Rosenblum et al. 2005; Silva et al. 2014), effects of nutrient supplementation in seabream, salmon and carp (Cajka et al. 2013; Robles et al. 2013; Anderson et al. 2014; Wagner et al. 2014), effects of dietary protein substitution and utilisation in salmon and carp (Bankefors et al. 2011; Jin et al. 2015), effects of using reduced fishmeal-based feed alternatives in cobia and charr (Schock et al. 2012; Abro et al. 2014), effects of newly introduced plant-derived contaminants in salmon feeds (Søfteland et al. 2014), and development of non-invasive dietary inspection techniques in various finfish species (Asakura et al. 2014).

To illustrate the use of metabolomics to investigate dietary performance we highlight a study by Abro et al. (2014) who tested the use of a protein-rich zygomycetes fungus (*Rhizopus oryzae*) as a substitute for the traditional fish meal protein in Arctic charr (*Salvelinus alpinus*) diets. The authors produced metabolite profiles of fish fed a commercial diet of unknown composition, a diet with mostly fish meal protein, and a diet with mostly zygomycetes protein. Analysis of metabolite profiles from liver samples indicated that the zygomycetes protein diet did not differ from the fish meal protein diet, and suggests similar physiological responses to these diets. However, significant metabolite differences were observed between fish fed the commercial diet and fish fed each of the other two protein-based diets, with the former being an inferior diet. The study

used liver samples to extract metabolite signatures since this organ is actively involved in metabolism of absorbed nutrients, such as proteins. The combination of a nuclear magnetic resonance (^{1}H-NMR) analytical platform and statistical analyses, including orthogonal projection to latent squares discriminate analysis (OPLS-DA), provided a powerful pathway to identify differences and similarities among the metabolite profiles to test for diet effects. In addition, the study highlights the possibility of using alternative protein sources, which may prove to be more sustainable for fish cultivation practices in the future.

In another study of fish diets, Robles et al. (2013) used the short-chain fatty acid butyrate as a diet supplement to increase body weight and enhance intestinal tract activity in sea bream (*Sparus aurata*). Using a high performance liquid chromatography mass spectroscopy (LC-MS) platform, the authors measured over 80 metabolites from fish intestine samples before and after feeding with the butyrate supplement diet. Initial samples were taken after a 12-h starvation period to obtain a basal metabolite profile with a non-active intestine. Three hours after the initial feeding, intestine samples were collected from fish that were fed the butyrate supplemented diet and fish fed diets without the supplement. Growth measurements of the remaining fish within each treatment were taken after 8 weeks. Results showed significant improvements with butyrate diets, including weight gains, increases in several essential amino acids and nucleotide derivatives, and potential increases in cell energy provisions through glucose and amino acid oxidation pathways. Based on these results, it appears that butyrate may be a good natural supplement to enhance fish growth and metabolic activity.

As an emerging tool in nutrition research, metabolomics offers a unique potential to unravel the complex intertwining mechanisms involved in nutrient utilisation, reproduction, growth and disease progression. Through the catabolic breakdown of macromolecules in foods and direct incorporation of smaller components, the

metabolome is the receiving depot for the raw materials required by cells to synthesise new products. These materials are essential for the formation and repair of body tissues, and the production of energy to support and maintain life. As signalling molecules and enzymatic cofactors, metabolites are involved in the synthesis, degradation and modification of proteins, which regulate gene expression and metabolic pathways. It is the intricate combination of these multifaceted processes which are required for biological systems to maintain homeostasis. Metabolomics can provide important mechanistic insights to identify how regulation of homeostatic control is disturbed in the early phases of diet-related diseases. This knowledge could be used to identify new metabolic biomarkers for health and nutritional status and to develop strategies for the dietary prevention and intervention of diseases. Future nutrition research in aquaculture will undoubtedly be radically advanced through application of metabolomic approaches.

2.6.3 Disease and immunology

The successful management of health and diseases is a major challenge for aquaculturists. Health is characterised by the optimal functioning of biological systems. Within this framework, disease can simply be defined as the lack of health. However, disease is commonly associated with interactions between host organisms and pathogens (bacterial, viral or parasitic). Disease outbreaks can arise due to transmission from wild-stocks, accidental transfer of diseased animals between farms, use of pathogen-infected feeds, poor water quality, lack of sanitary barriers, failure to identify and isolate unhealthy organisms, and impaired animal welfare as a result of overstocking and inadequate nutrition, among others. It is estimated that up to 20% of potential aquaculture production in China, which is responsible for over two thirds of the world's production, is lost due to the occurrences of diseases (Li et al. 2011). In some cases, mass mortalities as a result of severe disease epidemics have decimated certain sectors, leading to complete collapse

of the industry and having huge socioeconomic impacts (e.g., herpesvirus infections in oysters and white spot syndrome virus in shrimp) (Sánchez-Martínez et al. 2007; Peeler et al. 2012).

It was previously thought that diseases had single causes with single diagnostic targets. However, considerable research has established tight links with intricate metabolic imbalances. Subtle changes in entire metabolic pathways can be responsible for the onset and development of particular health conditions. Imbalances caused by genetic, nutritional or environmental factors can lead to suppression of immune function, which in turn can make organisms more susceptible to pathogen exposure than they might normally be. Host organisms may maintain healthy lives in the continuous presence of pathogens, and only when they experience stressful conditions will the equilibrium shift, favouring the dominance of the pathogen. Alternatively, the encounter of a healthy organism with a highly virulent pathogen can quickly overwhelm the immune system, leading to major instability in metabolic homeostasis and ultimately resulting in death. Due to the involvement of metabolic networks in the initiation and proliferation of diseases, metabolomics can provide unique insights into the effects of pathogen exposure and the mechanisms of resistance. Furthermore, metabolomics can be applied as a valuable tool to determine the efficacy of disease treatments and management.

Practical applications of metabolomics in aquaculture research to investigate host-pathogen interactions are developing rapidly. In general, these findings are revealing that pathogen exposure tends to cause severe disturbances to host energy metabolism, osmotic regulation, oxidative stress, cell-signalling pathways and respiratory mechanisms. A suite of recent metabolomics-based investigations on the immunological responses of bivalves to infections with various bacterial pathogens have also revealed that the molecular response-mechanisms are differentially regulated depending on bacterial strain (Ji et al. 2013; Wu et al. 2013; Liu et al. 2013a, 2014a,b), the host species and pedigree (Liu et al.

2013b; Wu et al. 2013), the sex of the host (Liu et al. 2014a), and between different tissues (Ji et al. 2013; Wu et al. 2013; Liu et al. 2014b). These results highlight and reinforce the importance that different disease management strategies may need to be developed for particular conditions. Metabolomics also has been used to investigate the interactions of nutrition, temperature, withering syndrome disease and disease treatments in abalone (Rosenblum et al. 2005, 2006), to discover metabolite biomarkers associated with enhanced host defence against bacterial infection in carp (Guo et al. 2014), to evaluate the effect of superintensive aquaculture systems on the health of shrimp (Schock et al. 2013), to identify metabolic responses of crabs to vibrosis (Schock et al. 2010), to characterise metabolic demands of hepatic tumors in flatfish (Southam et al. 2008), to enhance the health of cobia through dietary manipulation (Schock et al. 2012), and to develop nutritional and non-nutritional treatments for enhancing survival of diseased tilapia (Cheng et al. 2014; Zhao et al. 2015).

A good example of metabolomics applications to assess health parameters in cultured marine invertebrates is a study by Schock et al. (2013). In this case, the authors used NMR-based metabolomics to monitor health factors along the production line of shrimp (*Litopenaeus vannamei*) from nursery to harvest. Shrimp were grown in a superintensive aquaculture system with minimal water exchanges and a biofloc system to promote growth of beneficial bacterial communities. The aim of this superintensive system was to increase production (year-round high density farming) while reducing disease susceptibility and enhance water quality (via reduction of waste products). Weekly shrimp samples were collected throughout the nursery (about 2 months) and growout raceways (about 4 months) periods, snap frozen in liquid nitrogen and then stored at -80°C until analyses could be performed. A robust quality control procedure was performed to evaluate experimental biases in extraction process and timing with the use of standard reference material, extractions of different tissues from the same animal, and

various pooled and blank replicates as controls. Such protocols for verification of sound data collection procedures are essential for metabolomics project, and reporting on these results should be common practice in the literature, although seldom encountered. Comparison of NMR spectra of biological samples from nursery and raceway phases resulted in important differences that could be interpreted as stressful conditions to shrimp. Specifically, the compounds iosine and trehalose were found to be good biomarkers for stress in this species. From an industry perspective, these physiologically stressful conditions were attributed to three main events – a total ammonia nitrogen spike in the nursery, a period of reduced feeding due to surface scum build-up in raceways, and the transition of stock from the nursery to raceways. Clearly, this metabolomics-based study identified specific areas where the industry could focus to improve production efficiency. Such results are likely to have required considerably more time and financial investment with traditional experimental approaches.

Another study of health evaluation using metabolomics is provided by Guo et al. (2014). In this study crucian carp (*Carassius auratus*) were investigated for their ability to survive infections caused by the bacterium *Edwardsiella tarda*. Using a GC-MS platform, the authors compared metabolite profiles from infected fish that survived and died, and their respective controls (not infected). The results not only identified biomarkers that could be used to signal infection in the fish, but also biochemical pathways likely to be involved in immune responses to infections by *E. tarda*. Secondary bioinformatics analysis involved the use of the web-based MetaboAnalyst pipeline (Xia et al. 2015) for metabolomics data analysis and the pathway library of zebrafish (surrogate for carp) to identify biochemical pathways that were indicative of infections. The identified biomarkers for this infection include increased levels of palmitic acid and decreases in the amount of D-mannose. Relevant pathways included elevated unsaturated fatty acid biosynthesis and decreases in fructose and mannose metabolism. The broader

implications of these results are that metabolomics may be an effective approach to identify early signs of pathogens and infections that could be mitigated at the onset of a crisis. In addition, the improved understanding of biochemical pathways involve in immunological responses may be extremely valuable when selecting optimal conditions for growth of cultured species. It is important to re-iterate that the strength of metabolomics is that is provides a wide scan of physiological activities and identification of areas where problems may be encountered. Thus, this is a powerful approach to inform more detailed studies of production issues such as health threats.

The deleterious effects of pathogens and viruses are enhanced by exposure of aquatic organisms to immunotoxic contaminants, which supress the immune system and hasten the onset of disease (Morley 2010; Girón-Pérez 2010). Poor water quality is a major problem for the aquaculture industry in many regions. Rapid industrialisation and urbanisation has resulted in various organic and synthetic pollutants being introduced into aquatic ecosystems which pose serious threats to the health of cultured organisms (Li et al. 2011). The number of new and emerging contaminants being released into the environment is increasing and their biological fate and effects are often unknown. Metabolomics is progressively being used to characterise mechanisms of toxicity and develop novel methods to assess environmental contamination using fish and shellfish. For example, metabolomics-based studies have proven useful for: identifying physiological responses of various molluscs to heavy metals and endocrine disruptors (Wu et al. 2013; Hanana et al. 2014; Ji et al. 2014; Leonard et al. 2014; Zhou et al. 2014; Ji et al. 2015a), characterising the consequence of pesticide exposure in carp (Kokushi et al. 2015), assessing the effects of petrochemical contamination at industrialised sites harbouring caged mussels (Fasulo et al. 2012; Capello et al. 2013, 2015), detecting freshwater locations which have multi-contaminant sediment loadings (Watanabe et al. 2015), identifying new and highly sensitive bioindicator species of clams for monitoring

environmental contamination (Ji et al. 2015b), and developing non-invasive methods for pollution assessment by profiling metabolites in the excreted mucus of fish skin (Ekman et al. 2015). The results of these studies clearly demonstrate promising application. It is expected that metabolomics will be used extensively in the future to monitor the health of sentinel species, deliver forensic capabilities for evaluating pollution exposure, enhance the sensitivities of ecotoxicological assessments, and to provide authorities with information to set new regulatory guidelines. The exposure of aquatic organisms to environmental contaminants and disease-related factors during their cultivation has major implications for biosecurity, market opportunity, product quality, product value, and more importantly food safety.

2.6.4 Post-harvest product quality

One of the biggest challenges for the aquaculture industry is to achieve and maintain a high quality product from farm to market, and into the hands of consumers. One person's view of quality may be different from another's and numerous factors are normally involved in its classification. Nutritional value is an objective aspect based on the presence of essential amino acids, highly digestible proteins, vitamins, minerals and a high content of polyunsaturated fatty acids (e.g., omega-3 fatty acids). Meat quality is also defined by other compositional features, such as the lean to fat ratio and water content. Consumers' perception of quality is perhaps one of the most important aspects of aquaculture, and involves palatability factors, which can be highly subjective. These include visual appearance, smell, firmness, juiciness, tenderness and flavour. Product quality is a multifaceted term and there is still much to learn about how the various indices are perceived and interact.

In order to achieve a quality product prior to harvesting, it is critical that culture conditions are optimised and well-managed to enhance animal welfare and improve

nutritional and compositional traits. On the other hand, post-harvest processes, such as transport, storage and packaging have major impacts on product freshness and food safety. Due to the involvement of metabolites in flavour and aroma profiles, nutritional value and degradation processes, metabolomics provides an exciting opportunity for food quality assessment and development of new strategies for quality preservation and enhancement. Examples of metabolomics-based analysis in food quality, post-harvest processing and storage are growing rapidly. For instance, metabolomics has thus far proved useful for: identifying degradation of specific nutrients, flavours and aromas in cold-stored fish (Castejón et al. 2010; Leduc et al. 2012), examining interactions between culture conditions, post-harvest quality and storage (Savorani et al. 2010; Picone et al. 2011), development of rapid assessment techniques and discovery of biomarkers to determine fish meat freshness and quality during extended freezing (Duflos et al. 2010; Leduc et al. 2012; Heude et al. 2014), and investigating compositional changes in salmon during and after irradiation processing to reduce foodborne infection and enhance product safety (Villa et al. 2013).

A rapid and straightforward method to assess fish freshness and quality was used by Heude et al. (2014), based on indicative metabolite quantification using high resolution magic angle spinning (^{1}H HR-MAS) NMR spectroscopy. The procedure was aimed at quantifying two well-known indicators of fish freshness and quality (K value and trimethylamine nitrogen [TMA-N] content) in fish samples that had been stored at 0°C. The K value is a measure of autolytic process (spontaneous disintegration of cells or tissues that occurs after death) and TMA-N content is indicative of bacteria spoilage. These parameters are based on measurements of metabolite concentrations, which were recorded in fish from four commercially important species (sea bream, sea bass, trout, and red mullet). Both the NMR approach and traditional methods were compared, and produced similar results. However, NMR had the advantage that it could be used directly

on unprocessed fish without prior extraction procedures with an average time of 40 min per sample. Conversely, traditional analytical methods require extensive and complex extraction procedures that are time-consuming. Another advantage of this new approach is that small sample sizes (about 10–15 mg) are needed for metabolite analyses, and these amounts can be collected with a biopsy, thus, affording considerable benefits to the producer.

The same platform (^{1}H HR-MAS NMR) was used to test the effects of irradiation treatment on cold-smoked Atlantic salmon (*Salmo salar*) as a means to prevent bacterial and parasite infections (Villa et al. 2013). The authors used white salmon mussel samples from live fish from three irradiation treatments (0, 1, and 4 kGy) and non-irradiated controls to obtain NMR spectra that were statistically analysed (principal components analysis [PCA] and analysis of variance [ANOVA]) to identify differences in degradation of fish samples during storage. In this study, several compounds, including creatine, trimethylamine oxide and the sum of phosphorylcholine and glycerophosphorylcholine were found to be diagnostic of irradiation treatment. A huge advantage of this approach is that this is a non-destructive analysis to evaluate food processing and physiological changes (e.g., bioactive compounds) due to fish storage prior to market delivery.

Traceability is another important aspect of food quality control. Regulatory authorities may ask aquaculture producers to identify the origin of their product and stipulate the culture methods to prevent fraudulent activities and ensure food safety. Responsible farmers are concerned with protecting their market share with regards to products from unknown sources and of inferior quality. Methods for food authentication and traceability have been advanced through application of metabolomic-based approaches (reviewed by Castro-Puyana & Herrero 2013; Cubero-Leon et al. 2014). For example, metabolomics has been useful for discriminating between wild and farmed sea bream reared in different culture locations and conditions which are of various qualities,

but marketed as the same high-quality product (Melis et al. 2014). Similar techniques were also successfully used to classify wild-caught and cultured salmon and seabass and expose fraudulent labelling of seafood products (Aursand et al. 2007, 2009; Mannina et al. 2012). In Sardinia, Italy, dried and salted mullet row is processed using special methods which result in a unique high-value product. Metabolomics has been shown to be extremely valuable for distinguishing this locally-produced roe from other sources to protect Sardinia's market (Locci et al. 2011).

Indeed, the study by Locci et al. (2011) perfectly illustrates the use of metabolomics in product traceability. In this case, ^{1}H-NMR was used to obtain metabolite signatures that could differentiate salted and dried mullet (*Mugil cephalus*) roe samples from Sardinia (Italy) and those produced in other geographical regions. The study found that the different storage and manufacturing procedures from different mullet sources could be identified by several metabolites (e.g., free amino acids, organic acids) found in the aqueous phase extracted from samples. This traceability technique is crucially important to Sardinian communities who have a long tradition of processing a high quality roe from this species, called 'bottarga'. The uniqueness of this study is that it shows that this approach can be used not only to identify the providence of the fish samples (previously possible only with DNA and protein analyses), but also the manufacturing and processing style of this traditional Italian artisans. In today's global food markets, this rapid and straightforward metabolomics approach can be used effectively to provide authentication and traceability for the industry and consumers.

2.7 Future applications and directions

As we have discussed, improving nutrition to enhance larval production, animal health and meat quality will be important for the future of aquaculture. In order to advance research in feed formulation and discovery of alternative feed sources for improved

sustainability, an in depth understanding of nutrient requirement and utilisation is needed. Metabolomics is uniquely suited to this task. Individual molecules within test diets can be chemically labelled and traced during the digestive breakdown, uptake and the redistribution and energy conversion processes. Incorporation of comprehensive metabolomics-based tracer studies in nutritional research is an area which should be explored. Other nutrition-based challenges which require attention include enhancing low carbohydrate utilisation in fish, replacement or elimination of antibiotic and chemical use in feeds, improving flesh quality in some species, and lengthening the shelf life of fish and shellfish meat (Tincy et al. 2014).

Successful management of diseases is also a major challenge. Although development of vaccines to remediate many diseases have been indispensable for the successful expansion of the finish sector (Ringø et al. 2014), there are no effective treatments as yet for other organisms (e.g., viral infections in shrimp and oysters [Seibert & Pinto 2012]). Incorporation of metabolomics-based approaches to better-understand the reasons for disease susceptibility, and to develop methods for enhancing immunodiagnostics and host resistance, is an area which requires further exploration. New advances in invertebrate immunology is radically challenging the concepts of innate and adaptive immunity, and the line between them is starting to blur (Sun et al. 2014; Wang et al. 2015). Furthermore, large inter- and intraspecific variations in immune function and responses to pathogen exposure exist. Metabolomics has recently been shown to be extremely useful for investigating mechanisms of immunity and the physiological impacts of pathogen exposure in mammals (Dorrestein et al. 2014; Noto et al. 2014; Grey et al. 2015) and could easily be applied more widely to focus on cultured aquatic species.

Other relevant areas in which metabolomics could be applied in the future include climate change research, cryopreservation and selective breeding. The threats of global

warming and ocean acidification are increasingly becoming a major concern for aquaculture producers and stakeholders (Reid & Jackson 2014). Changes in sea surface temperatures and water pH can enhance the frequency of harmful algal blooms, alter primary productivity, escalate incidences of marine infectious diseases, and reduce growth rates and survival of cultured organisms (Burge et al. 2014; Gilbert et al. 2014; Richards et al. 2015). Metabolomics has recently been used successfully to investigate the effects of ocean acidification on oysters (Wei et al. 2015a,b), thermal stress on sea cucumbers (Shao et al. 2015) and the interactions between red-tide forming dinoflagellates and other phytoplankton species (Poulson-Ellestad et al. 2014). We anticipate there will be substantial growth in the application of metabolomics to this research area. Cryopreservation of gametes and embryos provides a means to preserve genetic material for future use. While there have been many successes in this field for some species and/or certain types of biological material, a number of challenges remain. For example, cryopreservation of fish and shellfish sperm has been achieved; however, cryopreservation of oocytes is still highly problematic (Herráez et al. 2011; Wang et al. 2014; Hassan et al. 2015). In molluscs, cryopreservation of fertilised embryos can be accomplished, but survival rates are often extremely low (Wang et al. 2011; Parades et al. 2012) and the reasons for poor success are unknown. Metabolomics could be used to better understand the physiological mechanisms involved in cellular damage during the cooling and warming processes, and help develop remedial strategies for cryoinjury (Koštál et al. 2011). Selective breeding in aquaculture has a long history based on morphological and, more recently, genetic traits. Recent applications of metabolomics in agriculture have resulted in the identification of new phenotypes for selective breeding of crops and animals to enhance production, disease resistance and product quality (Buitenhuis et al. 2013; Kushalappa et al. 2013; Pushpa et al. 2014; Sundekilde et al.

2014). This provides an exciting new avenue of research, which could be transferred to the aquaculture sector.

2.8 Conclusions

Metabolomics is a powerful newly emerging field that has huge application potential in aquaculture. Recent advances in analytical capabilities and bioinformatics have made it possible to acquire and analyse large amounts of metabolite data more effectively and efficiently. These procedures are now being applied successfully to identify biomarkers to detect a range of cultivation problems and issues in areas such as, larval production, nutrition, health and food safety and quality. Since this is a recently evolving approach, few examples are available to illustrate the relevance of metabolomics in aquaculture. However, these recent publications clearly highlight the benefits of this evolving field. It is envisaged that metabolomics will play an increasingly more important role in all aspects of aquaculture research. Indeed, metabolomics is well-placed to provide the biotechnological advances needed to meet our expected aquaculture growth in the coming years.

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Chapter 3 Literature review 2

Metabolomic strategies for aquatic research

Note: This chapter has been published with the following citation:

Young T., Alfaro A.C. 2016. Metabolomic strategies for aquaculture: A primer. *Reviews in Aquaculture*, DOI: 10.1111/raq.12146

Abstract

Metabolomics is a fast-evolving field that provides qualitative and quantitative analyses of metabolites within cells, tissues or biofluids. The recent applications of metabolomics approaches in aquaculture research reviewed in Chapter 2 have highlighted the huge potential for solving problems within all aspects of the production line, from hatchery production to post-harvest quality control. To assist with the growing application of metabolomics in aquaculture research, this contribution provides a review of techniques and steps necessary to conduct metabolomics research, from experimental design to data interpretation. Specifically, we target scientists who are new to the field of metabolomics, and we offer simple, but comprehensive steps and strategies to conduct this type of research. We conclude this primer and review with some advice on how to access relevant expertise and facilities for metabolomics-based aquaculture research.

3.1 Introduction

Within the last decade, the field of metabolomics has expanded, with a number of applications across the life sciences. In aquaculture alone, metabolite patterns have been successfully used to identify and resolve issues related to hatchery production (Young et al. 2015, 2016), nutrition and diet (Castro et al. 2015; Cheng et al. 2015), disease and immunology (Liu et al. 2015; Peng et al. 2015), and post-harvest quality control (Melis 2014; Chen et al. 2015), among others. There are some reasons why this approach has been so successful in such a relatively short amount of time. To begin with, metabolomics is an approach that can generate comprehensive datasets of metabolites to describe complex biological systems. Furthermore, the same analytical and computational tools used to generate and interpret data can be performed on any living organism, since metabolites are highly conserved in structure and function across species (in contrast to genes). With recent advances in analytical techniques and computational analysis, complete datasets that describe changes and/or differences in biological systems can be carried out in a rapid and cost-effective manner. However, results stemming from this approach do not necessarily provide mechanistic and/or causal information regarding the patterns observed. In other words, the exploratory nature of this approach is likely to generate new hypotheses, and further targeted experiments may lead to validation of the resultant biological markers. This process allows for unexpected information to be revealed, leading to innovation and discovery in a very efficient manner.

Compared to other areas of research, such as agriculture, food science, and medical science, the application of metabolomics to aquaculture research has only recently been realised. These applications have been reviewed in Chapter 2 (Alfaro & Young 2016). Thus, the scope of this Chapters' contribution is limited to a review of the techniques and steps necessary to conduct metabolomics research in aquaculture. Specifically, the purpose of this review is to provide aquaculture researchers and other aquatic scientists

who are new to the field of metabolomics with a simple, but comprehensive, primer on the various strategies that are involved in conducting a metabolomics-based investigation for the first time. This primer summarises information on experimental design, sample collection and preparation, choice of analytical platform, bioinformatics processing, statistical analyses, biological interpretation of the data, and reporting guidelines. Several aspects are outlined which require careful consideration, specifically for experiments involving aquatic organisms, and readers are directed to a range of specific aquaculture-related research studies to showcase the relevance of these topics. This review is concluded with some advice on how researchers can access the relevant expertise and facilities for conducting a metabolomics-based project, and we provide some perspectives on the development of future technological strategies for assessing the health and welfare of wild and cultured aquatic organisms.

3.2 Metabolomic Strategies

There are generally six steps involved in a metabolomics study: (i) robust experimental design, (ii) sample collection and preparation, (iii) analytical measurement and data acquisition, (iv) bioinformatics (data integrity checking and metabolite identifications), (v) statistical analyses, and (vi) biological interpretation and/or biomarker validation (Figure 3.1). Due to the wide range of fields encompassed by metabolomics studies (biology, biochemistry, analytical chemistry, bioinformatics and statistics), it is highly recommended that consultation with a metabolomics specialist is carried out in the early stages of experimental design.

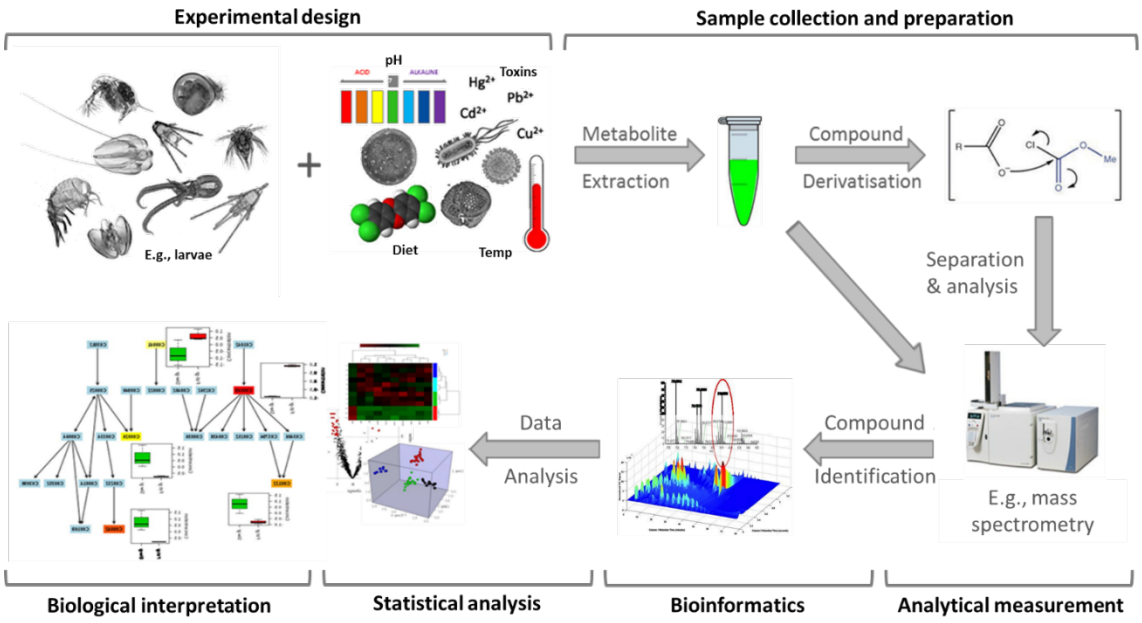

Figure 3.1. General workflow involved in a metabolomics study outlining the six main steps.

3.2.1 Experimental design and sampling

Along with good standard experimental design practices, there are a number of special considerations to keep in mind when planning a strategy for a metabolomics investigation. There are particular requirements, which mandate that samples be taken only after an experiment has been specifically designed and performed with a metabolomic-based analysis in mind. Samples which have previously been collected and stored for another purpose will unlikely be suitable for incorporation into a metabolomics-based study.

Collected samples must reflect and represent the biology in question, and be appropriate for the particular research questions of the study. It is critical that biological, technical and experimental variability be minimised, since the metabolome can change very rapidly in response to subtle changes in the environment. For example, the metabolic signatures of aquatic organisms can be affected by handling stress and air exposure (Karakach et al. 2009; Connor & Gracey 2012; Young et al. 2016), so this should be kept to an absolute minimum - even experimentally characterised if possible. The acute stress

of transferring fish and crustaceans between culture or storage tanks is reflected in the metabolome and, if not controlled, may influence results of a study (Schock et al. 2013; Mushtaq et al. 2014a). In the case of shellfish, metabolic responses to treatments can be masked when organisms are taken from their natural environments and into the laboratory for a period of acclimatisation (Hines et al. 2007). Therefore, sampling and tissue dissections should be performed in situ, when possible. Furthermore, in the case of time-course experiments, sampling at the same time of day can be important due to inherent effects associated with circadian rhythms (Gooley 2014; Li et al. 2015).

Selection of adequate control animals is crucial in all omics-based investigations. In most cases, controls and treatment groups should have the same genetic background and should be matched for gender, age, size-class and/or development stage. For example, male and female mussels from a homogenous population can easily be discriminated based on their metabolite profiles (Cubero-Leon et al. 2012), and have sex-specific physiological responses to environmental stressors, toxin exposures and pathogen infections (Ji et al. 2013; Liu et al. 2014a; Ellis et al. 2014). The metabolome is so sensitive that differences in the age of fish larvae can be detected within samples that are only a few hours apart in developmental stage (Huang et al. 2013), and marine invertebrate larvae of the same age but different size-class can be discriminated based on their metabolite profiles (see Chapter 4 [Young et al. 2015]. Thus, these features should be carefully managed to avoid potential experimental bias, unless they are the specific biological aspect under investigation.

Correct selection of sample material is also important. Different tissues (e.g., muscle, gills, liver, and pancreas) undergo specific metabolic processes by virtue of their distinct functional purpose. Recent studies of tissue-specific metabolism in aquatic organisms include digestive gland vs. gill response differences during pathogen infection in mussels, and differences measured under future climate change scenarios in oysters

(Liu et al. 2014b; Wei et al. 2015). In the case of biofluids, the serum and plasma components of blood contain significant chemical differences due to the way in which they are prepared (Yin et al. 2015). Thus, prior knowledge of the biological system is favourable in order to assess the suitability of particular tissues or biofluids for a given experiment.

Once the sample type has been decided, protocols for sampling should be developed. While there is limited information on how the speed of sampling affects the metabolite profile, we suggest that samples be taken rapidly and in a highly reproducible manner to minimise biological and technical variation. For example, if liver samples are to be taken from a number of fish, it would be prudent to make sure that the timing and procedures used to immobilise the organisms and to dissect the tissue be very similar between each animal. Application of anaesthetics during this process should be used with caution since they may disturb the metabolic baseline signature (Bando et al. 2010). The highly dynamic state of the metabolome continues in tissues and biological fluids even after they have been extracted from the organism. Therefore, in almost all metabolomics investigations it is vital that metabolic processes within samples be stopped, or quenched, as soon as possible during collection (reviewed by van Gulik et al. 2012). While other options exist, a typical method to quench metabolism in animal tissues involves snap-freezing samples in liquid nitrogen. Special considerations may need to be made for this, especially if sampling in the field. Furthermore, it is recommended that samples be stored at or below −80°C until metabolite extraction in order to maintain inactivation of enzymatic and chemical processes, which may influence the metabolite profile. Immediate access to appropriate facilities for sampling and storage is essential. The choice of containers in which the samples will be stored also requires attention due to potential introduction of contaminants, such as surfactants and plasticisers, which may cause severe interferences during analysis (Courant et al. 2014). See Álvarez-Sánchez et

al. (2010a) for additional information regarding appropriate selection of biological samples and a review of some practical aspects, which require consideration prior to sample preparation.

Techniques for preparing samples for analysis strongly depend on the type of biological material collected, and the analytical platform to be employed. Regardless of the approach, the metabolite extraction process should be rapid and robust, while minimising the potential for sample degradation and metabolite modification (Allwood et al. 2013). Special considerations may also be required for processing marine samples due to potential interferences from salts within the sample matrices (Keller et al. 2008), or presence of complex polysaccharides in the case of macroalgae (Goulitquer et al. 2012). Approaches are numerous and constant method development by chemists provide an array of options. These range from simple one-step solvent extraction processes to more complicated procedures involving multiple stages and/or organic synthesis reactions (derivatisation).

In general, the most commonly applied solvent extraction methods include: 1) extraction of polar and/or non-polar metabolites with a mixture of methanol, water and chloroform, 2) extraction of polar metabolites with methanol alone or in combination with water, and 3) extraction of polar metabolites with perchloric acid. There are many variations as to the solvent ratios which can be used, the temperature of extraction, the extraction duration, and the mechanical techniques used to disrupt tissue samples and lyse cells. Due to the diversity of possible techniques and wealth of excellent information already available in the literature, method particulars regarding sample preparation are outside the scope of this review. However, we have provided a sizeable table (Table 3.1) containing references to primary literature which have an aquatic metabolomics-based focus, and we highlight the various strategies employed by each study, including the extraction technique used. These studies may be useful to readers as guiding exemplars

for many of the strategies discussed in this article. For further details on the preparation of biological samples prior to metabolite detection, see Álvarez-Sánchez et al. (2010b). For comprehensive information on platform-specific sample preparation techniques for general biofluids and animal tissues, see Beckonert et al. (2007), Nováková & Vlčková (2009), Liebeke & Bundy (2012), Römisch-Margl et al. (2012), Vuckovi (2012) and Mushtaq et al. (2014b). For sample preparation techniques with a particular focus on fish and marine invertebrates, see Lin et al. (2007), Wu et al. (2008), del Carmen Alvarez et al. (2010), and Fernández-Varela et al. (2015).

3.2.2 Analytical platforms

A clear understanding of the analytical platform/s to be used is necessary before starting an experiment. Certain platforms have special requirements and may or may not be able to deliver the desired data/information. For example, to obtain broad metabolite coverage, including low abundance compounds, some procedures may require a tissue sample of only 2 mg wet weight, whereas others may require > 100 mg. Unfortunately, there is not yet a single platform which can analyse all metabolites within a sample, and some instruments are better-suited for the analysis of particular metabolite classes than others. Hence, multiple platforms may need to be used depending on the aims and scope of the investigation. The costs associated with employing different analytical platforms vary widely, and access to appropriate facilities for sample analysis may limit the decision making process. Therefore, selection of the most appropriate instrument for a given metabolomics-based study will depend largely on the type of sample material collected, the available sample mass, the accessibility of analytical platforms, the end-goals of the researchers, and the budget of the project.

The most commonly applied, high-throughput and high-resolution platforms to analyse samples in metabolomics studies are nuclear magnetic resonance (NMR) and

mass spectrometry (MS). In certain circumstances, lower resolution vibrational spectroscopy can also be used. See Figure 3.2 for usage trends of the various platforms employed over the past decade. The selection of which platform to apply for a particular metabolomics study is always a compromise between cost, sensitivity, speed, chemical selectivity, and metabolite coverage (Table 3.2). However, realistically, the choice of platform most-often comes down to the availability of analytical facilities and technical expertise through commercial or academic collaborations.

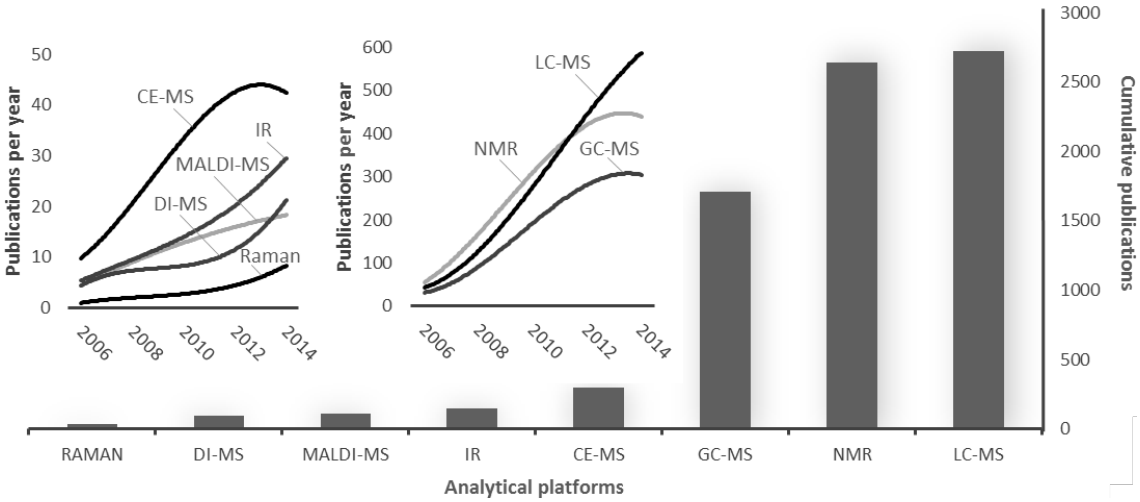

Figure 3.2. Bibliometric analysis in SciVerse Scopus abstract and citation database (March 24th, 2015). "Metabolom*" was used as a primary keyword in all searches and was combined with keywords for each analytical platform. Searches were limited to terms found within 'abstracts, titles and keywords', between the years 2006–2014, and to research articles only. The bar graph shows the cumulative number of peer-reviewed articles which performed metabolomics-based analyses using various analytical platforms. The inset line graphs show the general usage trends for each platform. These trends also reveal how quickly metabolomics has evolved into such a well-established field of biological research over this short period of time.

3.2.2.1 Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) detects the characteristic spin properties of atomic nuclei. When nuclei with particular magnetic attributes are immersed in an external magnetic field, they align themselves with (low energy state) or against (high energy state) that field. Application of very specific radio frequency pulses to the nuclei induces a change in the energy state called a 'spin flip' (Savorani et al. 2013). The presence of

other nuclei and chemical bonds in the immediate vicinity of a nucleus changes the intensity of the applied magnetic field by a small amount called nuclear shielding. As a result of this shielding, nuclei within a metabolite will absorb energy at slightly different frequencies, known as a chemical shift. The combination of all of these different frequencies produces a characteristic spectrum, or 'fingerprint' of the sample (Figure 3.3A–D). In addition, more complex interactions of the spins under various pulse conditions can provide rich sets of information about the chemical bonding and composition of a molecule or mixture.

All isotopes that contain an odd number of protons and/or neutrons can theoretically be assessed by NMR approaches. However, if they are not found in biological molecules, or have low NMR sensitivities or low natural abundances, they are not often used for metabolomic studies. ^{1}H NMR is frequently applied in metabolomics investigations to probe the molecular arrangements of hydrogen atoms. The ^{1}H isotope is highly abundant in nature (> 99.98%) and has a very high NMR sensitivity. See Schock et al. (2012) for an applied example of how ^{1}H NMR was employed to monitor the health of cobia in response to reduced fishmeal-based protein diets, and to identify differential regulation of metabolism indicative of thyroid disruption and variations in the composition of gut microflora. ^{13}C NMR can also be used, but is much less abundant (1.1%) and less sensitive. However, ^{13}C NMR has special applications in tracer studies to investigate metabolite transformations and metabolic flux (Tikunov et al. 2014). For example, molecules can be chemically labelled, or enriched, with the ^{13}C isotope and traced through metabolic processes, such as protein catabolism and lipid synthesis, to investigate the uptake and conversion of nutrients in fish (Conceição et al. 2007; Eckman et al. 2013).

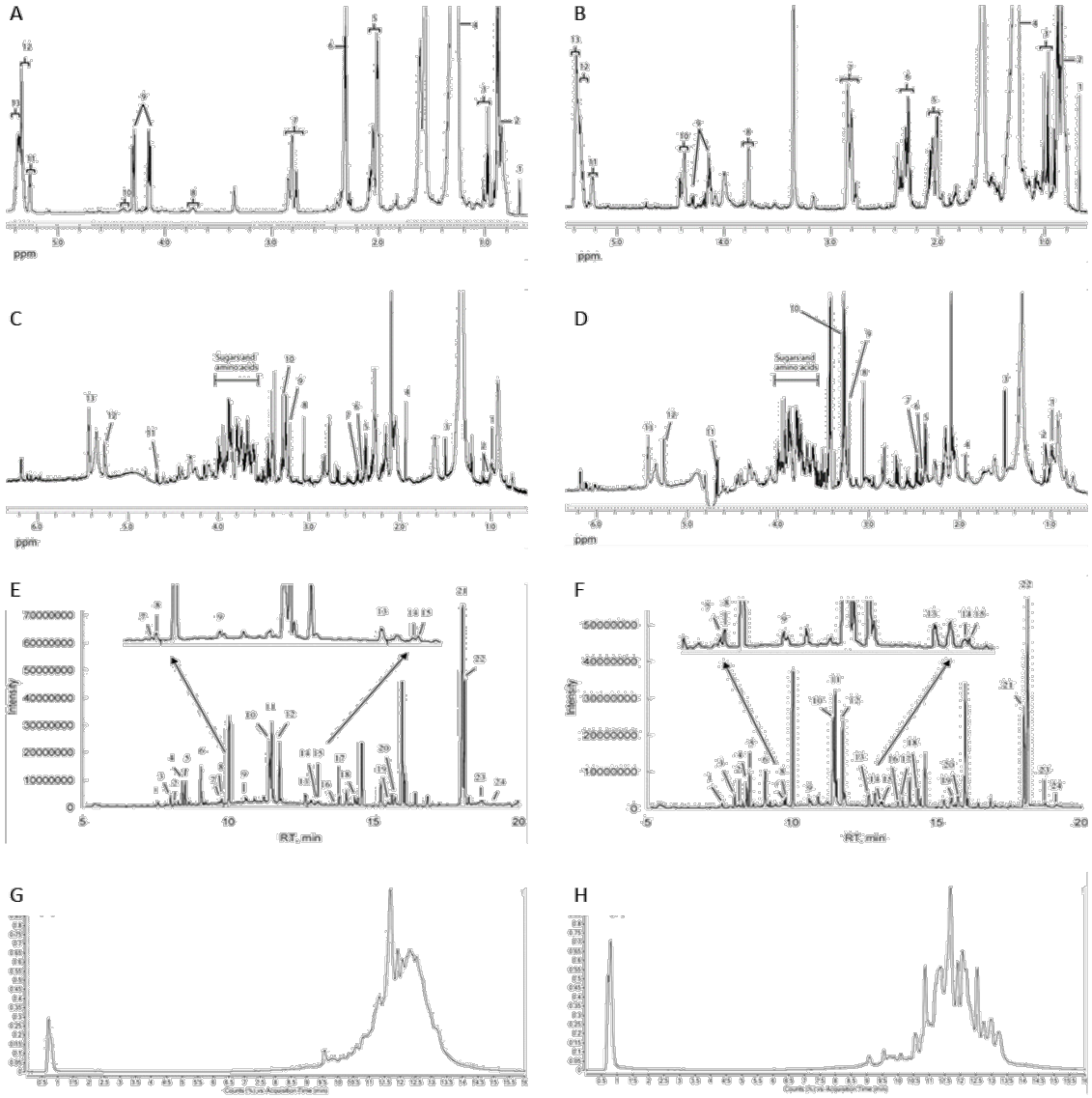

Figure 3.3. Multi-platform metabolomics-based analysis of fish (*Danio rario*) liver samples showing sex-specific differences in spectral fingerprints obtained from three platforms (NMR, CG-MS and LC-MS). ^{1}H NMR spectra of non-polar extracts from male **(A)** and female **(B)** fish. ^{1}H NMR spectra of polar extracts from male **(C)** and female **(D)** fish. GC-MS total ion chromatograms of non-polar extracts from male **(E)** and female **(F)** fish. LC-MS spectra of non-polar extracts from male **(G)** and female **(H)** fish. Numbered peaks represent reliably assigned metabolites after bioinformatics processing. Reprinted with permission from Ong et al. (2009).

Table 3.1. A selection of studies using metabolomics-based approaches with relevance to aquaculture. Although not a complete list of all the available literature, a broad range of references are provided which we think may be of interest to aquaculture researchers, and may be useful resources as guiding exemplars for the various strategies which can be employed, including: 1) methods for metabolite extraction in diverse organisms, tissues and biofluids; 2) use of different analytical platforms; 3) a range of primary bioinformatics software to process raw spectral data and assign metabolite identities; 4) a variety of databases for matching spectral signatures; 5) various pre-treatment techniques to prepare data for statistical analysis; 6) an array of univariate and multivariate statistical methods to identify sample group differences; and 7) some secondary bioinformatics software to aid interpretation of metabolite profiles within biologically meaningful contexts through use of global a priori knowledge stored in biochemical information databases.

Organism	Sample type	Experimental theme	Extraction method†	Metabolite component‡	Derivatisation method§	Analytical Platform/s¶	General approach	Metabolites Detected	Data pre-treatment methods applied\\	Bioinformatics & statistical software used††	Statistical analyses & data visualisation‡‡	Databases used§§	Reference
Fish	**Embryos**	Baseline developmental metabolism: Multiplatform metabolomics	MeOH	P	MSTFA	GC-MS, LC-MS	Fingerprinting & profiling	55+	Autoscaled	MZmine, SIMCA-P, MEV	PCA, OPLS-DA, HCA, Kruskal-Wallis test heatmap	NIST library, HMDB	Huang et al. 2013
	Larvae	Nutritional & thermal influence on larval physiology & growth	MeOH/H_2O	P	-	^{1}H NMR	Fingerprinting & profiling	28	Spectral area normalisation, mean centered	TopSpin, Chenomx NMR Suite, MATLAB, PLS Toolbox	PCA	CRL	Chauton et al. 2015
	Flesh/muscle	Nutrition & alternative feed development	MeOH/$CHCl_3$	P, NP	-	^{1}H NMR	Profiling	45	Spectral intensity normalisation, Pareto scaled	TopSpin, AMIX, SAS, SIMCA-P	PCA, OPLS-DA, ANOVA	HMBD, literature search	Cheng K. et al. 2015
		Culture conditions & post-harvest storage	$HClO_4$	P	-	^{1}H NMR	Fingerprinting & profiling	11+	Spectral intensity normalisation, mean centered	MestReC, R	PCA, *t*-test	Undefined	Picone et al. 2011
		Optimisation of sample preparation techniques	$HClO_4$, MeCN/H_2O, MeOH/H_2O/$CHCl_3$, MeOH/H_2O	P, NP	-	^{1}H NMR	Fingerprinting & profiling	26	Bin area normalisation, glog transformation, mean centered	XWINNMR, Chenomx NMR Suite, MATLAB, PLS Toolbox	PCA	Chemical shift data from the literature, CRL	Lin et al. 2007
		Effects of salmon farming on wild fish populations	$HClO_4$	P	-	^{1}H NMR	Fingerprinting & profiling	23	Undefined	MATLAB	RPCA, PLS-LDA	Chemical shift data from the literature	Marhuenda-Egea et al. 2015
		Nutritional history prediction & alternative feeds	C_6H_{12}/H_2O	P, NP		DART-MS	Fingerprinting & profiling	59+	Spectral area normalisation, log transformation, Pareto scaling	MassCenter, SIMCA, Excel	PCA, OPLS-DA	Undefined	Cajka et al. 2013
		Monitoring compositional changes in fillets during post-harvest cold-storage	TCA	P	-	^{1}H-^{13}C NMR	Profiling	51	Untreated	TopSpin, MestReC	N/A	Chemical shift data from the literature, HMDB, BMRB, YMDB, ECMDB	Shumilina et al. 2015
		Food authentication, forensics, providence	MeOH/H_2O/$CHCl_3$	NP	-	^{13}C NMR	Fingerprinting	N/A	Peak maximum normalisation, vast stability scaling	AI Trilogy, Tiberius	PNN, SVM	N/A	Aursand et al. 2009
	Liver	Nutrition & alternative feed development	MeOH/$CHCl_3$	P, NP	-	^{1}H NMR	Profiling	49	Spectral intensity normalisation, Pareto scaled	TopSpin, AMIX, SAS, SIMCA-P	PCA, OPLS-DA, ANOVA	HMBD, literature search	Cheng K. et al. 2015
		Nutrition & alternative feed development	MeOH/H_2O/$CHCl_3$	P, NP	-	^{1}H NMR	Profiling	23+	Spectral area normalisation, Pareto scaled	TopSpin, Chenomx NMR Suite, AMIX, SIMCA-P	PCA, OPLS-DA, ANOVA	CRL, HMBD, literature search	Wagner et al. 2014
		Enhancing disease resistance via simple metabolic modulation	MeOH	P	MSTFA	GC-MS	Profiling	60	Peak height normalisation, median centered, quartile range scaled, log transformed	AMDIS, R, SIMCA-P, SPSS, Prism, MetaboAnalyst (MetPA)	HCA, heatmap, PCA, ICA, MPEA	KEGG, NIST library	Peng et al. 2015
		Enhancing disease resistance via simple metabolic modulation	MeOH	P	MSTFA	GC-MS	Profiling	60	Peak height normalisation, median centered, quartile range scaled, log transformed	AMDIS, R, SIMCA-P, SPSS, Prism	PCA, OPLS-DA, heatmap	KEGG, NIST library	Cheng Z. et al. 2015
		Enhancing disease resistance via simple metabolic modulation	MeOH	P	MSTFA	GC-MS	Profiling	58	Peak height normalisation, median centered, quartile range scaled, log transformed	AMDIS, R, SIMCA-P, SPSS, Prism, MetaboAnalyst (MetPA)	PCA, OPLS-DA, MPEA, HCA, heatmap	KEGG, NIST library	Ma et al. 2015
		Nutrition & alternative feed development	MeOH/H_2O/$CHCl_3$	P	-	^{1}H NMR	Fingerprinting & profiling	12+	Centered (undefined), Pareto scaled	TopSpin, AMIX, SIMCA-P	PCA, OPLS-DA, ANOVA	CRL, BMRB, HMDB	Abro et al. 2014
		Enhancing disease resistance via simple metabolic modulation	MeOH, MeCN/H_2O	P	MSTFA	GC-MS, LC-MS	Profiling	64+	Peak height normalisation, median centered, quartile range scaled, log transformed	AMDIS, R, MarkerLynx, SIMCA-P, SPSS, Prism, MetaboAnalyst (MetPA)	PCA, ICA, MPEA, HCA, heatmap,	KEGG, NIST library	Zhao et al. 2015
		Optimisation of extraction methods	MeOH/H_2O/$CHCl_3$ (various protocols)	P, NP	-	^{1}H NMR, FT-ICRMS	Fingerprinting & profiling	8+	Bin or spectral area normalisation, glog transformation, mean centered	TopSpin. Chenomx NMR Suite, PLS Toolbox, MATLAB	PCA, ANOVA	Chemical shift data from the literature, CRL	Wu et al. 2008
		Utilisation of dietary protein: Growth-metabolic interactions	$HClO_4$	P	ECF	GC-MS	Profiling	12+	Peak height normalisation	SPSS, SIMCA-P	PLS-DA, *t*-test	NIST library, in-house library	Jin et al. 2005
		Health biomarkers & stress evaluation: Multi-platform, large *n* features	MeOH	P		LC-MS, LC-MS/MS, FI-MS/MS	Profiling	95+	Mean centered, Pareto scaled	MetaboAnalyst	PCA, PLS-DA, Mann-Whitney *U* test	N/A	Benskin et al. 2014
		Health biomarkers: Tumor diagnostics	MeCN/H_2O, MeOH/H_2O/$CHCl_3$	P	-	FT-ICRMS	Fingerprinting	4+	Bin area normalisation, glog transformation, mean centered	MIDAS, MSCalc, MATLAB, PLS Toolbox	PCA, PLS-R, *t*-test	N/A	Stentiford et al. 2005
	Kidney	Symptoms of anaemia & health biomarker identification	MeOH/H_2O/$CHCl_3$	P	-	^{1}H NMR	Fingerprinting & profiling	37+	Spectral area normalisation, mean centered, Pareto scaled	TopSpin, MestReNova, Chenomx NMR Suite, AMIX, SPSS	PCA, *t*-test, Mann-Whitney *U* test, Wilcoxon test	HMDB, BML	Allen et al. 2015
	Intestine	Feed additives to enhance growth & metabolism	MeCN/$CHCl_3$	P	-	LC-MS	Profiling	78+	Biomass normalised, autoscaled	Chemstation, EasyLCMS, SPSS, Metaboanalyst	PCA, HCA, heatmap, ANOVA, *t*-test,	Undefined	Robles et al. 2013

Table 3.1. Continued.

Organism	Sample type	Experimental theme	Extraction method†	Metabolite component‡	Derivatisation method§	Analytical Platform/s¶	General approach	Metabolites Detected	Data pre-treatment methods applied\\	Bioinformatics & statistical software used††	Statistical analyses & data visualisation‡‡	Databases used§§	Reference
Fish (cont.)	**Gut contents & faeces**	Effects of diet on microbial symbiosis & co-metabolism + non-invasive sampling	MeOH	P	-	^{1}H-^{13}C NMR	Fingerprinting & profiling	25+	Spectral area normalisation	TopSpin, SpinAssign, R, Gephi	PCA, PLS-DA	PRIMe DB, BMRB, in-house library	Asakura et al. 2014
	Humoral fluid	Mechanism of vaccine action against disease	H_2O	P	MSTFA	GC-MS	Profiling	65	Median centered, interquartile range scaled, Pereto scaled or autoscaled	XCalibur, NIST MS search, SPSS, SIMCA, MetaboAnalyst	OPLS-DA, Heatmap, Mann-Whitney *U* test, MPEA	NIST library, KEGG	Guo et al. 2015
	Haemolymph: Plasma (p) or serum (s)	Toxicological biomarkers & environmental monitoring	None required (p), MeOH/$CHCl_3$ for lipid fraction (p)	P, NP	-	^{1}H NMR	Fingerprinting (some limited profiling)	7+	Bin integral normalisation, mean centered, pareto scaled	NMR Processor, VNMR, SIMCA-P	PCA, PLS-DA	Chemical shift data from the literature	Sammuelsson et al. 2006
		Metabolic effects of food deprivation	None required (p)	P, NP	-	^{1}H-^{13}C NMR	Fingerprinting (some limited profiling)	4+	Bin integral normalisation, Pareto scaled	NMR Processor, SIMCA-P	PCA, OPLS-DA	Chemical shift data from the literature, HMDB	Kullgren et al. 2010
		Utilisation of dietary protein: Growth-metabolic interactions	None required (p)	P	BSTFA	GC-MS	Profiling	16+	Peak height normalisation	SPSS, SIMCA-P	PLS-DA, *t*-test	NIST library, in-house library	Jin et al. 2005
		Spawning-induced inappetence & stress: High resolution platform, large *n* features	MeCN (s)	P	MSTFA	2D GCxGC-MS	Profiling	137	Log transformed, autoscaled, quartile range filtering, KNN (missing variables)	ChromaTOF, MetPP, MetaboAnalyst	PCA, PLS-DA, *t*-test, MPEA	In-house MS library, KEGG	Cipriano et al. 2015
		Health & immunology: Predicting survival	None required (s)	P, NP	MSTFA	GC-MS	Profiling	67	Peal area normalisation, log transformation, autoscaled	XCalibur, SPSS, SIMCA, MetaboAnalyst	Kruskal-Wallis test, Mann-Whitney *U* test, OPLS-DA, MPEA,	NIST library, KEGG	Guo et al. 2014
		Health, nutrition & alternative feed development	None required (s)	P, NP	-	^{1}H-^{13}C NMR	Fingerprinting & profiling	34	Spectral area normalisation, mean centered, Pareto scaled	Chenomx NMR Suite, AMIX, Excel	PCA, Kruskal-Wallis test, ANOVA	CRL, BMRB, in-house library	Schock et al. 2012
	Primary cell culture: Cells (c), media (m)	Effects of plant-derived contaminants in fish feeds: A multiplatform study (c)	MeOH/H_2O/$CHCl_3$	P, NP	-	FT-ICR-MS, ^{1}H NMR	Fingerprinting & lipid profiling	40+	Probabilistic quotient normalisation, glog transformation, mean centered	ProMetab, MATLAB, PLS Toolbox, MI-Pack	PCA, PLS-DA, ANOVA	KEGG	Søfteland et al. 2014
		Nutritional supplementation & diet optimisation (m)	None required	P, NP	-	^{1}H NMR	Fingerprinting & profiling	17	Pareto scaled	Chenomx NMR Suite, SIMCA-P, Statistica	PCA, OPLS-DA, ANOVA	Undefined	Andersen et al. 2014
	Fin tissue	Identifying animal providence	None required	P, NP	-	FT-IR	Fingerprinting	N/A	Undefined	Undefined	PCA	N/A	Nurdalila et al. 2015
	Skin mucus	Minimally-invasive sampling & ecotox: Large *n* features	MeOH/H_2O	P	-	LC-MS/MS	Profiling	204	Peak height normalisation, autoscaled	SIEVE, SIMCA-P, Excel, Systat	PCA, PLS-DA, ANOVA, *t*-test	HMBD, Metlin, LipidMaps	Ekman et al. 2015
	Fish oil capsules	Food authentication, forensics & quality control	None required	NP	-	^{13}C NMR	Fingerprinting	N/A	Peak maximum normalisation	Undefined	PCA, KNNA, GTM, PNN, GRNN	Chemical shift data from the literature	Aursand et al. 2007
	Canned fish packing oil	Food authentication, forensics & quality control	None required	P, NP	-	FT-IR	Fingerprinting	N/A	Undefined	MATLAB, PLS Toolbox	PCA, PLS-DA	N/A	Dominguez-Vidal et al. 2016
Molluscs	**Larvae**	Identification of larval quality biomarkers during hatchery culture	MeOH/H_2O	P	MCF	GC-MS	Profiling	29	Peak height normalisation, metabolite ratios, log transformation, autoscaled	R, AMDIS, MetaboAnalyst	PLS-DA, HCA, heatmap, volcano plot, EBAM, SAM	In-house MS library	Young et al. 2015
		Handling stress & culture conditions	MeOH/H_2O	P	MCF	GC-MS	Profiling	27	Peak height normalisation, autoscaled	R, AMDIS, MetaboAnalyst, SPSS	PCA, PLS-DA, HCA, heatmap, *t*-test,	In-house MS library	Young et al. 2016
	Adductor muscle	Organ function & physiology: A multi organ study	$HClO_4$	P	-	^{1}H NMR	Profiling	37+	Peak area normalisation	NMR Processor, SpinWorks	N/A	HMBD	Tikunov et al. 2010
		Optimisation of extraction methods & animal providence: A multi organ study	$HClO_4$, MeCN, Ringer's solution	P	-	^{1}H NMR	Fingerprinting & profiling	32	Bin integral normalisation, Pareto scaled	TopSpin, NMR Processor, Chenomx NMR Suite, JMP, Excel	PCA, *t*-test	HMDB, BMRB	Hurley-Sanders et al. 2015a,b
	Mantle	Sex discrimination	MeOH/H_2O/$CHCl_3$	P	-	^{1}H NMR	Fingerprinting & profiling	16+	Spectral area normalisation, biomass normalisation, glog transformation, mean centered	TopSpin, MATLAB, Chenomx NMR Suite, Excel	PCA, LDA, *t*-test	CRL	Hines et al. 2007
		Ocean acidification, disease, thermal stress & sex differences	MeOH/H_2O/$CHCl_3$	P	-	^{1}H NMR	Fingerprinting & profiling	25	Probabilistic quotient normalisation, glog transformation	MATLAB, PRIMER	PERMANOVA, MDS, SIMPER	Undefined	Ellis et al. 2014
	Hepatopancreas	Health & immunology: Host responses to bacterial pathogens	MeOH/H_2O/$CHCl_3$	P	-	^{1}H NMR	Fingerprinting & profiling	27	Spectral area normailisation, biomass normalisation, glog transformation, mean centered	TopSpin, Chenomx NMR Suite, MATLAB, PLS Toolbox, Minitab	PCA, PLS-DA, OPLS-DA, ANOVA	Chemical shift data from the literature	Wu et al. 2013
		Ocean acidification: Integrated metabolomics & proteomics	MeOH/H_2O/$CHCl_3$	P	-	^{1}H NMR	Fingerprinting & profiling	32	Spectral area normalisation, glog transformation	TopSpin, Chenomx NMR Suite, MATLAB, SIMCA-P	PLS-DA, OPLS-DA	CRL	Wei et al. 2015
	Foot	Health: Biomarkers for toxicology, hypoxia & food limitation	MeCN/H_2O	P	-	^{1}H NMR	Fingerprinting & profiling	20	Box-Cox transformation	SpecManager, Excel, Minitab, GenStat	PCA, LDA, ANOVA	HMBD	Tuffnail et al. 2009
	Gonad	Sex discrimination & reproductive physiology	SPE & fractionation	P, NP	-	LC-MS	Fingerprinting & profiling	21+	Spectral area normalisation, mean centered	MarkerLynx, MassLynx, SIMCA-P	PCA, PLS-DA, OPLS-DA, Mann-Whitney *U* test	HMBD, KEGG LDB, BiGG DB, PubChem, NLDB, MBDB	Cubero-Leon et al. 2012

Table 3.1. Continued.

Organism	Sample type	Experimental theme	Extraction method†	Metabolite component‡	Derivatisation method§	Analytical Platform/s¶	General approach	Metabolites Detected	Data pre-treatment methods applied\\	Bioinformatics & statistical software used††	Statistical analyses & data visualisation‡‡	Databases used§§	Reference
Molluscs (cont.)	**Gills**	Identification of thermal stress biomarkers	MeOH/H_2O	P	Undefined	GC-MS	Profiling	52	Biomass normalisation	JMP	DFA	Undefined	Dunphy et al. 2015
		Toxicology: Integrated metabolomics & proteomics	MeOH/H_2O/$CHCl_3$	P	-	^{1}H NMR	Fingerprinting & profiling	28	Spectral area normalisation, glog transformation, mean centered	TopSpin, Chenomx NMR Suite, MATLAB, SIMCA-P	PLS-DA, OPLS-DA	Chemical shift data from the literature	Ji et al. 2013
		Toxicology: Integrated metabolomics & proteomics	MeOH/H_2O/$CHCl_3$	P	-	^{1}H NMR	Fingerprinting & profiling	25+	glog transformation	TopSpin, Chenomx NMR Suite, SIMCA-P, MATLAB	PCA, PLS-DA, OPLS-DA	CRL, KEGG	Song et al. 2016
		High resolution NMR: Coastal marine pollution & toxicology	MeOH/H_2O/$CHCl_3$	P	-	HR-MAS ^{1}H NMR	Fingerprinting & profiling	27+	Spectral area normalisation, glog transformation, mean centered	XWIN-NMR, Chenomx NMR Suite, MATLAB, Unscrambler X, Excel	PCA, *t*-test	HMBD, CRL	Cappello et al. 2013
		Toxicology: Integrated metabolomics & proteomics	MeOH/H_2O/$CHCl_3$	P	-	^{1}H NMR	Fingerprinting & profiling	9+	Spectral area normalisation, glog transformation, mean centered	TopSpin, Chenomx NMR Suite, MATLAB, SIMCA-P	PLS-DA, OPLS-DA	Chemical shift data from the literature	Ji et al. 2016
	Central nervous system & glands	Baseline molecular phenotyping: Multiplatform metabolomics	MeOH/H_2O/$CHCl_3$	P, NP	BF_3MeOH (lipids)	GC-MS, LC-MS (RPLC & HILIC)	Fingerprinting & profiling	73+	Selected peak normalisation, total protein content normalised	DataAnalysis, ProfileAnalysis, SPSS, Excel, SIMCA-P	PCA, PLS-DA, OPLS-DA, HCA, ANOVA	Undefined	Tufi et al. 2015a
		Neurotoxicity of pesticides in aquatic environments: Multiplatform metabolomics	MeOH/H_2O/$CHCl_3$	P, NP	BF_3MeOH (lipids)	GC-MS, LC-MS	Profiling	73	Total protein content normalised	Compass DataAnalysis, DataAnalysis, PathwayScreener, ProfileAnalysis, IMPaLA, SPSS, Metamapp, Cytoscape	ORA, BNM, *t*-test	Reactome, EHMN, KEGG, Wikipathways, SMPDB, HumananCyc	Tufi et al. 2015b
	Gastrointestinal tract and/or digestive gland	Bioindicator species for pollution monitoring	MeOH/H_2O/$CHCl_3$	P	-	^{1}H NMR	Fingerprinting & profiling	19+	Spectral area normalisation, glog transformation, mean centered	MATLAB, PLS Toolbox	ANOVA, PCA, PLS-DA	Chemical shift data from the literature	Liu et al. 2011
		Metabolic effects of food deprivation & extraction method optimisation	MeOH/H_2O/$CHCl_3$	P	-	^{1}H NMR	Profiling	28	Log transformation, median centered	Chenomx NMR Suite, TopSpin, R, Unscrambler	PLS-DA, Mann-Whitney *U* test	CRL	Sheedy et al. 2016
	Whole soft tissue	Dual platform metabolomics: Toxicological mechanisms	MeOH/H_2O/$CHCl_3$	P	MSTFA	GC-MS, ^{1}H NMR	Fingerprinting & profiling	NMR: 17+ GC-MS: 24+	Spectral area normailsation, autoscaled (GC-MS), mean centered & Pareto scaled (NMR)	SpecManager, SIMCA-P	PCA, PLS-DA	Chemical shift data from the literature, NIST library	Spann et al. 2011
		Toxicological mechanisms	MeOH/H_2O/$CHCl_3$	P	-	^{1}H NMR	Fingerprinting & profiling	25+	Bin integral normalisaiton, glog transformation, mean centered	TopSpin, MATLAB, PLS Toolbox	PCA, PLS-DA, ANOVA	Undefined	Wu & Wang 2010
		Unique extraction & platform: Method assessment	SPME	P, NP	-	2D GCxGC-MS	Fingerprinting & profiling	63+	Fourth root transformation	ChromaTOF, PRIMER	PERMANOVA, PCoA, HCA	WMSL, NIST library	Rocha et al. 20013
		Coastal marine pollution & toxicology	MeOH/H_2O/$CHCl_3$	P	-	^{1}H NMR	Fingerprinting & profiling	24	Pareto scaled	Chenomx NMR Suite, MATLAB, SIMCA-P, R	PCA, OPLS-DA, PLS-DA	CRL	Kwon et al. 2012
	Haemolymph: whole blood (wb) or plasma (p)	Mechanical shaking & salinity stress	None required (wb)	P, NP	-	FT-IR	Fingerprinting	N/A	Undefined	Undefined	MANOVA, PCA, CVA	N/A	Bussell et al. 2008
		Developing strategies for identifying stress	None required (wb)	P, NP	-	FT-IR	Fingerprinting	N/A	Untreated	Undefined	MANOVA, PCA, CVA	N.A	Gidman et al. 2007
		Health/stress biomarkers: Toxicology	None required (p)	P, NP	-	^{1}H NMR	Fingerprinting & profiling	18	Spectral area normalisation, glog transformation, mean centered	MATLAB, PLS Toolbox, Chenomx NMR Suite	ANOVA, PCA	CRL	Zhou et al. 2015
Crustaceans	**Claw muscle**	Nutritional composition & quality assessment, 3 spp.	MeOH/H_2O/$CHCl_3$	P, NP	-	^{1}H NMR	Fingerprinting & profiling	24+	Spectral area normalisation, mean centered, areto scaled	TopSpin, AMIX, SIMCA-P	PCA, PLS-DA	Chemical shift data from the literature	Zotti et al. 2016
	Tail muscle & other	Identification of health & stress biomarkers in shrimp during intensive culture	MeOH/H_2O/$CHCl_3$	P	-	^{1}H-^{13}C NMR	Fingerprinting & profiling	50+	Spectral area normalisation, mean centered, Pareto scaled	AMIX, Chenomx NMR Suite, Excel	PCA, *t*-test	HMDB, BMRB, CRL, in-house library	Schock et al. 2013
	Hepatopancreas	Mechanisms of white spot virus syndrome in shrimp	Undefined	Undefined	-	^{1}H NMR	Fingerprinting & profiling	27+	Undefined	TopSpin, SIMCA-P	PCA, OPLS-DA	Chemical shift data from the literature	Liu et al. 2015
	Haemolymph	Pathogen-induced oxidative stress responses in crabs: New biochemical insights	None required	P, NP	-	^{1}H-^{13}C NMR	Fingerprinting & profiling	20	Spectral area normalisation, mean centered	AMIX, SIMCA-P	PCA, sPCA, PLS-DA, ANOVA	Chemical shift data from the literature, in-house library	Schock et al. 2010
Echinoderms	**Muscle**	Thermal stress responses	MeOH/H_2O/$CHCl_3$	P	-	^{1}H NMR	Fingerprinting & profiling	31	Spectral area normalisation, glog transformation, mean centered	TopSpin, Chenomx NMR Suite, MATLAB, SIMCA-P	PCA, OPLS-DA	Chemical shift data from the literature	Shao et al. 2015
Macroalgae	**Thallus**	Effects of food processing on nutrient composition	MeCN/H_2O	P	-	^{1}H NMR	Fingerprinting & profiling	32+	Bin integral normalisation	AMIX, SIMCA-P, MATLAB, SPSS	PCA, OPLS-DA, heatmap, *t*-test	Chemical shift data from the literature	Ye et al. 2014
		New insights into metabolism	MeOH, H_2O	P	-	^{1}H NMR	Profiling	27	N/A	Chenomx NMR Suite	N/A	PRIMe DB, HMDB, BMRB	Gupta et al. 2013
	Stipe & blades	Seasonal variations in metabolism: Multiplatform metabolomics	None required (FT-IR), MeOH, H_2O (NMR)	P, NP	-	FT-IR, ^{1}H-^{13}C NMR	Fingerprinting & profiling	51	Specific peak intensity normalisation (FT-IR), spectral area normalisation (NMR)	Excel, TopSpin, SpinAssign, OMNIC, R, Amos, Gephi, Fityk	PCA, ICA, SOMs, CNA, SEM, MCR-ALS	PRIMEe DB	Ito et al. 2014

Table 3.1. Continued.

Organism	Sample type	Experimental theme	Extraction method†	Metabolite component‡	Derivatisation method§	Analytical Platform/s¶	General approach	Metabolites Detected	Data pre-treatment methods applied\\	Bioinformatics & statistical software used††	Statistical analyses & data visualisation‡‡	Databases used§§	Reference
Microalgae	**Cells or extracts**	Screening microalgae to identify commercially useful mutants	None required (FT-IR), MeOH/H_2O/$CHCl_3$ (LC-MS)	P, NP	-	FT-IR, LC-MS	Fingerprinting & lipid profiling	11+ lipid classes	Biomass normalised	MATLAB, Xcalibur, Unscrambler	PCA, PC-DFA, PLS-DA, PLS-R	MMD, HMDB, KEGG, BioCyc, LIPIDMAPS, DrugBank	Bajhaiya et al. 2016
		Profiling diatoms for bioenergy & feedstock	MTBE/MeOH/H_2O	P, NP	MSTFA	GC-MS, LC-MS	Profiling	96+	Cell density normalised, median scaled, log transformed	Expressionist Refiner MS, ChromaTOF, R	PCA, HCA, heatmap, *t*-test	In-house MS library	Bromke et al. 2015
		Dual platform metabolomics: Natural products research	MeOH/H_2O/$CHCl_3$ (GC-MS), MeOH (LC-MS)	P	MSTFA	GC-MS, LC-MS	Profiling	128	Peak area & cell density normalisation	AMDIS, SIMCA-P, MeV	PLS-DA, HCA, heatmap, MPEA, WGCNA	KEGG	Yu et al. 2015
		Photobioreactor culture conditions & bioresource development	None required (FT-IR), MeOH & EtOH/H_2O (LC-MS)	P, NP	-	FT-IR, LC-MS	Fingerprinting & profiling	13+	Second derivative calculation (FR-IR), log transformed, Pareto scaled	OpusLab, XCMS, Xcalibur, R, Spectrum Database, Statistica Data Miner, MetaboAnalyst, SIMCA-P	Kruskal-Wallis test, HCA, Mann-Whitney *U* test, heatmap, PLS-DA	In-house MS library	Courant et al. 2013
		Chemical interactions between bacteria & diatoms	MeOH/EtOH,$CHCl_3$ (GC-MS)	P	MSTFA	GC-MS	Fingerprinting & profiling	19+	Cell density normalised	MassLynx, AMDIS, MET-IDEA, SigmaPlot, Excel	RM-ANOVA, PCoA, CAP, heatmap	NIST library, GMDB	Paul et al. 2013
Bacteria	**Cell extract**	Mechanisms of white spot syndrome virus in shrimp: Nutritional treatment	MeOH, H_2O	P	MSTFA	GC-MS	Fingerprinting & profiling	3+	Untreated	Xcalibur, NIST MS search	Wilcoxon rank-sum test,	NIST library	Zhu & Jin 2015
		Restoring pathogen susceptibility to antibiotics: Simple metabolic modulation	MeOH	P	MSTFA	GC-MS	Profiling		Undefined	Xcalibur, NIST MS Search, MetaGeneAlyse	ICA	NIST library	Su et al. 2015

† **Extraction solvents:** MeCN (acetonitrile), MeOH (methanol), EtOH (ethanol), MTBE (methyl-tert-butyl ether), H_2O (water), $CHCl_3$ (chloroform), $HClO_4$ (perchloric acid), C_6H_{12} (cyclohexane), TCA (trichloroacetic acid), SPME (solid phase microextraction), SPE (solid phase extraction)

‡ **Metabolite components:** P = polar component, NP = non-polar component

§ **Derivatisation:** MSTFA = silylation with *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide, BSTFA = silylation with *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide, MCF = alkylation with methyl chloroformate, ECF = alkylation with ethyl chloroformate

¶ **Analytical platforms:** FI-MS/MS (flow injection tandem mass spectrometry), FT-IR (Fourier transform infrared spectroscopy), ^{1}H NMR (proton nuclear magnetic resonance), ^{1}H-^{13}C NMR (two dimensional proton and carbon NMR for assisting metabolite identifications), HR-MAS NMR (high resolution magic angle spinning NMR), FT-ICRMS (Fourier transform ion cyclotron resonance mass spectrometry), DART-MS (direct analysis in real time mass spectrometry), GC-MS (gas chromatography mass spectrometry), LC-MS (liquid chromatography mass spectrometry), RPLC (reverse phase liquid chromatography), HILIC (hydrophilic interaction liquid chromatography)

\\ **Note:** Whilst not explicitly stated within the table, most metabolomics-based investigations will also include normalisation of data to an internal standard as a data pre-treatment method to compensate for potential technical variations (e.g., variable metabolite recoveries during sample preparation and processing).

†† **Software:** AI Trilogy (Ward Systems Group Inc., US), AMDIS (Automated Mass Deconvolution and Identification System [The National Institute of Standards and Technology, US]), Amos (IBM Corp., US), AMIX (Bruker Corp., Germany), Chemstation (Agilent, US), Chenomx NMR Suite (Chenomx Inc., Canada), ChromaTOF (LECO Corp., US), Compass DataAnalysis (Bruker Corp., Germany), Cytoscape (Shannon et al. 2003), DataAnalysis (Bruker Corp., Germany), EasyLCMS (Fructuoso et al. 2012), Excel (Microsoft, US), Expressionist Refiner MS (Genedata, Switzerland), FityK (Wojdyr 2010), Gephi (Bastian et al. 2009), GenStat (VSN Internaitonal, UK), IMPaLA (Integrated Molecular Pathway Level Analysis [Kamburov et al. 2011]), JMP (SAS Institute Inc., US), MarkerLynx (Waters Corp., US), MassCenter (JEOL, Japan), MassLynx (Waters Corp, US), MATLAB (Mathworks, US), MestReC (Mestrelab Research, Spain), MestReNova (Mestrelab Research, Spain), Metaboanalyst (Xia et al. 2015), MetaGeneAlyse (Daub et al. 2003), MetaMapp (Barupal et al. 2012), MET-IDEA (Lei et al. 2012), MetPA (Xia & Wishart 2010 [now a component of MetaboAnalyst]), MetPP (Wei et al. 2013), MEV (Multi Experiment Viewer [Howe et al. 2010]), MIDAS (Wang et al. 2014), Minitab (Minitab Inc., US), MSCalc (SoftShell International Ltd., US), MZmine (Katajamaa et al. 2006), NMR Processor (ACD/Labs, Canada), OMNIC (Thermo Fisher Scientific, US), PathwayScreener (Bruker Corp., Germany), PLS Toolbox (Eigenvector Research Inc., US), PRIMe DB (Platform for RIKEN Metabolomics Database, RIKEN Yokohama Institute, Japan), PRIMER (Plymouth Routines In Multivariate Ecological Research [PRIMER-E Ltd, UK]), Prism (GraphPad Software Inc., US), ProfileAnalysis (Bruker Corp., Germany), ProMetab (Parson et al. 2007), R (R Core Team 2014), SAS (Statistical Analysis System [SAS Institute Inc., US]), SIEVE (Thermo Fisher Scientific, US), SIMCA-P (MKS Unimetrics, Sweden), SpecManager (ACD/Labs, Canada), Spectrum Database (ACD/Labs, Canada), SpinAssign (Platform for RIKEN Metabolomics, RIKEN Yokohama Institute, Japan), SpinWorks (University of Manitoba, US), SPSS (IBM Corp., US), Statistica Dataminer (StatSoft, US), Systat (Systat Software, US), Tiberius (Tiberius Data Mining, Australia), TopSpin (Bruker Corp., Germany), Unscrambler (CAMO, Norway), VNMR (Varian Inc., US), Xcalibur (Thermo Fisher Scientific, US), XWINNMR (Bruker Corp., Germany).

‡‡ **Statistical analyses:** ANOVA (analysis of variance), RM-ANOVA (repeated measures ANOVA), MANOVA (multivariate ANOVA), PERMANOVA (permutation MANOVA), BNM (biochemical network mapping), CAP (canonical analysis of principal coordinates), CNA (correlation network analysis), CVA (canonical variates analysis), DFA (discriminant function analysis), EBAM (empirical Bayes analysis of metabolites), GRNN (general regression neural networks), GTM (generative topographic mapping), HCA (hierarchical cluster anlaysis), ICA (independent component analysis), KNNA (Kohonen neural network analysis), MDS (multi-dimensional scaling), MCR-ALS (multivariate curve resolution-alternating least squares), MPEA (metabolite pathway enrichment analysis), ORA (over-representation analysis), PNN (probabilistic neural networks), PCA (principal components analysis), SPCA (supervised PCA), RPCA (robust PCA), PC-DFA (principal component discriminant function analysis), PCoA (principal coordinate analysis), PLS-DA (projection to latent structures discriminant analysis), OPLS-DA (orthogonal projection to latent structures discriminant analysis), PLS-LDA (projection to latent structures linear discriminant analysis), PLS-R (projection to latent structures regression), SAM (significant analysis of metabolites), SEM (structural equation modelling), SIMPER (Similarity Percentage analysis), SOMs (self-organising maps), WGCNA (weighted gene correlation network analysis).

§§ **Databases:** BiGG DB (Database for Biochemically, Genetically and Genomically structured genome-scale metabolic network reconstructions), BML (Birmingham Metabolite Library), BMRB (Biological Magnetic Resonance Data Bank), CRL (Chenomx Reference Library), ECMDB (E. coli Metabolome Database), EHMN (Edinburgh Human Metabolic Network), GMDB (Golm Metabolome Database), HMDB (Human Metabolome Database), KEGG (Kyoto Encyclopaedia of Genes and Genomes), KEGG LDB (KEGG Ligand Database), NIST (The National Institute of Standards and Technology) library, MBDB (Massbank Database), MMD (Manchester Metabolomics Database), NLDB (Nature Lipidomics Database), SMPDB (The Small Molecule Pathway Database), WMSL (Wiley Mass Spectral Libraries), YMDB (Yeast Metabolome Database).

A range of other NMR-based techniques are also available which have various applications and levels of analytical sensitivity, such as two-dimensional and hyphenated platform approaches (reviewed by Simpson & Bearden 2013; Bharti & Roy 2014; Larive et al. 2014). NMR was originally the workhorse of metabolite profiling in the early days, but recent advances in mass spectrometry based approaches offer alternative methods of analyses. These platforms are often used in combination, since they have their own individual merits (Ong et al. 2009; Zhang et al. 2012). For example, NMR is a non-destructive technique and acquires highly robust and reproducible measurements. Separation of metabolites prior to detection is not necessary and minimal sample preparation is required. NMR is generally cheaper to perform, but unfortunately has comparatively low sensitivity in relation to MS-based platforms, which means only metabolites that are present in significant quantities can be detected.

3.2.2.2 Mass spectrometry

Mass spectrometry (MS) is a method which involves the measurement of molecular weights of molecules (reviewed by El-Aneedet al. 2009; Viant & Sommer 2013). There are three components to a mass spectrometer: the ion source, the mass analyser, and the detector (Glish & Vachet 2003). There are many different types of these components (see Dettmer et al. 2007; El-Aneed et al. 2009; Junot et al. 2014). At the ion source, metabolites within a sample are ionised by a variety of processes. For metabolomics work, the most commonly used ionisation techniques are electron ionisation and electrospray ionisation (Lei et al. 2011). In most cases, the molecules become sufficiently excited to fragment into a number of electrically charged ions. These ions move into the mass analyser where they are separated based on their mass to charge (m/z) ratio by accelerating them and subjecting them to various combinations of electric, magnetic or electromagnetic fields or in a ‘time of flight’ mass spectrometer which assesses how fast they are travelling. Fragments with different m/z ratios travel at different speeds and are

deflected from their forward trajectory to different degrees; lighter ions deflect more than heavier ions, and the higher the ionic charge, the greater the deflection. This allows the various types of mass analysers to filter the ions. The ions are then directed into a device that counts the number of ions at each different mass. This information is plotted in a spectrum of the ion abundance as a function of the m/z ratio. The identity of a metabolite can be putatively elucidated by comparing the fragmentation patterns against open access and/or proprietary databases which contain mass spectra of known compounds. Depending on the particular instrument, some (high resolution mass spectrometers) are capable of determining the actual elemental composition of each ion, thus providing an extra dimension of information for validation of metabolite identity.

MS-based methods are becoming highly sophisticated and newly-developed platform variations are increasingly being showcased in the scientific literature. MS can be performed directly on samples without pre-separation of metabolites (reviewed by Ibáñez et al. 2014). While direct MS techniques are rapid, they also suffer from low ionisation efficiencies and ion suppression. Thus, to decrease the complexity of the sample matrix and enhance the sensitivity and selectivity of the analysis, MS-based metabolomic approaches usually involve separation of metabolites via chromatography or electrophoresis prior to MS detection. The benefits of pre-separation are that a significant amount of information is available from the pre-separation process, and metabolites with the same mass can easily be distinguished since they are introduced into the MS system at different times. In addition, higher quantitative accuracies can be achieved since problems associated with ion suppression and other interferences are greatly reduced. Gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis (CE) are the most commonly applied methods for this purpose. When coupled, these instruments are called hyphenated platforms (GC-MS, LC-MS and CE-

MS). Each of these platforms have their own unique advantages, and can be used in combination to obtain very broad coverage of the metabolome (Lei et al. 2011).

3.2.2.2.1 Gas Chromatography Mass Spectrometry

Gas Chromatography (GC) separates metabolites which are volatile and thermally stable, or which become volatile and thermally stable after functional group modifications (e.g., alkylation or silylation via chemical derivatisation [Villas-Bôas et al. 2011]) (reviewed by Garcia & Barbas 2011). Once the sample extract has been prepared, it is injected into a hot gas stream flowing through a long and very small diameter tube in the GC instrument. The inside walls of the tube, called a column for historical reasons, are coated with material that has some affinity for the various components in the mixture. The different interactions of the metabolites with the gas stream and the column walls result in differential flow speeds through the column and they exit the column at different times (producing a chromatogram [Figure 3.3 E–F]); thus entering the mass spectrometer in a unique sequence. This combination of unique entrance times and associated information on the physicochemical properties of metabolites provides an enhanced means of profiling and identification. See Figure 3.4 for an illustrated overview of the analytical processes involved using pre-separation techniques combined with MS for the metabolomic analysis of complex sample matrices. GC-MS has the advantage that it produces very stable metabolite retention times within the column, does not have drawbacks associated with ion suppression, and generates highly reproducible fragmentation patterns. These features mean that metabolite identifications can more easily be authenticated by matching spectra against those contained within numerous open-access spectral libraries. However, if samples need to be derivatised, are thermally unstable, or have too high a molecular weight, GC-MS may not be suitable. See Zhao X. et al. (2015) for an applied example of how GC-MS was used to identify biomarkers for

temperature stress in tilapia, and to discover that an exogenous supply of L-proline into the culture water led to higher disease resistance against bacterial pathogens.

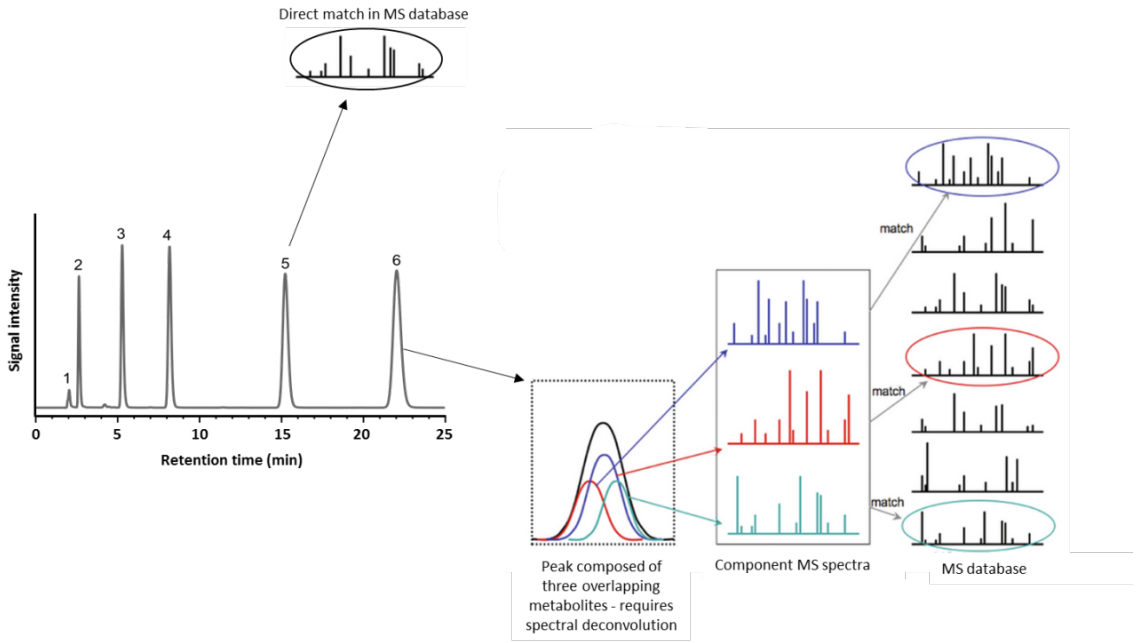

Figure 3.4. Overview of the processes involved using pre-separation techniques combined with mass spectrometry. Gas chromatography, liquid chromatograph or capillary electrophoresis is used to separate metabolites in the sample extract to produce a chromatogram. Compounds within the peaks are then sequentially analysed by mass spectrometry, and their ion m/z ratios are compared to those stored in mass spectral databases for identification. In some cases, peaks may comprise multiple metabolites with similar physicochemical properties which are unable to be separated by the pre-separation device and deconvolution of the spectra is required.

3.2.2.2.2 Liquid Chromatography Mass Spectrometry

High Performance Liquid Chromatography (HPLC or LC) is based on similar chromatographic principals as GC, but the sample is not heated to high temperatures (reviewed by Xiao et al. 2012). The most important distinction between GC and LC is that GC largely separates metabolites based on their boiling points with secondary retention by polarity, whereas metabolite size or polarity are the main mechanisms of LC. The column in this case is a tube a few millimetres wide and a few centimetres long and packed with extremely fine powder coated with material that has some affinity for the various metabolite components in the sample. A suitable solvent mixture is pumped at

very high pressure through the column. The sample is introduced into the column as a solution and, like GC, the various components of the mixture travel through the column at different speeds and exit in sequence to produce a chromatogram (Figure 3.3 G–H). From there the outlet stream is directed into a suitable detector which, for metabolomic work, is typically a high-resolution mass spectrometer. Therefore, LC-MS can analyse a wider range of metabolites since non-volatile and thermally sensitive compounds can be separated in the liquid phase. However, LC-MS suffers from greater ionisation and matrix effects, and lower chromatographic reproducibility. These features make assigning metabolites through spectral library matching considerably more difficult. Nevertheless, LC-MS is a very popular metabolomic platform and the analytical technology, spectral libraries, and software for processing spectra are continually being updated to improve metabolite identifications. See Yan et al. (2012) for an applied example of how LC-MS was used to identify species-specific metabolic stress responses of fish immediately after a tropical cyclone at various cage-farming sites, and to determine the physiological mechanisms which resulted in high mortalities during the following month of grow out.

3.2.2.2.3 Capillary Electrophoresis Mass Spectrometry

Capillary electrophoresis (CE) is an alternative pre-separation technique which separates metabolites based on their ionic charge characteristics, or electrophoretic mobility (reviewed by Ramautar et al. 2009). In many respects this technique is similar to LC, but molecules are separated based on their ionic affinities and size rather than on their solid phase solubilities (Hiryama et al. 2014). In CE, sample extracts enter a column which contains electrolytes. Charged metabolites migrate through the column and exit at different times under the influence of an electric field, and can be further concentrated using gradients in conductivity and pH. CE-MS is an efficient platform that does not require rigorous sample pre-treatment, is useful for small samples, is good at separating

highly polar metabolites, has separation power and sensitivities which are comparable to GC-MS and LC-MS, and can quantify certain metabolites that other hyphenated MS platforms cannot. On the other hand, CE is unable to separate non-charged compounds, and suffers more than GC or LC from poor reproducibility. However, recent advances in CE-MS technologies are contributing to the increasing usage of the technique in metabolomics studies (Ramautar et al. 2015). See Koyama et al. (2015) for an applied example of how CE-MS was used to gain detailed metabolic insights into salinity adaption of brackish-water clams from four commercial fishery grounds in Japan with different water chemistries.

The sensitivity, or at least the detection limits, of MS techniques can be extremely high. As long as a substance can be separated, detection limits in parts per million or even better are possible. If pre-concentration techniques are used, molecules can easily be detected in concentrations of parts per trillion or better.

3.2.2.3 Vibrational spectroscopy

The analysis of complex sample matrices can also be performed using lower resolution instruments which measure the vibrational signatures of broad metabolite functional groups (Moore et al. 2014). Such analyses generally do not provide detailed information for identifying particular metabolites, but can still be very useful for obtaining an overall 'metabolite fingerprint' of a sample. This fingerprint is based on the holistic composition of functional group chemistries across all metabolites within the sample, and can be used to classify samples from different conditions when significant variations are observed. However, the drawback is that biological interpretation of spectra can be difficult because of this non-specificity. The application of vibrational-based technologies, such as Fourier transform infrared (FT-IR), near infrared (NIR), and Raman spectroscopy are growing in

popularity due to their rapid and high through-put analysis capabilities, their ability to work with very small samples, and their very low cost compared to other platforms.

3.2.2.3.1 Infrared Spectroscopy

Infrared techniques work on the principal that when a sample is exposed to light, or electromagnetic radiation, the different chemical bonds within metabolite functional groups absorb energy at different wavelengths and vibrate in characteristic ways. A plot of the absorbance or transmittance of light at different wavelengths produces a spectrum which represents the overall metabolite composition of the sample to provide a snapshot, or fingerprint, of the organism's metabolome (Figure 3.5). Infrared platforms are categorised into Near-Infrared (NIR) 0.78–3 µm and Mid-Infrared (MIR) 3–50 µm depending on the wavelength of light used to analyse the samples. Modern instruments commonly use Fourier transform techniques (a mathematical process which converts the raw data from the instrument into a spectrum) so the expression FT-IR is often seen when discussing MIR spectroscopy. MIR analysis examines the absorptions of bond vibrations and other molecular movements, whereas NIR evaluates the overtones and combinations of strong MIR absorptions, which, while not as specific as the sharper, stronger MIR absorptions, can be characteristic and more easily quantified for a range of biologically important functional groups such as sugars, fats, and proteins. Unlike MIR, NIR can penetrate many millimetres through water and the instruments can use glass optics. Infrared platforms have proven useful for a range aquaculture-related purposes. For example, to identify pathogenic bacteria responsible for disease in farmed salmon (Wortberg et al. 2012), to determine the causation for post-harvest variations in shrimp quality based on the methods used for culling (Fu et al. 2014), to identify fraudulently marketed fish from different origins (Vidal et al. 2014), to assess the meat quality of various fish species (Cheng et al. 2013; Qu et al. 2015), to develop new food safety and

authentication techniques for classifying shelled shrimp based on their post-harvest storage conditions (Qu et al. 2015), and to develop fast and cost-effective methods for proximate chemical analysis of cultured shellfish for the purposes of monitoring animal condition and assisting in selective breeding programs (Brown et al. 2012), among others.

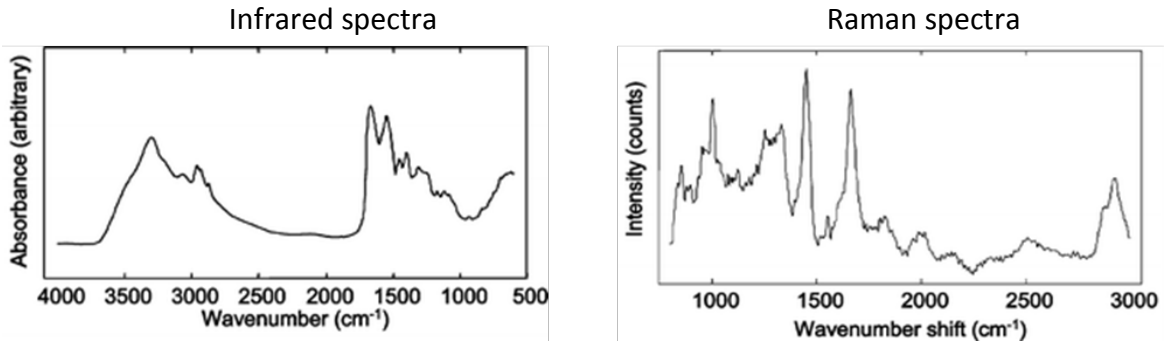

Figure 3.5. An example of comparative IR and Raman spectra obtained from the analysis of blood serum (reproduced with permission from Ellis & Goodacre 2006).

3.2.2.3.2 Raman Spectroscopy

Raman spectroscopy is a technique closely related to MIR. When a laser beam hits a molecule, approximately 1 in 10^7 photons will interact with electrons in the chemical bonds resulting in the scattered laser light having extra wavelengths (a few nm) added to it and subtracted from it which correspond to the vibration frequencies of the bonds in the molecule. These shifts in photon wavelengths are called the 'Raman effect'. The original laser colour can be subtracted using filters and the remaining frequencies provide information about the vibrational, rotational and other low frequency transitions within metabolites. Raman spectra are closely related to MIR spectra and look very similar (Figure 3.5). The principal difference is that the sorts of chemical bonds that give weak MIR absorptions are usually very strong in the Raman spectrum, and vice versa, so the two techniques are complementary. Although its big drawback is the very weak Raman signal, Raman spectroscopy has several major advantages. Glass optics can be used and

since Raman spectroscopy is based on the scattering of incident light rather than on absorption, it does not suffer from interferences caused by water. Thus, measurements can be made directly on biofluids and aqueous extracts, minimal to no sample preparation is required, and spectra can be obtained very quickly. See Ishigaki et al. (2014) for an applied example of how non-invasive Raman spectroscopy was used on live fish eggs to predict and monitor their quality and viability to ensure successful fertilisations.

Table 3.2. Comparisons between different analytical platforms for processing metabolomics samples.

Platform	Advantages	Disadvantages	Processing Cost†
NMR	Rapid analysis time (5–10 mins) Simple sample preparation No derivatisation needed Provides detailed structural information Low chemical bias Very reproducible Can be high resolution Excellent metabolite recovery (no suppression) Highly quantitative (without standards)	Low sensitivity Convoluted Spectra Libraries of limited use due to complex matrix More than one peak per component Peak overlap common pH adjustment required	Cheap $30–100 USD per sample
GC-MS	Very sensitive Very robust Large linear range MS provides some structural information Many available libraries for metabolite identification Pre-separation provides additional information Does not suffer from ion suppression Reproducible retention times Quantitative (with appropriate standards)	Slow analysis time (30–60 mins) Extensive sample preparation Derivatisation required Destructive to sample Some metabolites cannot be made volatile Some metabolites are too large for analysis Cannot detect some thermally unstable metabolites	Expensive $100–200 USD per sample
LC-MS	Very sensitive Can detect a very wide range of metabolites MS provides some structural information High mass accuracy Many modes of pre-separation available Pre-separation provides additional information Quantitative (with appropriate standards)	Analysis time can be slow (10–60 mins) Lack of comprehensive spectral libraries Ion suppression & adduct formation problems Destructive Metabolite identification is difficult Low retention time reproducibility	Very expensive $150–400 USD per sample
FT-IR, NIR, Raman	Very rapid analysis time (10–60 secs) Low chemical bias Can be used directly on samples No derivatisation required Complete fingerprint of sample composition Useful for identifying functional groups	Extremely convoluted spectra More than one peak per component Metabolite identification almost impossible Often requires sample drying	Very Cheap $10–50 per sample

† Per sample processing costs vary between service providers and depend highly on the number of samples to be analysed within a particular project, and the resolution of the specific platform to be employed. The price ranges displayed are not strictly defined limits but are typical of the current rates charged for commercial samples, and generally will include metabolite extraction (and derivatisation when required), instrumental analysis, and some primary bioinformatics processing. Additional statistical analysis, secondary bioinformatics processing and/or biological interpretation of the data can usually be provided as an extra service by most facilities (e.g., $50–200 USD per hour for a metabolomics specialist). Academic-based metabolomics service providers may offer discounted rates for collaborative projects.

3.3 Metabolite fingerprinting vs. profiling

There are generally two approaches to generation and examination of metabolomics data – metabolite fingerprinting and metabolite profiling. The approach utilised depends largely on the objectives of the investigation and the facilities available.

Metabolite fingerprinting compares the overall nature of samples based on the entire set of signals generated by the analytical platform. These signals, or features, are analysed using statistical techniques to discern patterns in the data for the purpose of sample classification. This approach usually involves the analysis of a very large number of signals, which represents the total compositions of metabolites, and does not necessarily require metabolite identification. Data obtained from NMR and vibrational spectroscopy platforms are particularly well-suited for metabolite fingerprinting. However, interpretations of results within particular biological frameworks are limited unless further analyses of relevant features within the fingerprints are performed. Nevertheless, metabolite fingerprinting can be convenient for situations when only sample-class discrimination is required. For example, Aursand et al. (2007) used metabolite fingerprinting to reliably identify fraudulently mislabelled fish oil products to ensure food safety and develop novel techniques for food traceability and quality assurance.

Metabolite profiling evaluates all of the signals generated by the analytical platform so that they may be characterised and matched to spectra of known metabolites in reference libraries. Once identified, data analyses are then performed on the abundances of the metabolites within the samples. This approach provides data which can be more easily interpreted across various biological frameworks since features are ascribed an identity with often well-known biochemical roles. Metabolite profiling frequently leads to discovery of biomarkers and development of novel and testable hypotheses. For example, Guo et al. (2014) used metabolite profiling to identify early-

warning biomarkers to predict fish health, and to better-understand the mechanisms of defence against bacterial infection.

Metabolite fingerprinting combined with profiling is sometimes used when very large numbers of signals are present within the raw spectral data so that only those features statistically different between samples, or otherwise deemed important, are subsequently identified. This approach can be used to reduce the computational and resource demands of processing noisy or large and complex datasets. In most metabolite fingerprinting applications, signals that are different between samples are usually identified to aid interpretation of the data. For example, Savorani et al. (2010) and Picone et al. (2011) used combined approaches to identify factors responsible for meat quality variation in fish reared under different culture environments, and stored under different post-harvest conditions.

NMR-based metabolomics usually involves fingerprinting as an initial step, whereas use of MS-based hyphenated platforms may involve profiling only. It is important to note that the definitions and term usage for these two approaches tend to be flexibly applied in the literature and as yet there are no standardised descriptions.

3.4 Data Analysis

Although metabolomic datasets are often very large and complex, recent advances in bioinformatics and streamlined statistical workflows provide simple strategies for coping with the high dimensional data (Johnson et al. 2015). Bioinformatics is an interdisciplinary field incorporating computer science, database management, mathematics and statistics. Primary bioinformatics processing involves analysis of the raw data obtained from the analytical platform and incorporates all procedures which are required to generate a list of features or metabolites. The resulting data can then be analysed by a range of classical and applied statistical procedures.

A number of steps are involved in the primary bioinformatics processing and usually includes data conversion, spectral processing (e.g., deconvolution, alignment, and noise reduction), feature selection, metabolite identification via database matching, metabolite quantification, and quality control procedures. Although a variety of freely or commercially available software packages exist to perform these tasks, many laboratories employ their own proprietary programs and algorithms, which have been custom designed for their unique situations and analytical set-ups. The methods used for primary bioinformatics processing vary widely and depend on data type and the analytical platform employed. Thus, it is impossible to provide general advice. However, at the end of this section we direct readers to a wide range of relevant literature, which covers these topics in more depth.

Prior to statistical analysis of metabolite profile/fingerprint data, data scaling, normalisation and/or transformations are often performed to enhance extractability of biologically relevant information from the dataset. Metabolite concentrations have huge dynamic ranges, and variance is typically larger at higher concentrations. Because many statistical procedures rely on homoscedasticity or distributional assumptions, it is important to alleviate the dependency of the variance on the concentration through variance-stabilising transformation or transformation to normality. Furthermore, the relative abundances of different metabolites are not proportional to the biological importance that they may represent, and many data analysis techniques fail to take this into consideration. Some of the more commonly applied pre-treatment methods for metabolomics data include centering, autoscaling, pareto scaling, range scaling, log transformation, and power transformation (reviewed by van den Berg et al. 2006). The pre-treatment method chosen may vary between different metabolomics datasets; hence, a solid understanding of how the implemented method affects the outcome of subsequent statistical analyses is essential for reliable interpretations.

Statistical data analysis can be achieved using general statistical software (e.g., Minitab [Minitab Inc.: PA, USA], SIMCA [Umetrics, Umea, Sweden], SPSS [IBM Corp.: NY, USA], STATISTICA [Statsoft Inc.: OK, USA]) or dedicated metabolomics-based data analysis packages (e.g., DeviumWeb [Grapov 2014], MeltDB [Kessler et al. 2013], Metaboanalyst [Xia et al. 2015]). A basic knowledge of programming is useful for employing, modifying, or writing script in certain data analysis environments (e.g., Matlab [Mathworks Inc.: MA, USA], R [R Core Team 2014]). However, recent development of easy-to-use graphical user interfaces for these environments have substantially reduced the need for advanced programming skills. See Mishra and Van der Hoot (2016) for further information regarding the latest advances in available computational tools and resources for the analysis of metabolomics data. OMICtools (http://omictools.com/) is also a useful and growing online repository of web-accessible tools related to omics-based data analysis.

Similar to other –omics disciplines, it is common for the number of measured variables (genes, proteins or metabolites) within each sample to far exceed the number of samples analysed. Metabolomics data are by their very nature multivariate in design and lend themselves particularly well to multivariate statistical analyses. However, univariate techniques can also be employed to extract valuable information from the data. Use of both approaches in combination is routinely performed and recommended because they can expose different characteristics of the samples (Sugimoto et al. 2012).

3.4.1 Univariate methods

Univariate methods involve analysis of single variables (metabolites) at a time. The *t*-test and ANOVA (analysis of variance), or their non-parametric equivalents (e.g., Mann-Whitney U test, Kruskal-Wallis test), are the most commonly applied univariate techniques to identify differences in metabolite abundances between samples. However,

due to the high number of variables, it is important to correct for multiple hypothesis testing to protect against the likelihood of identifying false-positives (Broadhurst & Kell 2006).

SAM (Significant Analysis of Microarrays/Metabolites) and EBAM (Epirical Bayes Analysis of Microarrays/Metabolites) (Xia et al. 2009) are examples of univariate methods which are able to account for correlations between metabolites and do not assume independence, unlike the *t*-test and ANOVA. These algorithms were originally developed for examining large sets of gene expression data and are of particular value for the analysis of datasets where *n* features are considerably greater than *n* samples; as is usually the case with data from transcriptopmic, proteomic and metabolomic studies. SAM is designed to address false discovery rate problems (FDR) when running multiple tests on high-dimensional metabolomics data. The algorithm first assigns a significance score to each variable, based on its change relative to the standard deviation of repeated measurements. Then, it selects variables with scores greater than an adjustable threshold and compares their relative difference to the distribution estimated by random permutations of the class labels. For each threshold, a certain proportion of the variables in the permutation set will be found to be significant by chance. The proportion is used to calculate the FDR. EBAM is a variation of the SAM algorithm based on moderated *t*-statistics. The advantages of EBAM are that it also addresses FDR and is well-suited to analysis of data where *n* samples or biological replication is low (e.g., $n = 3–8$).

Volcano plots are often used for the univariate analysis and visualisation of gene, protein and metabolite expression data (Li 2012). Volcano plots are scatterplots which incorporate a measure of statistical significance (e.g., *t*-test *p*-values) with information about the magnitude of metabolite change (fold-change) (Figure 3.6). Volcano plots allow quick identification of metabolites, which are not only statistically different between two sample conditions, but which also co-display large variations in abundance. See Young

et al. (2015) (Chapter 4) for an applied example of how a volcano plot and SAM was used to assist construction of a multivariate classification model for assessing the quality of hatchery-reared mussel larvae.

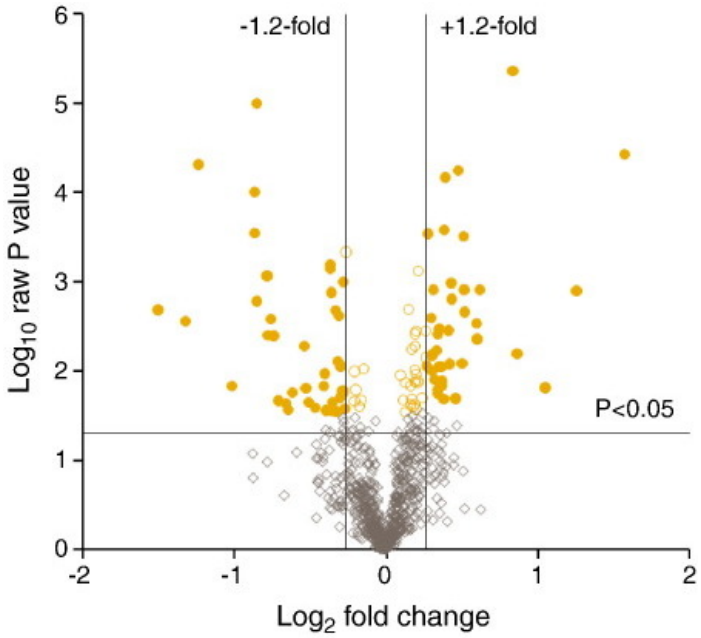

Figure 3.6. Example of a volcano plot. Solid yellow circles represent metabolites which are significantly different between sample groups ($p < 0.05$), as well as have large variation (> 1.2 fold-change) in their mean abundances.

Univariate methods are attractive because they are generally simple to apply and the results are easily interpreted and communicated across various levels of expertise. However, they cannot detect group differences when only minor variations exist on a single molecule level. Associations between metabolites and low variations in abundance can be highly important on a systems level due to the orchestrated flux of metabolites within common biochemical networks.

3.4.2 Multivariate methods

Since univariate techniques may not account for interrelations between metabolites, multivariate methods are applied to compensate, and to provide additional and complementary information for assisting interpretation of the data. Multivariate

techniques can be used to reduce complexity and identify patterns, group structure, and relationships among metabolites and samples (Worley & Powers 2013). Commonly applied procedures include Principal Components Analysis (PCA), Projection to Latent Structures Discriminant Analysis (PLS-DA), and clustering.

3.4.2.1 Principal Components Analysis

PCA is a mathematical procedure that aims to capture and extract most of the important information in a high-dimensional data matrix and re-express it in fewer dimensions (Abdi & Williams 2010). In doing so, the data can be more easily visualised, described and analysed. PCA does this by combining the multiple correlated variables into a number of smaller uncorrelated variables called principal components. A different data matrix is constructed in which the first 2–3 new variables account for the vast majority of the total variance in the original data. The samples can then be projected and visualised on a 2D or 3D score-plot (Figure 3.7). PCA is an unsupervised statistical technique which incorporates only the independent metabolite information. Dependant variables are not required for modelling and information of sample class membership is not included in the analysis. As an unsupervised technique, patterns among the independent variables are discerned and groups of samples are formed based solely on the structure of the metabolite data. The PCA algorithm therefore achieves unbiased dimensionality reduction and only exposes group structure when within-group variation is substantially less than between-group variation. PCA is very useful for visualising multi-dimensional data, identifying outliers, conducting classification studies, identifying a subset of original variables which explain most of the variation between samples, and for exploratory data analysis before building predictive models. See Kokushi et al. (2015) for an applied example of how PCA was used to identify differential regulation of metabolic pathways due to insecticide exposure in freshwater carp.

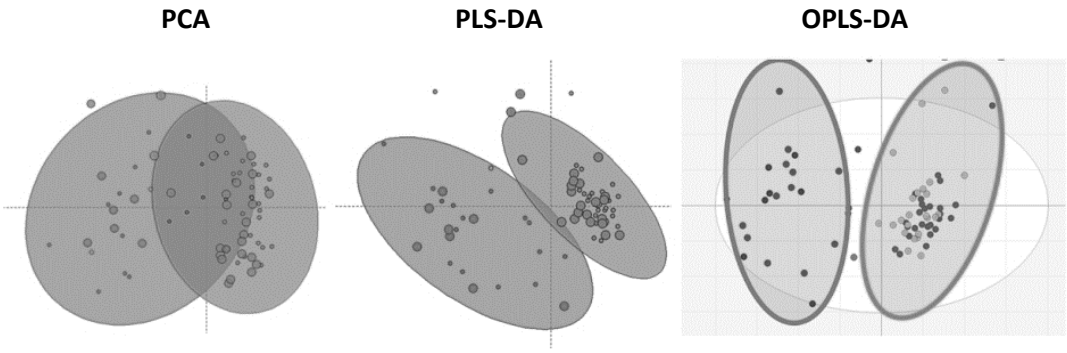

Figure 3.7. Comparison of multivariate data reduction techniques for assessing sample groupings using non-supervised principal component analysis (PCA) and supervised projection to latent structures discriminant analysis (PLS-DA), and its orthogonal extension (OPLS-DA) (reproduced from Grapov 2014 with permission). Discrimination power: PCA < PLS-DA < OPLS-DA.

3.4.2.2 Projection to Latent Structures Discriminant Analysis

Similar to PCA, PLS-DA is a technique, which can be used to reduce dimensionality and help visualise and analyse multivariate data (Worley & Powers 2013). However, PLS-DA is a supervised statistical technique, which incorporates information about the sample classes. Using this information, PLS-DA rotates the data within the newly created latent variable subspace in a way that maximises separation between groups of samples. This can result in much clearer separations than when PCA is applied (Figure 3.7). PLS-DA can be very useful for identifying and ranking metabolites which contribute most towards sample group separations and, when applied correctly, to assist construction of predictive classification models. Orthogonal PLS-DA is a related technique, which can further enhance separations due to its ability to distinguish between predictive and non-predictive (orthogonal) variation (Bylesjö et al. 2006) (Figure 3.7).

PLS-DA and its extensions have a tendency to over-fit the model to the data. Therefore, validation is important when using these algorithms in predictive capacities. Model validation is the process of defining a model's performance and is a critical requirement for predictive modelling (Szymańska et al. 2012). This ensures that the model's internal variable rankings are truly informative. Commonly used methods to test a model's performance include permutation-based tests and cross validation (Worley &

Powers 2013). The ideal scenario involves the use of a training dataset to build the model, and a separate validation dataset to assess its predictive capacity. See Liu et al. (2015) for an applied example of how PLS-DA and OPLS-DA were used to identify metabolites associated with white spot syndrome virus infection in shrimp, and provide preliminary information for developing biomarkers for diagnosing the pathophysiology of the disease.

3.4.2.3 Clustering

Clustering is a collection of statistical procedures, which aims to group samples together that are most similar in their metabolite profile (reviewed by Andreopoulos et al. 2009). Like PCA, most clustering techniques involve unsupervised approaches to group samples and the goal of clustering is to identify the actual groups based on the underlying structure of the data. Where PCA selects the variables with the most variation to form a reduced data matrix for partitioning samples, cluster analysis algorithms do not lose variance through dimensionality reduction in the same way and generally use all variables equally to display sample similarity/dissimilarity. Although clustering can be used to discover structures within the data irrespective of sample-class membership, it does not explain why they exist. Nevertheless, clustering is a very useful exploratory technique for uncovering patterns, finding natural groupings, confirming known groupings, identifying outliers, and discovering groups of metabolites with similar expression patterns across a wide range of biological conditions by clustering the variables rather than the samples. The most commonly applied clustering algorithms in metabolomics-based investigations are Hierarchical Cluster Analysis (HCA) and k-means clustering.

HCA is a method which seeks to construct a hierarchy of clusters and arrange them into a binary tree-structured graph called a dendrogram (Meunier et al. 2007). HCA does this by successively merging comparable groups based on the similarity/dissimilarity, or distance, between them. Visualising this tree provides a useful

summary of the data. HCA can be combined with data visualisation techniques to provide new ways of looking at the data, and to enhance the extraction of important information (Figure 3.8). See Courant al. (2013) for an applied example of how HCA was combined with heatmap analysis to assist visualisation of metabolite-group expressions, and to identify biomarkers for fine-scale monitoring during continuous culture of microalgae under different nitrogen regimes.

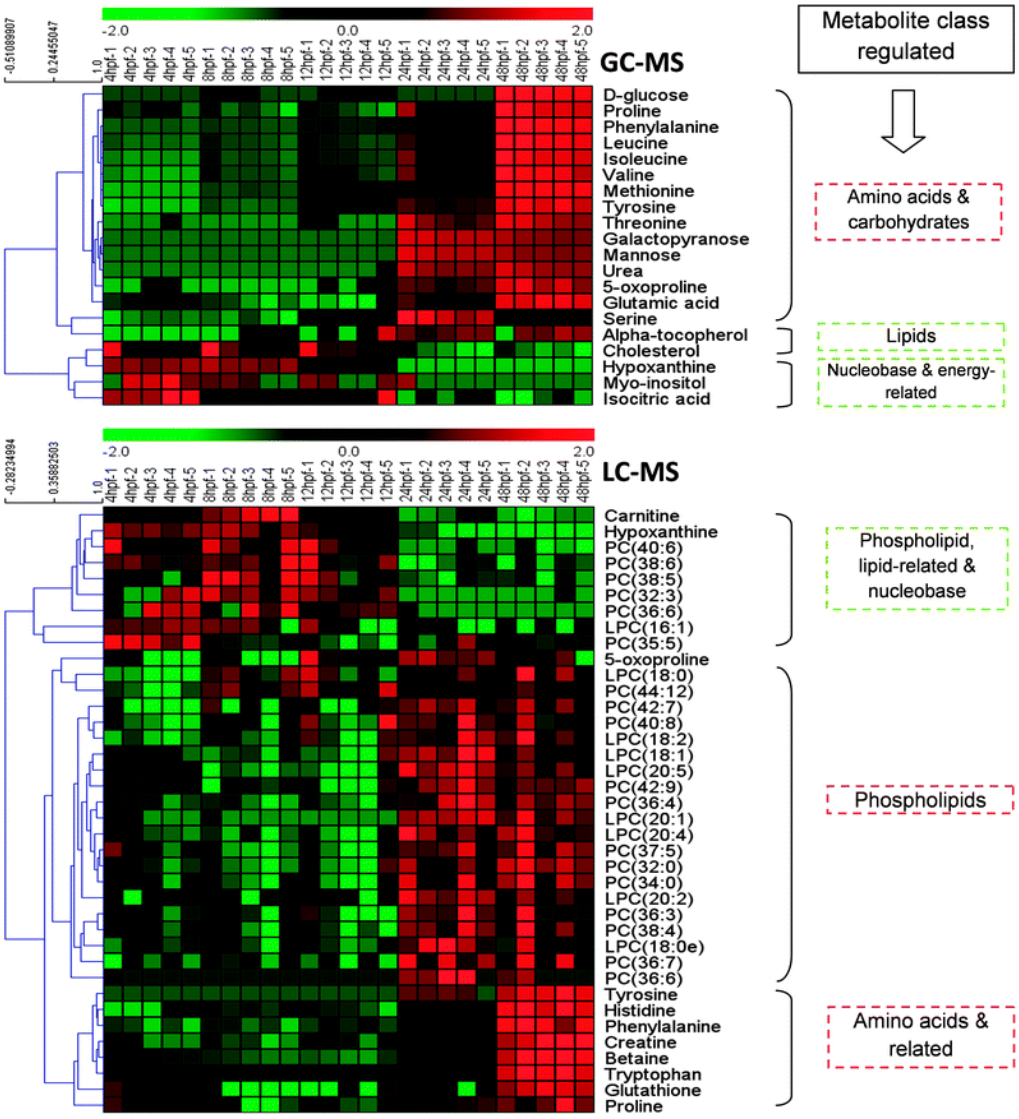

Figure 3.8. Combined heatmap and hierarchical cluster analysis of metabolites in developing zebrafish during embryogenesis *via* GC/MS- and LC/MS-based metabolomics (reproduced with permission from Huang et al. 2013). Each column represents a sample (five biological replicates for each of the five development stages). Each row represents the abundance of a particular metabolite (red = high abundance, green = low abundance). Metabolites cluster naturally into groups which, in this case, have functional relationships (labelled metabolite classes in dotted boxes).

K-means clustering (*k*-MC) is a non-hierarchical unsupervised vector quantisation and partitional clustering approach (Verma et al. 2012). Although sample class membership information is not incorporated into the analysis, the researcher must initially define how many clusters (*k* number of clusters) into which the samples are to be partitioned. Like other clustering techniques, the aim of *k*-MC is to gather samples into groups so that those in the same group are most similar to one another, and those in different groups are as different as possible. Working within an *n*-dimensional subspace of true vectors (number of variables), the algorithm performs this task through an iterative sequence of minimising the sample distances to a centroid point within each of the *k* number of clusters, and reallocating the samples to the cluster with the closest centroid so as to minimise the within-cluster sum of squares. Initially, the first centroid points are randomly placed and samples are assigned to a cluster. Then, the true centroid points of those clusters are calculated and repositioned, samples are reassigned, and the clusters are redefined. This is performed repeatedly until convergence is found and effectively results in a partitioning of the data space into Voronoi cells.

Use of *k*-MC in aquaculture-related metabolomics research is limited thus far. However, see Yu et al. (2013) for a relevant example of how *k*-means was used to identify groups of genes with similar expression profiles in fish which had been fed a diet contaminated with the persistent organic pollutant BDE-47, and to determine potential enzymatic and metabolic mechanisms of toxicity defence. *k*-MC (combined with HCA and PCA) is also used in Chapter 6 as an independent unsupervised measure to discern natural sample groupings of oyster larvae infected with Ostreid herpesvirus microvariant and thus help identify mechanisms of the host-virus interaction.

While three widely used multivariate techniques to analyse metabolomics data have been discussed, one should be aware that an array of other procedures are available

which may be better-suited for the analysis of particular datasets in some cases. These include: multivariate analysis of variance, linear discriminant analysis, partial least squares regression, support vector machines, *k*-nearest neighbour, random forests, soft independent modelling of class analogies, and self-organising maps, among others. For further information on these alternative data analysis approaches, see Steuer et al. (2007), Liland (2011), and Xi et al. (2014). For platform-specific reviews on various bioinformatics processes see Smolinska et al. (2012), Sugimoto et al. (2012), Du & Zeisei (2013), Engel et al. (2013), and Wei et al. (2012 & 2014).

3.5 Biomarker discovery and validation

The aim of many metabolomics-based investigations is to discover novel metabolite biomarkers, which correlate with specific diseases or health states. These molecules can then be used as early diagnostic tools, or in conjunction with other assessments for confirmation of pathology. Metabolite biomarkers are also very useful within the aquaculture industry to assist in the evaluation of pre- and post-harvest meat quality, and for food safety and traceability purposes (reviewed in Chapter 2 [Alfaro & Young 2016]). Biomarkers may be single metabolites, multiple metabolites, ratios of metabolite pairs, particular features (e.g., ion fragments), or entire unannotated spectral fingerprints.

The initial step in biomarker discovery is often to perform an exploratory experiment with different treatments or animal conditions, and to identify features which are substantially different between the sample groups using one or more statistical procedures outlined in the previous section. Once determined, these features can be considered as ‘candidate biomarkers’. The purpose of initial biomarker discovery is to identify the most salient features for further investigation, and may involve low biological sample replication ($n < 10$), although higher replication is usually preferred. The results of these studies can be very useful for generating hypotheses, and gaining preliminary

mechanistic insights into metabolic factors responsible for, or involved in, particular health states or other conditions. However, when the ultimate goal is to develop practical biomarkers with useable applications and minimal risks for Type II errors occurring, they must have extremely reproducible performances. Thus, in order to ensure that the identified candidate biomarkers have high sensitivity and specificity for the particular condition under investigation, it is important to validate them.

The process of biomarker validation was born from the medical research field where the misdiagnosis of a health condition might result in a disastrous outcome for a patient. Biomarker validation is a quality assurance process of defining the performance of a biomarker within acceptable limits, whilst understanding and minimising the rate of false discovery. Biomarker validation usually involves one or more additional experiments where the candidate metabolite/feature is targeted more specifically for quantification using complementary or alternative analytical platforms with a high selectivity for that analyte. Such experiments will typically also involve much higher sample replication (n = 100–1000), experimental replication, a broadening of scope in some cases (e.g., incorporation of multiple sexes, development stages, and environmental conditions), and rigorously refined statistical approaches (e.g., permutation based cross-validation or using different sub-sets of the samples to construct, validate, and test the performance of a predictive model [Westerhuis et al. 2008; Szymańska et al. 2012; Xia et al. 2013]). Accordingly, biomarker validation within the framework of a high-quality clinical study can involve substantial costs and time, which may not be viable for an environmental study or commercial aquaculture exercise. However, what constitutes validation is a subjective measure and is scalable within the confines of the researcher.

In a practical scenario where time and funds are limited, an alternative approach might be to employ a particular candidate biomarker whilst accepting its potential vulnerability, and continually adding new data to the predictive model as it becomes

available. In this way, quality control limits on the model's performance can be set and monitored as it is updated. Thus, expenses are diluted over time and performance-based milestones can be implemented based on cost-benefit analyses to guide management decisions in an empirically data driven context. Validation is an important concept when identifying and implementing new biomarkers, and should be a carefully considered component within the general strategy of a metabolomics-based biomarker discovery and development project. For further information on biomarker discovery and validation procedures, see Xia et al. (2013).

3.6 Biological interpretation and secondary bioinformatics

The discovery and identification of biomarkers do not always necessitate in-depth functional explanations for their presence and/or roles. For example, a simple metabolomics-based study using nuclear magnetic resonance and a fingerprinting approach combined with pattern-recognition tools (e.g., PCA, PLS-DA) could be used for food authentication purposes to identify biomarkers to classify an adulterated product, or determine its provenance (reviewed by Cubero-Leon et al. 2014). However, for many investigations, more detailed insights into the reasons for sample group separations are required, and the procurement of mechanistic biochemical explanations are highly desirable. In such cases, it becomes necessary to interpret the data within biologically meaningful frameworks.

The past 100 years of biological research has provided us with an amazing wealth of knowledge concerning cellular metabolism across a wide range of taxa. Rigorous empirical experimentation by a multitude of pioneers during this period has established the major biochemical pathways. Not only do we know which genes, enzymes, cofactors, substrates, products, and intermediates are involved in these pathways, in many cases we also know about individual enzyme kinetics and have detailed information about vast

arrays of endogenous and exogenous factors which influence their pathway flux (German et al. 2005). Information such as this provides us with a rich source of knowledge which can be used to assist the interpretation of biochemical data. Nevertheless, interpretation of metabolite expression data can be one of the most challenging aspects of a metabolomics study.

In many cases, concentrations of particular metabolites within a tissue, biofluid, or organism may correlate very well with our current understanding of biochemical networks and the functional relationships among metabolites, enzymes, and genes within normal or perturbed systems. For example, classic signs of stress caused by pathogen or toxin exposure in aquatic animals include increased levels of reactive oxygen species (ROS), and differential co-expression of metabolites (e.g., glutathione, NADPH) and enzymes (e.g., glutathione reductase, superoxide dismutase, catalase) involved in regulating excess ROS production in order to maintain redox homeostasis (Parrilla-Taylor et al. 2013; Macías-Mayorga et al. 2015). The results of recent omics-based investigations provide data which corroborate the presence of such mechanisms in various taxa, as well as offer new information on associated regulatory pathways (Srivastava et al. 2013; Barth et al. 2014; Shi et al. 2015). On the other hand, a number of studies (particularly those containing metabolomics-based components) are providing data which are shedding light on unfamiliar biochemical associations which cannot be explained by our current theses of molecular biology and biochemistry (Steuer 2006). For example, unlike genes and proteins, it is relatively common for metabolite levels within a particular pathway to be highly correlated with metabolites from other pathways for which a mechanistic connection is not currently known. Furthermore, in some cases precursor-product metabolites in enzyme-linked reactions may correlate well, whereas in other cases they do not. It is intriguing and unexpected results like these that are starting to deliver new information that is helping to push forward our understanding of metabolic networks at

an astonishing rate, and also highlights the usefulness and efficiency of omics-based approaches for generating novel data to assist new interpretations. The continual development of metabolomic techniques to characterise larger and larger sets of metabolites requires new methods to analyse these data in order to obtain biologically meaningful information. Here, we briefly outline a few methods that can be used to help researchers interpret their metabolomics data beyond more conventional scenarios, involving assessments of single metabolite variations based on a priori biochemical knowledge.

If biological replication is sufficient, a simple method involves correlation analysis in which construction of a correlation matrix of pairwise metabolite level comparisons are made. Such matrices can be useful for identifying potentially important relationships requiring further investigation. For example, consider the following hypothetical situation where levels of metabolite X and metabolite Y are not significantly different between control and treatment groups. However, metabolite X is positively correlated with metabolite Y in the control group, and negatively correlated in the treatment group (Figure 3.9).

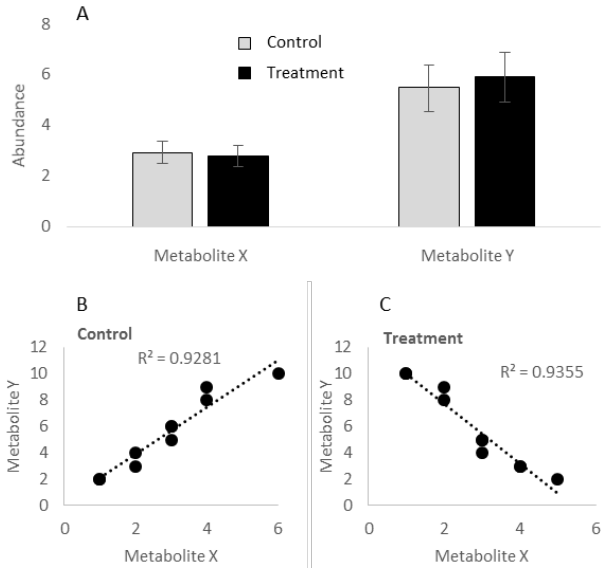

Figure 3.9. An example of a situation where mean levels of metabolite X and metabolite Y are not significantly different between groups of samples **(A)**, but are differentially correlated within each group **(B & C)**.

Such a scenario would indicate that some major perturbation of the underlying network was taking place, and would have gone undetected had the correlations not been investigated. Differential non-linear correlation patterns may also be present which would require alternative methods of detection. For further information on the interpretation of linear and non-linear correlations in metabolomics data, see Camacho et al. (2005) and Steuer (2006).

While our knowledge is relatively comprehensive compared to only a few decades ago, much of our understanding to date has come from highly targeted analyses of specific pathway components, and it is increasingly becoming clear that there are many gaps to be filled. With more of a focus on the interconnections between pathway components, we are starting to uncover new insights into metabolism which are much more integrated than ever before. An alternative method for identifying metabolite association patterns is called correlation network analysis.

Correlation networks are increasingly being used in omics-based applications to visually capture the overall network of interconnections between biomolecules and to describe the correlation patterns, to identify relationships between entire biochemical pathways, to discover new modules or clusters of relationships, and to assist data interpretation (Langfelder & Horvath 2008; Hero & Rajaratnam 2015). Applied to metabolomics, correlation network analysis is a technique that maps the relationships between every metabolite pair onto a metabolite network. Lines between metabolites typically are descriptive of the relationship between them (e.g., a solid line for a positive correlation and a dotted line for a negative correlation), and may also be quantitative (e.g., defined by the width of the line). The positions of the metabolites within the network map may be placed manually to enhance visualisation, or for additional interpretive purposes they may be positioned using algorithms to identify and define metabolite modules that

cluster together. For an applied example of a study involving an aquatic organism, see Southam et al. (2008), who used a combination of correlation analysis techniques to identify key metabolic differences in hepatic tumors of flatfish compared to control tissues, and to assist detection and interpretation of the underlying mechanisms involved in the diseased phenotype (Figure 3.10).

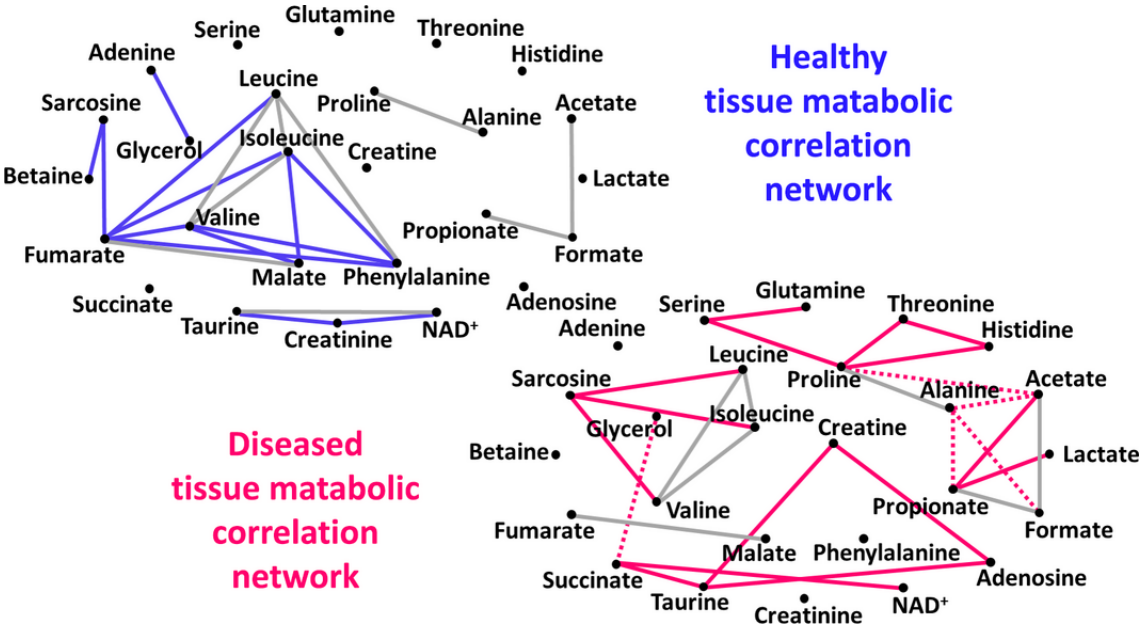

Figure 3.10. An example of two correlation networks constructed using NMR-based metabolomics data from samples of healthy and diseased fish livers (reproduced with permission from Southam et al. 2008). Solid lines represent positive correlations between metabolites, and dotted lines represent negative correlations. Grey lines represent similarly shared relationships between the healthy and diseased phenotypes, and coloured lines represent those which are dissimilar. Clear differences in the underlying biochemical networks are easily visualised using this technique.

Correlation network analysis can additionally be used to integrate transcriptomic, proteomic and metabolomic datasets to help identify functional roles at different biochemical levels (e.g., gene–gene/protein interactions and relationships between enzymes and metabolites) (Higashi & Saito 2013). There are a number of software packages available to perform correlation network analysis, such as DPClus (Altaf-Ul-Amin et al. 2006), Metscape (Karnovsky et al. 2012), COVAIN (Sun & Weckworth

2012), 3Omics (Kuo et al. 2013), and MetaMapR (Grapov et al. 2015). For further information on correlation network analysis and various applications, see Steuer (2006), Adourian et al. (2008), Hüning et al. 2013, and Kotze et al. (2013).

Other procedures useful for supporting data interpretation include a variety of 'pathway enrichment analysis' techniques. Rather than focusing on individual metabolites which may be responsible for discriminating groups of samples, pathway enrichment analysis techniques aim to discover predefined metabolic pathways or biological networks that are altered in an orchestrated manner. Such analyses make use of large amounts of biochemical information collated over decades and stored in publicly accessible depositories, such as the Kyoto Encyclopaedia of Genes and Genomes (KEGG) (Kanehisa et al. 2000), and can be considered as secondary bioinformatics processes. There may be cases where levels of individual compounds are not identified as being statistically different between samples using conventional statistical approaches. However, when analysed together as functional groups, or metabolite sets within their known pathways, it might be revealed that particular pathways as a whole are being differentially regulated under certain experimental conditions. The recent development of secondary bioinformatics tools (reviewed by Booth et al. 2013) to analyse biochemical data within the context of predefined metabolite sets are changing the way that the results of metabolomics projects are interpreted. Pathway Activity Profiling (PAPi) is one example of such a technique (Aggio et al. 2010).

PAPi is an algorithm developed into an R package which can be used to analyse sets of functionally-related metabolites, and quantitatively compare the activity of metabolic pathways between different groups of samples. PAPi performs this task by calculating 'activity scores' based on the number of metabolites identified from each pathway and their relative abundances. Pathways for which each detected metabolite is involved in is collected from KEGG, and each is given a score based on the absolute

abundance/relative abundance of the metabolite to which it is linked. The pathways are ranked by the total number of metabolites they comprise, and the percentage of detected compounds within them are calculated. The sum of the scores for each pathway are then calculated and normalised (dividing by the proportion of metabolites detected from within the respective pathway) (Aggio et al. 2010). This simple yet effective method can be used to help determine the likelihood of a particular biochemical process being up- or down-regulated under certain circumstances. To our knowledge, PAPi has not yet been applied to studies involving aquatic organisms. However, it has been successfully applied in a number of other biological systems (Han et al. 2012; Portella et al. 2014; Zhao C. et al. 2015).

Another useful pathway analysis tool is called Metabolite Set Enrichment Analysis (MSEA) (Xia & Wishart 2010; Kankainen et al. 2011; Persicke et al. 2012). MSEA is an algorithm designed to detect subtle, but consistent changes among groups of metabolites within the same biological pathway. Using an analysis package with MSEA capabilities (e.g., MarVis-Pathway [Kaever et al. 2014], MeltDB [Kessler et al. 2013], Metaboanalyst [Xia et al. 2015]), a quantitative dataset of annotated metabolites can be cross-referenced with information in the KEGG database, and metabolite sets belonging to reference pathways from various model organisms (e.g., human, mouse, zebrafish, drosophila, nematode) can be analysed together as a group. The ability to examine biochemical information for different animal models is a key advantage of MSEA, and options also exist to use proprietary/customised background sets of data from any organism. Pathway enrichment analysis techniques, which use software to interrogate databases that contain global biochemical knowledge, are tremendously powerful data interpretation tools. For applied examples in the literature, see Zhao X. et al. (2015) and Ma et al. (2015) who utilised MSEA to identify differentially enriched pathways in Tilapia infected with two pathogenic Streptococcus species, and to develop remedial

strategies to enhance disease resistance. MSEA-type techniques are also applied in Chapters 6 and 7 of this thesis to help identify perturbed biochemical pathways of early life-stage bivalves in response to pathogen infection and toxic heavy metal exposure, respectively.

3.7 Reporting guidelines in metabolomics

The final stage of a metabolomics project is to disseminate the findings, either internally through technical reports, or externally through peer-reviewed publication. Whichever route is taken, it is advised that researcher's follow to the best of their abilities a number of 'minimum reporting standards' which have been developed over the past decade by the wider metabolomics community (the Metabolomics Standards Initiative [Fiehn et al. 2007a]). These readily available standards are 'highly recommended' guidelines for the reporting of various aspects of a metabolomics project, and provide a framework to ensure scientific rigour, allow study replication, support data sharing, and enable a better-informed process of assessment and interpretation. In accordance with other biological science investigations, typical areas of focus include detailed descriptions of the biological sample/s involved in the study, descriptions of the environment/s involved in the study, and descriptions of biologically-relevant processes involved in the study (Table 3.3) (see Morrison et al. 2007).

Table 3.3. Summary of sample-specific topics (prior to chemical analysis) which should be described in detail when reporting the results of a metabolomics project.

Focus area	Descriptions
Sample	Taxonomic classifications, common name/s, genotype/s, ecotype/s, sample composition, sample type, specimen condition (phenotypic characteristics, weight, age, sex, development stage, health)
Environment	Any field environment: Geographic location, habitat, depth, meteorological conditions (e.g., precipitation, wind speed/direction, humidity), lunar/solar phase, other measured parameters (e.g., pollutant concentrations)
	Any aquatic environment: Water temperature, tidal phase (or submergence/emergence information), other measured parameters (e.g., salinity, pH, dissolved inorganic/organic content, oxygen concentration)
	Any laboratory environment: Details not covered elsewhere, laboratory address and contact information
Process (biological)	Maintenance and acclimation of organisms: Procedure and means (e.g., cage, aquaria, static/flowthrough tanks, continuous culture), reasons for maintenance/acclimation, other parameters (e.g., feeding regime, lighting regime, tank/cage dimensions)
	Manipulation of organisms/samples: Controlled manipulation as part of the study (e.g., exposure to a toxicant, environmental perturbation or dietary manipulation etc.), dissection of a specific organ/tissue, capture/sampling means and procedures (e.g., netted, electrically stunned, anaesthetised, razor cut), reason for capture, other capture parameters (e.g., handling/stress aspects, time to capture, air exposure duration)
	Sample handling and storage/preservation: Procedure and means (e.g., snap frozen and stored in liquid nitrogen or on dry ice, sample container material), reasons for storage/preservation, temperature and duration of storage
	Organism/sample transportation: Procedures and means (e.g., live/dead, submerged/emerged, refrigerated container, dry shipper, temperature, transport duration)

Additional aspects to consider when reporting metabolomics-derived information include prescribing to the use of specific standard terms (ontology), providing particular details of a wide range of platform-specific instrumental parameters and data processing methods (computational, bioinformatics, statistical), quality-scoring metabolite identifications, and, among others, participating in the standards initiative to advance the future of the field by reporting and exchanging various levels of metadata with others. We strongly advise that all metabolomics researchers, from aspiring to seasoned investigators, become familiar with the recommended reporting guidelines for each component of a metabolomics project (summarised in Figure 3.11).

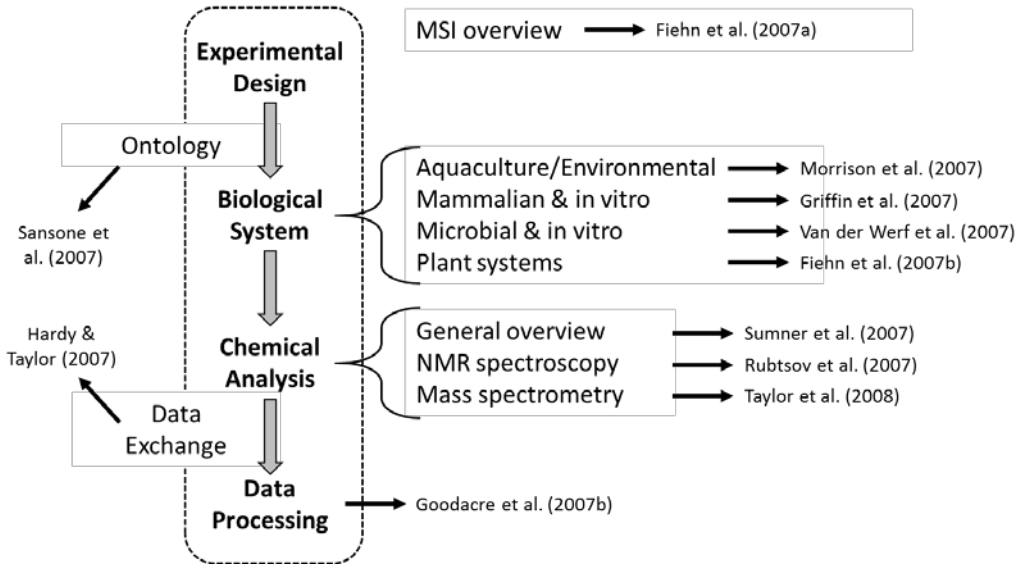

Figure 3.11. Overview of the metabolomics workflow showing the different components for which the Metabolomics Standards Initiative (MSI) have developed recommended minimum reporting standards (modified with permission from Goodacre 2014).

3.8 Incorporating metabolomics

Two main avenues exist for researchers who wish to conduct metabolomics investigations, or add a metabolomics component to an existing research project. There are a number of commercial metabolomics laboratories worldwide that offer streamlined services. Core facilities at various universities and centres house a combination of infrastructure and expertise to carry out a range of advanced metabolomics studies. These organisations can provide excellent support from consultation on experimental design to data analysis and interpretation of results. Inevitably, significant costs are usually associated with such commercial services. Alternatively, access to metabolomics facilities can be gained through academic institutions for substantially reduced charges based on collaborative agreements. For scientists wanting to conduct metabolomics research for the first time, it is important to note that running a successful metabolomics project requires an adequate experience in chemistry, statistics, bioinformatics and the advice from a metabolomics expert on hand. For researchers with sufficient chemistry knowledge and access to appropriate equipment and facilities, extraction and initial

identification of metabolites may be relatively easy. However, there are some specific constraints in sample collection/preparation and experimental design that need to be considered. In addition, the bioinformatics required for data analysis and interpretation are significantly complex and may require the involvement of a bioinformatics expert. Regardless of the approach, we suggest that new metabolomics projects incorporate the appropriate expertise from the start. Furthermore, we urge scientists to give appropriate consideration to the expected results and implications of findings, since this approach is exploratory by nature.

3.9 Summary

In summary, metabolomics is a relatively new approach that has the potential to make a huge contribution to the field of aquaculture. With a wide range of analytical platforms available today and the rapidly evolving computational and bioinformatics capabilities, we are likely to see a growing number of studies using metabolomics in all aspects of cultivating aquatic organisms. However, it is important to be aware of the potential limitations of this approach, especially with regard to sensitivity to external influences during sample collection and complex bioinformatics procedures required to obtain meaningful biological interpretations.

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Chapter 4 Mollusc larval quality

Quality assessment of hatchery-reared mussel larvae

Note: This chapter has been published with the following citation:

Young T., Alfaro A.C., Villas-Bôas S.G. 2015. Identification of candidate biomarkers for quality assessment of hatchery-reared mussel larvae via GC/MS-based metabolomics. *New Zealand Journal of Marine and Freshwater Research,* 49(1): 87–95.

Abstract

To ensure environmental and economic sustainability of future aquaculture growth, large-scale hatchery production of mollusc larvae is required. However, variation in larval quality currently limits potential maximum yields. Identification of biomarkers which reflect the immediate physiological condition of larvae during hatchery production could help monitor and determine causes of variation. Metabolomics is well-suited to this task due to its capacity for providing an instantaneous snapshot of the physiology of an organism through analysis of its metabolite profile. As a first test, GC/MS-based metabolomics was applied in this chapter to characterise the larval metabolomes of mussel larvae displaying differential growth rates. Using a variety of univariate and multivariate feature selection methods, we identified four metabolite–metabolite ratios involving levels of succinate, glycine, alanine, pyroglutamate and myristic acid as candidate biomarkers for assessing mussel larval quality. These metabolites are known to have roles in energy metabolism, osmotic regulation, immune function and cell–cell communication. We anticipate that further investigation of these metabolites and their associated biochemical pathways will yield a more complete understanding of the factors responsible for larval production variability.

4.1 Introduction

Global mollusc production is rapidly increasing and is one of the largest aquaculture activities in the world. Most of the seed requirements are obtained from wild populations where seasonal and regional reliability of quality and quantity is inconsistent. Furthermore, these natural stocks are approaching, or have exceeded, maximum sustainable yields for some species (Helm et al. 2004). In recent years considerable attention has been placed on the production of hatchery-reared juveniles for natural stock enhancement and grow-out (O'Connor et al. 2012). However, a number of bottlenecks present problems for commercial viability, such as broodstock management and gamete quality, mass production and variable quality of microalgal diets, establishment of successful and reproducible larval rearing practices, improvement of settlement rates and metamorphosis synchronisation, and identification of causes and development of remedial strategies for poor larval health and growth.

In New Zealand, mussel (*Perna canaliculus*) exports represent the largest aquaculture sector by value and volume. Small-scale hatchery production of spat currently contributes only marginally towards industry's seed requirements. However, substantial research over the past decade has led to the ongoing development of selective breeding lines (MacAvoy et al. 2008), establishment of a cryopreservation programme (Paredes et al. 2012), optimisation of microalgae culture and larval rearing procedures (Ragg et al. 2010; Kaspar et al. 2014) and future strategies for extensive growth and upscaling of hatchery facilities (Roberts 2013). The ability to provide consistency in larval quality and quantity is an important step towards reaching the commercial goal of successful large-scale production.

'Larval quality' is a term which refers to the physiological condition of larvae and is related to growth and survival during different developmental stages and under various environmental conditions (Racotta et al. 2003). For marine invertebrates, factors

responsible for variation in larval quality may include parental genetics (Meyer & Manahan 2010), maternal size and life-history experience (Marshall & Keough 2004, 2006), level of maternal provisioning (Smith & Bolton 2007), sperm environment prior to fertilisation (Ritchie & Marshall 2013), sperm/egg ratios during fertilisation (Marshall & Keough 2003), embryo and larval rearing densities (Galley et al. 2010; Deng et al. 2013), availability of food (Aarab et al. 2013), presence of pathogens (Kesarcodi-Watson et al. 2009) and physical environmental parameters (Sánchez Lazo & Martínez-Pita 2012). However, even with attempts to control such factors within hatcheries, inter- and intra-cohort variation in larval quality is still commonly observed. The effects of this variation have far-reaching implications for mollusc aquaculture due to difficulties in predicting larval production volumes and potential carry-over effects on post-metamorphic survival, growth and reproduction (Emlet & Sadro 2006; Przeslawski & Webb 2009). Thus, continual monitoring of larval quality is critical to provide information for determining causation of poor production levels, which may lead to better management decisions. Identification of biomarkers which reflect the immediate physiological condition of larvae has the potential to provide industry with valuable tools to improve the commercial viability of hatchery operations.

Metabolomics is a non-targeted approach to comprehensively profile a broad range of metabolites within biological samples, such as cells, tissues and whole organisms. Presenting unique opportunities for novel biomarker discovery, metabolomics is one of the latest fields gaining a stronghold in the various approaches towards molecular biology. Since metabolites are end products of gene and protein expression, and are exceptionally sensitive to genetic and environmental perturbations, metabolomics offers a revolutionary framework for phenotyping organisms at the molecular level (Kuehnbaum & Britz-McKibbin 2013). Using such a hypothesis-free approach has yielded excellent information without a priori knowledge of biological processes.

Furthermore, this approach routinely demonstrates value in corroborating data from gene and protein expression studies, as well as providing additional and complimentary evidences (e.g., Wilmes et al. 2013). Examples of applications are widespread across diverse areas of research from human medicine (Jin et al. 2014) to deep-sea microbial ecology (Kimes et al. 2013).

While only limitedly applied to aquaculture research thus far, specific metabolomics-based investigations are starting to provide useful information for various sectors within the aquaculture industry (reviewed in Chapter 2). For example, identification of biomarkers and development of new techniques for monitoring health (Schock et al. 2012, 2013; Ji et al. 2013a), growth (Almli 2012), nutritional condition (Cajka et al. 2013; Liu et al. 2013; Abro 2014; Wagner et al. 2014), culture environment (Savorani et al. 2010; Picone et al. 2011) and meat quality (Erikson et al. 2012) in fish, shellfish and crustaceans are now available. However, despite its wide applicability, the use of metabolomics in mollusc larval culture has not yet been realised. Here, we utilise GC/MS-based metabolomics to identify candidate biomarkers for assessing mussel larval quality using differential growth rates as a quality measure.

4.2 Methods

4.2.1 Broodstock spawning

Mussel (*Perna canaliculus*) broodstock (~60 individuals) from a selectively bred family line (F2) maintained at the Cawthron Institute Aquaculture Park (CAP; Nelson, New Zealand) in collaboration with Spat Production & Technology New Zealand Ltd (SPATnz; Nelson, New Zealand) were transferred from a nursery facility to a specialised CAP breeding laboratory 24 hrs prior to spawning. Mussels were kept dry overnight under a damp cloth ~16°C ready for spawning the next day. Spawning was induced via thermal cycling; broodstock were fully immersed in warm seawater (20–24°C) for 2 hrs, before

30 min cycles of cold seawater (8–9°C) changes were initiated. Spawning began after 2.5 to 3 hrs.

4.2.2 Gamete collection and fertilisations

Upon oocyte release, female mussels were removed from the spawning trays, briefly immersed in freshwater and then placed in 1 L plastic containers filled with 8°C 1 µm filtered seawater (FSW). The seawater was exchanged several times during the first 10 min to remove potential sperm contamination. Newly released oocytes were subsequently collected and held in 4°C FSW in 70 mL plastic containers (Labserv – Thermo Fisher; NZ). In order to protect organisms from unnecessary stress and damage, wide bore pipette tips (Axygen, USA) were used wherever oocytes, embryo or larvae were pipetted during this sampling, and throughout the experiment. Each oocyte collection was assessed via microscopy (Olympus CR30, ×10, ×20 objective) to ensure quality, and that no oocytes were fertilised. Accepted quality oocyte collections were pooled in a 250 mL glass Schott bottle and held for 1 hr at 4°C whilst oocyte counts were undertaken in BD Falcon™ 24-well Tissue Culture Plates (BD Biosciences: NSW, Australia) via microscopy (Olympus: model CK-X31). The pool consisted of gametes from 20 individual females.

Upon sperm release, male mussels were collected from the spawning tray, blotted dry, and individually placed (vertically-orientated) into 50 mL plastic containers at ambient room temperature (~16°C). Sperm was collected every half hour and stored at ~4°C. The final pool consisted of sperm from 14 individual males. In order to calculate the volume of pooled sperm required for subsequent fertilisations, sperm counts were performed using a Neubauer counting chamber via microscopy after immobilising the gametes with 10% Lugol's solution. Male and female gametes were distributed among eight fertilisation tanks containing 15 L of FSW incorporating 4 µM EDTA (according to recommended protocols [Buchanan 1999; Gale et al. 2016]) at an approximate density of

3500 oocytes mL^{-1} and 50 sperm egg^{-1}. Successful fertilisation was confirmed via microscopy after 1 h by the presence of polar bodies.

4.2.3 Larval production and sampling

Newly fertilised zygotes were pooled and transferred to a large cylindrical static tank containing 5000 L of continuously aerated FSW. Embryos were reared at 17–18°C to the D-larval stage (2 days post-fertilisation [dpf]), then graded on an 80 µm sieve to retain healthy individuals from dead or undeveloped embryos and trochophores. A portion of these larvae were equally distributed among three 150 L conical flow-through tanks and fed *ad libitum* with a mixed diet of *Chaetocerous calcitrans* and *Isochrisis galbana* (at a 4:1 ratio) for two days. Umbo-stage larvae (4 dpf) were then separated on a mesh screen based on their relative sizes (> or < 120 µm in diameter) to divide poor quality (slow growing) and high quality (fast growing) larvae. Six samples including pooled individuals (about 80,000) from each of the two size fractions and three replicate rearing tanks were transferred to BioStor™ 2.0 mL cryovials (National Scientific Supply Company: CA, USA), snap frozen in liquid nitrogen, and stored at −80°C until metabolite extraction.

4.2.4 Metabolite extraction and derivatisation

Metabolites were extracted using a cold methanol-water method (Villas-Bôas et al. 2011), with modifications for marine invertebrate embryo and larval tissues. The cryovials containing the frozen larvae were placed on dry ice and 1000 µL of a cold (–20°C) 1:1 MeOH:H_2O solution was added (MeOH [Merck: Darmstadt, Germany]; Milli-Q filtered H_2O), followed by 20 µL of internal standard (10 mM L-alanine-2,3,3,3-*d*4 [Sigma-Aldrich: St. Louis, MO, USA]). Samples were partially thawed and vortexed for 1 min, then placed through a series of three freeze-thaw cycles with vortexing in between. Samples were cold-centrifuged (2–4°C) at 20,800*g* for 10 min. Supernatants were transferred to 15 mL Falcon tubes and cold-stored on dry ice. A second extraction was

performed on the leftover pellets by adding 800 µL of cold 4:1 MeOH:H_20, followed by two further freeze-thaw cycles. Samples were centrifuged as previously described and the paired supernatants were combined from both extractions. Remaining biological materials were re-frozen and stored at −80°C for subsequent protein analysis. The total volume of the methanol-water extracts were brought up to 6.5 mL with cold Milli-Q water, vortexed briefly to mix, and then frozen at −80°C. Sample extracts were lyophilised in a 12 L freeze dryer (Labconco Corporation: Kansas city, MO, USA) at −80°C and 0.03 mbar for 24 hrs, then derivatised by alkylation.

Methyl chloroformate (MCF) derivatives were prepared to convert amino and non-amino organic acids into volatile carbamates and esters. Lyophilised samples were re-suspended in 400 µL of 1 M sodium hydroxide (Merck: Darmstadt, Germany) and 68 µL of pyridine (Sigma-Aldrich: St. Louis, MO, USA). Mixtures were transferred to Kimble™ silanized borosilicate glass tubes (12 × 75 mm) (ThermoFisher: Auckland, NZ) containing 334 µL of methanol. 20 µL of MCF reagent (Sigma-Aldrich: St. Louis, MO, USA) were added and samples were vortexed for 30 sec. Another 20 µL of MCF was added, followed by vortexing for 30 sec. To separate the MCF derivatives from the mixture, 400 µL of chloroform (Merck: Darmstadt, Germany) were added, vortexed for 10 sec, then followed by addition of 400 µL of 50 mM sodium bicarbonate (Merck: Darmstadt, Germany) solution and vortexed for a further 10 sec. The upper aqueous layer was discarded and a small amount of anhydrous sodium sulphate (BDH Chemicals: Poole, UK) was added to remove residual H_2O. The chloroform phase containing the MCF derivatives was transferred to 2 mL amber CG glass vials fitted with inserts (Sigma-Aldrich: St. Louis, MO, USA). A sample blank containing 20 µL of L-alanine-2,3,3,3-*d*4 was similarly derivatised for QC purposes, along with a separate standard amino acid mix (100 µl, 20 mM [Merk: Darmstadt, Germany]).

4.2.5 GC-MS analysis

Immediately after derivatisation, the MCF derivatives were injected into a GC-MS system (GC7890 coupled to a MSD5975 [Agilent Technologies], with a quadrupole mass selective detector [EI] operated at 70 eV). The system was equipped with a ZB-1701 GC capillary column (30 m × 250 µm id × 0.15 µm with 5 m stationary phase [86% dimethylpolysiloxane, 14% cyanopropylphenyl]) (Phenomenex: Torrance, CA, USA). The instrumental setup parameters were conducted according to Smart et al. (2010). Samples (1 µL) were injected under pulsed splitless mode with the injector temperature at 260°C. The helium gas flow through the GC-column was set at a constant flow of 1 ml min^{-1}. The GC-oven temperature was initially held at 45°C for 2 min, and then raised with a gradient of 9°C min^{-1} to 180°C; after 5 min the temperature was increased at 40°C min^{-1} to 220°C. After a further 5 min, the temperature was increased at 40°C min^{-1} to 240°C and held for 11.5 min. Finally, the temperature was increased at 40°C min^{-1} until it reached 280°C where it was held for a further 2 min. The interface temperature was set to 250°C and the quadrupole temperature was set at 200°C. The mass spectrometer was operated in scan mode (starting after 6 min; mass range 38–650 a.m.u. at 1.47 scans sec^{-1}). A derivatised sample blank containing the internal standard, a derivatised standard amino acid mix, a non-derivatised standard alkane mix, and a sample of pure chloroform solvent were also injected and analysed for QC purposes.

4.2.6 Data pre-processing and metabolite identification

Deconvolution of raw chromatographic data was performed using the Automated Mass Spectral Deconvolution and Identification System (AMDIS v2.66) software (online software distributed by the National Institute of Standards and Technology, USA—http://www.amdis.net/). Metabolite identifications and peak integrations (relative quantification) were conducted using Chemstation Software (Agilent Technologies) and customised R xcms-based scripts (Aggio et al. 2011) to interrogate an in-house mass

spectral library of MCF derivatised commercial standards. Analyses were carried out in 'R' platform version 2.15.0 (http://www.r-project.org/). Compound identifications were based on matches to both the MS spectrum of the derivatised metabolite and its respective chromatographic retention time. The values are generated from the maximum height of the reference ion for the compound peak. The reference ion used as a measure of abundance for each compound is usually the most abundant fragment, and is not the molecular ion.

A Microsoft® Excel file containing peak height data for each metabolite was generated and manually checked for the presence of contaminants (e.g., MCF derivative artefacts and plasticisers). Aberrant records were removed and the resulting QC-filtered peak intensity values were normalised by the internal standard (D4-alanine) to compensate for potential technical variations (e.g., variable metabolite recoveries). Twenty-nine metabolites from different chemical classes (e.g., amino acids, fatty acids, organic acids, cyclic alcohols, vitamins) were reliably identified. Relative metabolite abundances ($\Sigma peaks_{i-j}/peak_i$) were calculated for each sample. Six metabolites with low abundance values (< 0.1%) were manually filtered and removed as a data integrity precaution, and every possible remaining pair-wise metabolite level ratio (n = 253) was determined. All ratios were log-transformed to ensure independence of the metabolite order in which they were calculated. The dataset matrix was converted to a comma-separated values (.csv) file and subjected to a variety of feature selection methods to identify potential biomarkers for discrimination of larval quality classes.

4.2.7 Statistical analyses

All statistical analyses were performed using Metaboanalyst 2.0, a comprehensive web-based analytical pipeline for high-throughput metabolomics studies (Xia et al. 2013), and ROCCET, a web-based tool for common receiver operating characteristic (ROC) curve

analyses on metabolomics datasets (Xia et al. 2013). Each of the statistical approaches applied here have previously been described in Chapter 3. To visualise feature differences between larval quality classes, the top 50 metabolite ratios (ranked by their *t*-test statistics) were used to perform agglomerative hierarchical cluster analysis (distance = Pearson's correlation; aggregation = Ward's criterion). The constructed dendrogram is co-displayed with the results of heat map analysis to reveal metabolite ratio differences and assist conception of between-class/-feature clusters. Four methods of feature reduction were used independently to minimise selection bias and provide robust criteria for assisting candidate biomarker identification:

1. Volcano plot analysis was performed to simultaneously display fold-change and *t*-test statistics to maximise utilisation of statistical information from the data. Since the aim of this study is to identify low numbers of accurate biomarkers with potential for commercial applicability, the *t*-test significance level and fold-change cut-off thresholds were set at 0.01 and 3, respectively.

2. Supervised projection to latent squares discriminant analysis (PLS-DA) was performed to help identify features which contribute most towards the class separation in the PLS-DA model. Leave-one-out cross-validation (LOOCV) was used to assess the performance of the PLS-DA model. Feature selection was based on the variable importance in projection (VIP) scores (a weighted sum of squares of the PLS loadings taking into account the amount of explained class-variation in each dimension). Features displaying VIP scores > 1.2 were considered as potential biomarkers of larval quality.

3. Significant Analysis of Microarrays/Metabolites (SAM) was performed to supply supportive evidence for the selection of features identified by PLS-DA. A delta value

of 2.0 was selected after analysis of FDR versus the number of significant features as a function of various delta values. An acceptable biomarker selection criteria was considered when the FDR *q*-value < 0.05.

4. Empirical Bayesian Analysis of Microarrays/Metabolites (EBAM) was used as a final independent feature selection method. The default delta value of 0.9 was used and metabolite ratios with FDR *q*-values < 0.05 were considered for further analysis.

To establish the best candidate biomarkers of larval quality, multivariate ROC analyses were performed (PLS-DA algorithm) using the common metabolite ratios identified from the four feature selection methods. ROC analyses which employ Monte-Carlo cross-validation with multiple iterations are generally considered the method of choice for evaluating the performance of potential biomarkers. Using the ROC Explorer module to carry out repeated model testing, the two most robust predictive models were determined which incorporated the minimum number of the most stable features.

4.3 Results

Unsupervised hierarchical clustering (Figure 4.1A) revealed that the metabolite ratio profiles of the samples (shown on the *y*-axis) can be distinguished clearly and robustly. Furthermore, the three biological replicates from each of the two larval quality classes are clearly clustered. Clustering of the features (shown on the *x*-axis) revealed several groups of ratios that were either substantially higher or lower in the poor quality group compared with those in the high quality group.

The results of the hierarchical clustering were confirmed in the PLS-DA model (Figure 4.1B), with the first two components explaining 73.5% and 4.1% of the variation, respectively. The performance of the model was assessed via LOOCV, which showed a good level of predictive ability (Q^2 = 0.80; R^2 = 0.95). The metabolite ratios with the greatest influence on the clustering in the PLS-DA plot were identified by their first component VIP scores, resulting in selection of 47 features.

SAM analysis (Figure 4.1C) identified 25 ratios, with an FDR of 3.2%, that were significantly different between the larval quality classes. Of these features, 20 were lower in the poor quality group and five were higher. Using a variation of the SAM algorithm, EBAM analysis (Figure 4.1D) identified 26 ratios with an FDR of 4.2%, 20 of which were the same as those identified by SAM. Volcano plot analysis (Figure 4.1E) identified 29 ratios that satisfied the stringent criteria placed on feature selection (i.e., ratios having a fold-change of > 3 and simultaneously having a *t*-test statistic of < 0.01). The inclusion of this test removed potentially less biologically relevant features (i.e., those with small between-class differences) that may otherwise have been selected due to very low within-class variation. Of the metabolite ratios independently selected from the four methods, 19 ratios were commonly identified (Figure 4.1F), all of which were lower in the poor quality cohort.

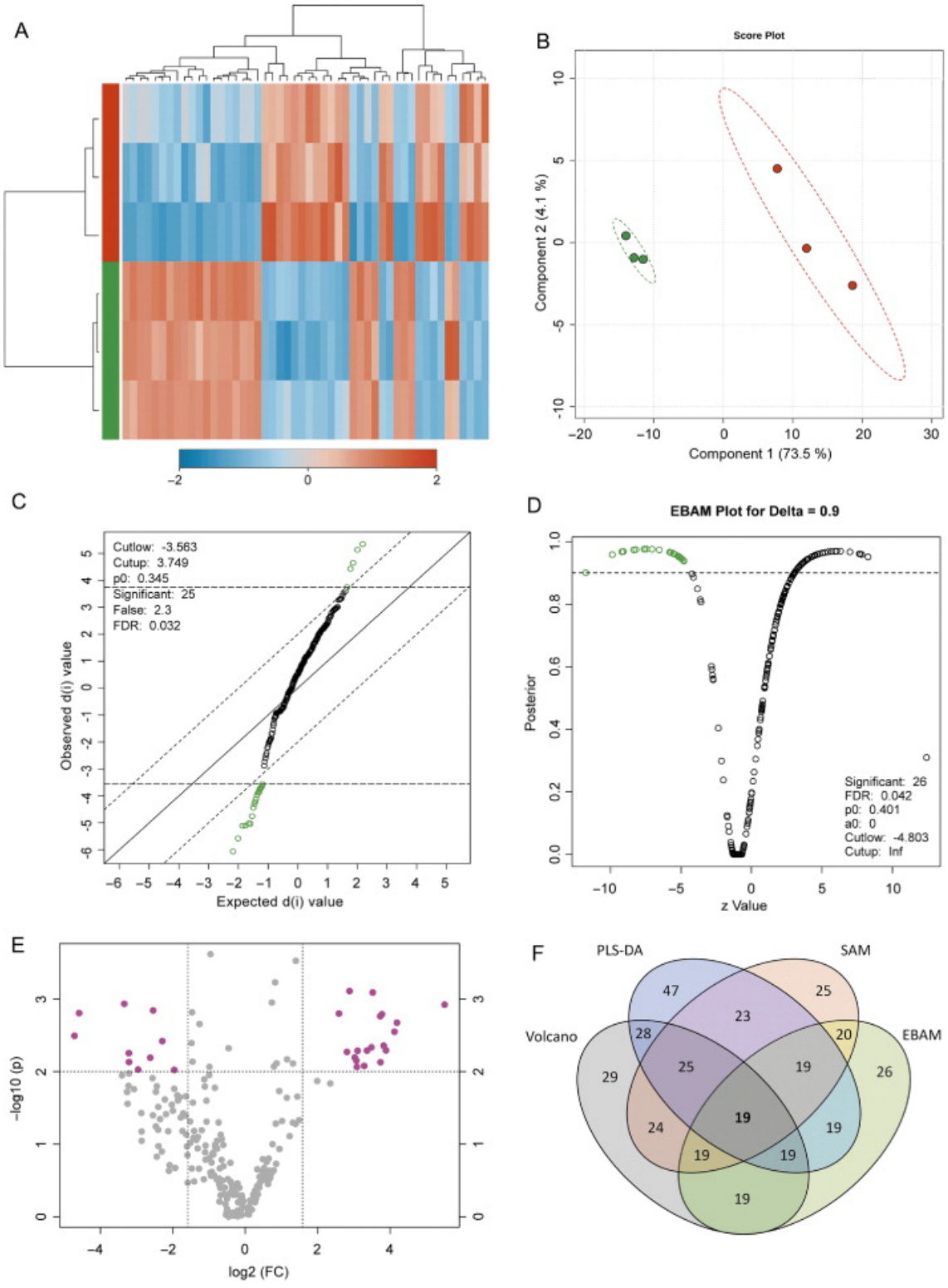

Figure 4.1. Feature reduction analyses for candidate biomarker selection: **A)** Hierarchical clustering and heat map of poor (■) and high (■) quality larvae based on the top 50 features ranked by their T-test statistic (samples on the y-axis, features on the x-axis); **B)** PLS-DA scores plot of poor (●) and high (●) quality larvae; **C)** SAM plot of significantly different features (○) between quality classes (upper right = ratios that were higher in poor quality group; lower left = ratios that were lower); **D)** EBAM plot of significantly different features (○) between quality classes (all lower in the poor quality group); **E)** Volcano plot of features (●) with a between-class fold-change >3 and a *t*-test statistic <0.01 (upper left = ratios that were higher in the poor quality group; upper right = ratios that were lower); and **F)** Venn diagram displays the counts of commonly identified ratios by the four independent feature selection methods.

Multivariate ROC analyses (Table 4.1) of the 19 selected features resulted in identification of four candidate biomarkers. Using balanced subsampling, two thirds of the samples were used to evaluate and rank the important features, which were then used to build classification models. These models were validated on the samples that were left out and the procedure was repeated multiple times to construct a series of most stable n-feature PLS-DA models. Although the proportions that the features were selected for in the iterated models differed (i.e. their rank frequencies), the four ratios identified were similarly incorporated in the two- and three-feature models. Candidate biomarker ratios and associated relative metabolite abundances for each larval quality class are presented in Table 4.2.

Table 4.1. Degree of importance and rank frequency of candidate biomarkers in models for larval class separation.

Candidate biomarker	Importance in models	Two-feature model rank frequency	Three-feature model rank frequency
Pyroglutamate/succinate	1.39	0.68	0.90
Glycine/succinate	1.28	0.54	0.92
Alanine/succinate	1.27	0.38	0.78
Myristic acid/succinate	1.16	0.40	0.40

Table 4.2. Candidate biomarker ratios and associated relative metabolite abundances in high and poor quality larvae.

	Log_2 ratio ± SE			Relative abundance ± SE	
Candidate biomarker	High	Poor	Metabolite	High	Poor
Pyroglutamate/succinate	0.20 (0.15)	−5.58 (0.69)	Pyroglutamate	0.36 (0.01)	0.18 (0.03)
Glycine/succinate	7.37 (0.21)	3.01 (0.58)	Glycine	51.20 (1.03)	31.15 (5.28)
Alanine/succinate	6.08 (0.20)	1.75 (0.63)	Alanine	20.99 (1.37)	13.37 (3.12)
Myristic acid/succinate	1.05 (0.13)	−3.12 (0.71)	Myristic acid	0.65 (0.08)	0.44 (0.16)
			Succinate	0.31 (0.04)	3.27 (1.05)

4.4 Discussion

Of the initial 253 metabolite ratios analysed, we used four independent feature selection methods (Volcano plot, PLS-DA, SAM and EBAM) to identify candidate biomarkers for assessing mussel larval quality. These methods resulted in the selection of between 25 (SAM) to 47 (PLS-DA) potentially important features. The 19 common features selected by the four tests were subjected to ROC-based analyses to reduce the number of final candidate biomarkers and assess their performance. Four metabolite ratios were identified as being good candidates for assessing larval quality: alanine/succinate, glycine/succinate, myristic acid/succinate and pyroglutamate/succinate. The use of ratios can have the advantage of relying on fluctuations of two metabolites rather than one, thereby substantially increasing biomarker sensitivity when these changes occur in opposite trajectories (Budczies et al. 2012). Ratios of reaction-linked metabolites within a biochemical pathway can also help to identify functional changes not immediately apparent by revealing important associations at the enzyme level, which can be very useful in hypothesis-free studies (Petersen et al. 2012). Each of the identified ratios were substantially lower in the poor quality larvae. Comparing this group with the faster-growing cohort, the trend is characterised by elevated levels of succinate and simultaneous reductions in relative metabolite abundances of alanine, glycine, pyroglutamate and myristic acid.

Succinate is a tricarboxylic acid (TCA) cycle intermediate and is common across all candidate biomarkers. Although many of the 19 potential biomarkers initially identified by the four feature selection methods did not contain succinate, incorporation of this metabolite into all ratios within the most stable two- and three-feature PLS-DA models highlights its significance in predicting larval quality. Furthermore, the approximate 10-fold difference in succinate abundance with concomitant $\leq$ 2-fold differences in the other four metabolites suggests that succinate is primarily responsible

for the variation in biomarker ratios between larval quality classes. The accumulation of succinate in the poor quality larvae may indicate overall metabolic repression and lower energy production (Müller et al. 2012). Many marine molluscs have evolved well-adapted strategies for coping with dynamic environmental fluxes and stressful conditions, such as variations in salinity, pH and oxygen availability. For example, the capacity to easily convert between aerobic and anaerobic respiration under duress provides a mechanism for molluscs to produce enough energy to preserve critical metabolic function without wasting energy on other non-critical processes, such as growth. Under aerobic conditions, succinate is readily oxidised to fumarate by succinate dehydrogenase/ respiratory complex II (SDH/CII). Interestingly, the fumarate/succinate ratio was identified as a potential biomarker candidate during our initial statistical screening in all four of the feature selection methods applied. As a classic example of a metabolite ratio with a direct substrate–product link, further integrated analysis of this reaction and its associated pathways at the transcript and/or protein level may provide important mechanistic insight into the physiological basis for intraspecific growth variation in mussel larvae.

Myristic acid is a C:14 fatty acid and is found in high concentrations (13–15% of lipids) in the microalgae diet fed to larvae during the rearing process (Ragg et al. 2010). The mean relative abundances of free myristic acid in the poor quality larvae were lower than in the high quality larvae (0.49% versus 0.65% respectively). This may reflect slight differences in lipid digestive abilities between the two classes. The fact that this discrepancy was small shows that the variation in the myristic/succinate ratios were dominated by large differences in succinate content, providing strong support for the potential importance of the TCA cycle intermediate as a marker of larval quality.

The free amino acid glycine was one of the most abundant metabolites found across all samples, which is consistent with its role as a common osmolyte in marine bivalves (Kube et al. 2006). Our findings reveal that glycine content was significantly

reduced in the poor quality cohort. Recent metabolomic and gene expression studies have demonstrated that glycine content is related to the health state of oysters and clams, with reductions of this amino acid occurring after infection with pathogens (Liu et al. 2013), exposure to arsenic (Ji et al. 2013b) and under hypo-osmotic conditions (Meng et al. 2013). Levels of alanine (also serving osmolytic function) in the poor quality larvae mirrored the lower relative abundance of glycine. These observations may indicate the occurrence of stress-induced disruption in osmotic regulation.

Pyroglutamate is the cyclic lactam of glutamic acid and, despite its existence being known for over a century, its functions remain poorly understood. When protein-bound, the inclusion of a terminal pyroglutamate residue stabilises proteins by making them resistant to degradation by amino peptidases (Kumar & Bachhawat 2012). In its free form as a cellular metabolite, pyroglutamate is an intermediate in glutathionine degradation, can act as an osmoregulator and is also readily interconverted to glutamate, which itself serves various biological roles (Kumar & Bachhawat 2012). For example, glutamate is a molluscan neurotransmitter (Hatakeyama et al. 2010), a common osmolyte in marine bivalves (Kube et al. 2006) and the precursor to proline and alanine (Wang et al. 2012). Thus, variations in pyroglutamate levels between larval quality classes may indicate differential immune function and capacities for protein stabilisation, osmoregulation and cell–cell communication.

4.5 Conclusion

In conclusion, the results of this study demonstrate the potential application of metabolomic-based approaches in aquaculture research to: 1) classify mollusc larvae based on their quality; 2) construct prediction models for larval quality assessment; and 3), identify biochemical pathways which may be under differential regulation to reveal important mechanistic insights for future investigation. Indeed, it is anticipated that

further analysis of the single metabolites and their ratios will reveal additional information and, when integrated with gene and protein expression data, could provide new avenues for selective breeding programmes to consistently yield high quality larvae. Supplementary experiments incorporating metabolomics-based approaches to investigate other measures of larval quality (e.g., health after immunological or toxicological challenges) and in response to different culture conditions and other stresses have the potential to offer industry with a suite of biomarkers for monitoring the physiological state of larvae throughout the rearing process. Such a toolkit could help identify causes of larval batch failures and develop remedial strategies to enhance overall larval production within hatcheries. In the next chapter, metabolomics is applied to characterise potential differences in mussel larval physiology during a hatchery production run, before and after a water exchange (handling stress), and between two larval rearing conditions (flowthrough vs. static tank culture).

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Chapter 5 Mollusc larval quality

Effect of handling and culture conditions

Note: This chapter has been published with the following citation:

Young T., Alfaro A.C., Villas-Bôas S.G. 2016. Metabolic profiling of mussel larvae: Effect of handling and culture conditions. *Aquaculture International*, 24(3): 843–856.

Abstract

Gas chromatography mass spectroscopy was applied to characterise the metabolic profiles of hatchery-reared mussel (*Perna canaliculus*) larvae before and after a prolonged handling and water exchange process, and to investigate the effect of culture conditions. A decrease in succinate and an increase in alanine were observed after the water exchange, which indicated alterations in energy production and osmotic balance. However, these variations were subtle and it is unlikely that the water exchange practice had any lasting negative effects on larval physiology and performance. Multivariate pattern recognition tools (hierarchical clustering, principal component analysis and projection to latent squares discriminant analysis) were used to assess metabolite variations in larvae reared in low-density static and high-density flow through systems and to construct a culture condition classification model. Twelve metabolites contributed most towards the model, which indicated differences in energy, protein and lipid metabolism. The clear group separations were not represented by observable variations in morphological traits. This suggests that growth performance is metabolically buffered through an adaptive physiological mechanism to provide similar developmental characteristics under these conditions.

5.1 Introduction

Increased hatchery culture of marine molluscs is necessary to reduce the industry's dependence on wild populations for stocking farms, to provide a reliable high-quality source of spat for on-growing, and to meet future global requirements for marine-based food protein in an environmentally sustainable manner. Mussel aquaculture represents one of the fastest growing seafood sectors, but current product supplies are unable to meet market demands. Unfortunately, inconsistent sources of juveniles have hindered industry development (Martell et al. 2013; Carrasco et al. 2014). The vast majority of mussel farms around the world are dependent on wild-caught spat either by collecting juveniles attached to seaweed which has been washed onshore, or by deploying spat-catching ropes within the water column which free-swimming larvae settle on. However, accurate predictions of the location, timing, and size of any spat-fall is practically impossible, since a large number of complex and poorly understood environmental variables need to be accounted for (Wilson 2008). Furthermore, the effect of spat-catching on natural population dynamics for many species is unknown. Hatchery production of larvae can provide an alternative supply. While higher production overheads are generally associated with hatchery-reared juveniles compared to wild-caught spat, such expenses can be offset by using ultra-high-density larval rearing practices, seeding ropes with more spat, incorporation of selective breeding programs to achieve higher-value products, and the possibility for growing triploids to enhance meat yields, among others (Kamermans et al. 2013).

Many regions with high mussel production, such as Europe, China and Chile, have not yet invested significantly in the development of mussel hatchery technologies. However, clear global interest is growing among producers and stakeholders (Kamermans et al. 2013; Carrasco et al. 2014). Small-scale hatchery production of larvae for commercial research purposes has recently been conducted with a number of

important advances coming from the west coast USA, Canada, South Australia and New Zealand (SARF 2014). These ventures are providing solid working foundations for the up-scaling of production, and highlight the effectiveness of such facilities. For example, in New Zealand, strong government support and highly successful R&D by two leading aquaculture research organisations (Cawthron Institute and SPATnz; Nelson, New Zealand) has led to the current development of the world's largest mussel hatchery, which is set to provide industry with substantial quantities of Greenshell™ mussel (*Perna canaliculus*) spat (Capson and Guinotte 2014).

As with other bivalves, numerous challenges face the successful larval production of mussels, which include factors associated with broodstock conditioning, gamete quality, larval nutrition, disease (bacterial, viral and parasitic pathogens), water quality, variations in growth, and synchronisation of developmental timing (e.g., settlement and metamorphosis). While many advances have been made over the past decade, such as optimised feeding regimes (Ragg et al. 2010), use of probiotics (Kesarcodi-Watson et al. 2012a,b), and identification of larval settlement inducers (Young et al. 2011, 2015a), high mortality events during larval culture and variable spat production yields are commonly experienced for some species. Undoubtedly, there is still much to learn about the fundamental biology and requirements of marine bivalves during early development, and considerable scope exists to further optimise and fine-tune the larval rearing conditions.

Metabolic profiling, or metabolomics, is a newly emerging approach for studying biological systems. With foundations in other post-genomic fields, such as transcriptomics and proteomics, metabolomics aims to capture and characterise the profile of small metabolites in cells, tissues and organisms (Villas-Bôas et al. 2007). Metabolites reflect the final products of gene expression, but they are also tightly regulated by external environmental influences and thus more closely represent the

phenotype of an organism (Patti et al. 2012). As the molecules of metabolism, differential metabolite profiles can provide important insights into how an organism actually responds metabolically to different situations, such as temperature changes (Dunphy et al. 2015), variations in salinity (Koyama et al. 2015), exposure to poor water quality (Ji et al. 2015), dietary manipulation (Jin et al. 2015), long-term handling stress (Karakach et al. 2009), and super-intensive culture conditions (Schock et al. 2013), among others. Use of metabolomics-based approaches in aquaculture research is starting to gain tract (reviewed in Chapters 2 & 3). However, thus far, only one other early life-stage investigation during hatchery culture of shellfish larvae has been performed (Young et al. 2015b).

In Chapter 4, metabolic profiling was used to identify a suit of candidate biomarkers for assessing mussel (*P. canaliculus*) larval quality, based on growth variations (Young et al. 2015b). Results demonstrated that the quality of larvae could be modelled using multivariate pattern recognition tools, and provided good evidence that visually different phenotypes within a larval cohort are represented by the composition of the identified metabolites. In the current study, metabolic profiling is used to investigate whether physiological larval traits, which are not morphologically observable, can be distinguished as a result of short-term handling stress during prolonged water exchanges, and when organisms are reared under different culture conditions. The development and incorporation of new methods to assess and monitor the physiological condition of larvae, which cannot be measured with currently-used microscopic techniques would be highly valuable for the developing mussel industry.

5.2 Methods

5.2.1 Larval rearing

D-stage larvae were reared to 10 days post-fertilisation (dpf) (veliger stage) under two culture conditions (low density static vs. high density flow through) using standard industry protocols developed by Spat Production & Technology New Zealand Ltd (SPATnz). Briefly, *Perna canaliculus* zygotes were produced at the Cawthron Aquaculture Park (CAP; Nelson, New Zealand) as described in Chapter 4, but using different F2 broodstock individuals. Zygotes were pooled and equally distributed between two large cylindrical static tanks containing 5000 L of continuously aerated 1 µm filtered seawater (FSW). After 2 days incubation at 17–18°C, embryos had developed into D-stage larvae and the first water exchange was performed. Organisms were filtered through an 80 µm mesh screen and the bulk of them were placed back into clean 5000 L tanks containing fresh FSW. A portion of larvae ($n \approx 60$ million) were removed from the static culture system and equally distributed among three 150 L flow through conical tanks containing 100 L of continuously aerated FSW. Larvae were fed *ad libitum* (routinely checked via fluorimeter) with a mixed diet of *Chaetocerous calcitrans* and *Isochrisis galbana* and reared for a further 8 days under the two different culture regimes. Larval rearing conditions are summarised in Table 5.1.

5.2.2 Larval sampling

To test the effects of handling stress, pooled samples of larvae ($n \approx 100{,}000$ individuals) were taken from each of the 5000 L tanks at 10 dpf, before and after a 100 % water exchange. Draining of the tanks took approximately one and a half hours, during which time larvae were collected on mesh screens positioned at the outflows. Once finished, larvae were placed back into tanks containing fresh FSW and left to equilibrate for 1 h before being re-sampled.

Table 5.1. Larval rearing parameters used during low-density static and high-density flow through culture

Parameter	Incubation	Static system	Flowthrough system
Water volume	5000 L	5000 L	100 L
Density	60 embryos mL^{-1}	20 larvae mL^{-1}	200 larvae mL^{-1}
Temperature	17 ± 0.5°C	18 ± 0.5°C	18 ± 0.5°C
Continuous flowrate	N/A	N/A	1000 mL min^{-1}
100% water exchange	N/A	Every two days	Every two days
Water exchange duration		90 min	10 min
Diet	N/A	*C. calcitrans* & *I. galbana* (4:1 ratio), batch fed daily with an increasing number of cells per larva to provide ad libitum feeding (2,000–10,000 cells.larva.day^{-1})	*C. calcitrans* & *I. galbana* (4:1 ratio), continuously introduced *via* pump to provide organisms with the same daily dietary ration as in the static system

To sample the larvae, 10 L of water were taken from the top of the tanks and concentrated on an 80 μm mesh screen. Organisms were transferred via pipette into 2 mL cryovials, excess water was removed, and then samples were immediately snap frozen in liquid nitrogen and transferred to a −80°C freezer until metabolite extraction. To test the effect of culture conditions, larval samples were also taken from each of the flow through tanks at 10 dpf. A water volume of 1250 mL was filtered through a mesh screen to provide equal numbers of larvae as in the samples obtained from the static system, and similarly processed. A vital stain, neutral red, was used to confirm that only live organisms were included in the larval samples. Following the protocol by Young et al. (2015a), ten replicate subsamples (each containing approximately 20 organisms) from every tank were assessed.

5.2.3 Metabolomics

Metabolite extractions, derivatisations, GC-MS analysis, data pre-processing and metabolite identifications were conducted as previously described in Chapter 4. Twenty seven metabolites from different chemical classes were reliably identified by comparing retention times and *m/z* ratios against an in-house library of spectra. Metabolite peak intensities were normalised against the internal standard to compensate for potential technical variations (e.g., variable metabolite recoveries), adjusted for biomass and the relative metabolite abundances were calculated for each sample. The dataset matrix was converted to a .csv file and the data were standardised via autoscaling before being subjected to a variety of feature selection methods to assess group differences.

5.2.4 Statistical analyses

SPSS v.22.0 software (SPSS, Chicago, IL, USA) and MetaboAnalyst v.3.0 (Xia et al. 2015) were used to analyse data. Each of the statistical approaches applied here have previously been described in Chapter 3. To investigate the effect of handling stress on the metabolite pool, paired *t*-test statistics and fold-change (FC) values were calculated, where: FC = $\overline{x}$ $\log_2$ post-handled/pre-handled (unstandardised data). To examine whether the culture conditions had an influence on larval metabolic profiles, unpaired *t*-test statistics, FC values, multivariate clustering, and multivariate projection-based techniques were employed.

Agglomerative Hierarchical Cluster Analysis (HCA) was initially used as an unbiased method to identify inherent sample groupings, based on the underlying structure of the data. Pearson's correlation and Ward's criterion were selected as the measure of distance and aggregation, respectively. Unsupervised principal components analysis (PCA) was performed to reduce dimensionality, assess the between-sample variation, and investigate natural sample groupings. Supervised projection to latent structures

discriminant analysis (PLS-DA) was performed to identify metabolites which contributed most towards partitioning of samples between the culture conditions.

PLS-DA uses a multivariate regression technique to extract via linear combination of original variables the information that can predict the sample class membership. Leave-one-out cross validation (LOOC) was used to assess the performance of the PLS-DA model. Metabolites contributing most towards the classification model were identified based on the variable influence on projection (VIP) scores (a weighted sum of squares of the PLS loadings taking into account the amount of explained class-variation in each dimension). To visualise differential expressions of metabolites in larvae from the static and flow through systems, the top 12 metabolites (ranked by their *t*-test statistics) were used to perform HCA (distance = Pearson's correlation; aggregation = Ward's criterion). The constructed dendrogram is co-displayed with the results of heat map analysis to reveal differences in metabolite abundances and assist conception of between-class and between-metabolite clusters.

5.3 Results

5.3.1 Effect of handling and water exchange

The effect of handling and water exchange on the metabolic profile of larvae after 1 h re-acclimatisation is displayed in Table 5.2. The majority of detected metabolites before and after the process did not vary significantly. However, succinate decreased (FC = –1.93), whereas alanine showed a slight increase (FC = 0.29) (paired *t*-tests; $p < 0.05$) (Figure 5.1).

Table 5.2. Metabolic profiles of larvae before (pre-handled) and after (post-handled) a prolonged handling and water exchange process. Positive foldchange (FC) values represent mean metabolite abundances which were higher in the post-handled samples and negative FC values represent those that were lower

Metabolite	Mean relative abundance (± SD)		Log_2 FC	T-test *p*-value
	Pre-handled	Post-handled		
Alanine	$1.60^{E+01} \pm 5.24^{E-02}$	$1.95^{E+01} \pm 4.02^{E-03}$	0.29	0.007
Aspartic acid	$2.07^{E-02} \pm 2.93^{E-02}$	$8.55^{E-02} \pm 1.85^{E-02}$	1.54	0.260
Benzoic acid	$4.29^{E-02} \pm 2.48^{E-03}$	$2.55^{E-02} \pm 2.14^{E-02}$	-1.06	0.417
Decanoic acid	$1.30^{E-01} \pm 2.87^{E-02}$	$6.41^{E-02} \pm 3.05^{E-02}$	-1.09	0.361
Fumarate	$1.06^{E+01} \pm 3.58^{E+00}$	$9.84^{E+00} \pm 1.05^{E+00}$	-0.07	0.854
Glycine	$6.25^{E+01} \pm 3.61^{E+00}$	$6.33^{E+01} \pm 2.11^{E+00}$	0.02	0.884
Heptadecanoic acid	$2.71^{E-01} \pm 9.62^{E-02}$	$1.89^{E-01} \pm 1.53^{E-02}$	-0.48	0.487
Hexanoic acid	$1.68^{E-01} \pm 1.21^{E-01}$	$1.79^{E-01} \pm 1.69^{E-01}$	-0.13	0.969
Isoleucine	$5.53^{E-02} \pm 2.15^{E-02}$	$6.76^{E-02} \pm 1.40^{E-02}$	0.33	0.261
Itaconic acid	$3.74^{E-02} \pm 1.67^{E-02}$	$2.79^{E-02} \pm 2.85^{E-03}$	-0.35	0.509
Lactate	$4.72^{E-01} \pm 1.19^{E-01}$	$2.44^{E-01} \pm 6.56^{E-02}$	-0.96	0.331
Leucine	$9.45^{E-02} \pm 4.29^{E-03}$	$1.85^{E-01} \pm 5.37^{E-02}$	0.94	0.234
L-Norvaline	$1.01^{E+00} \pm 2.53^{E-01}$	$2.02^{E+00} \pm 4.95^{E-01}$	1.00	0.307
Myristic acid	$1.27^{E-01} \pm 5.23^{E-02}$	$1.22^{E-01} \pm 1.45^{E-02}$	0.00	0.874
Nicotinic acid	$8.91^{E-02} \pm 1.47^{E-02}$	$9.04^{E-02} \pm 8.47^{E-03}$	0.03	0.950
Octadecanoic acid	$8.38^{E-01} \pm 4.89^{E-01}$	$5.62^{E-01} \pm 1.69^{E-01}$	-0.48	0.659
Oleic acid	$8.22^{E-02} \pm 2.95^{E-02}$	$6.46^{E-02} \pm 6.11^{E-03}$	-0.30	0.481
Oxalic acid	$3.91^{E-01} \pm 1.91^{E-01}$	$2.66^{E-01} \pm 6.86^{E-02}$	-0.49	0.620
Palmitelaidic acid	$3.74^{E-02} \pm 7.38^{E-03}$	$4.69^{E-02} \pm 4.89^{E-03}$	0.34	0.116
Palmitic acid	$5.32^{E-01} \pm 1.23^{E-01}$	$4.12^{E-01} \pm 5.21^{E-02}$	-0.35	0.512
Palmitoleic acid	$3.83^{E-02} \pm 6.67^{E-03}$	$6.23^{E-02} \pm 7.11^{E-03}$	0.71	0.246
Pentadecanoic acid	$2.49^{E-02} \pm 1.64^{E-02}$	$1.33^{E-02} \pm 4.96E^{-03}$	-0.77	0.586
Phenylalanine	$2.33^{E-02} \pm 1.82^{E-03}$	$3.82^{E-02} \pm 1.10^{E-02}$	0.69	0.259
Proline	$3.30^{E-01} \pm 1.07^{E-01}$	$4.82^{E-01} \pm 1.68^{E-03}$	0.59	0.291
p-Toluic acid	$3.80^{E-01} \pm 2.15^{E-01}$	$2.72^{E-01} \pm 1.18^{E-01}$	-0.43	0.724
Pyroglutamate	$2.21^{E-01} \pm 2.13^{E-02}$	$2.81^{E-01} \pm 9.66^{E-03}$	0.35	0.221
Succinate	$2.74^{E+00} \pm 5.59^{E-01}$	$7.89^{E-01} \pm 4.83^{E-01}$	-1.93	0.018

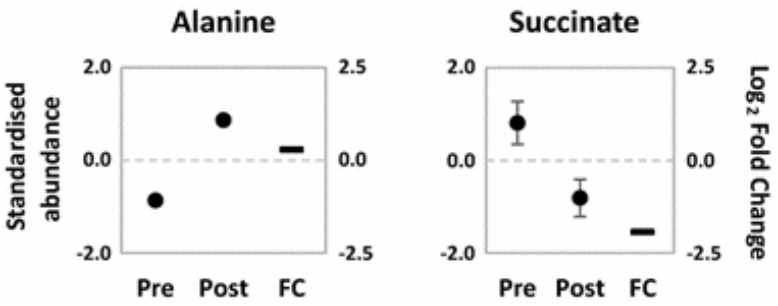

Figure 5.1. Metabolites that were identified as being statistically different (*t*-test; $p < 0.05$) before (pre) and after (post) a prolonged handling and water exchange process (mean ± SD). The primary *y*-axis represents a standardised (autoscaled) data measure which places abundances of different metabolites on the same scale since absolute variations in abundance are not necessarily proportional to the biological relevance of metabolites. The secondary *y*-axis represents foldchange (FC) values which indicates the relative magnitude of difference between the sample groups in the unstandardised data

Table 5.3. Metabolic profiles of larvae reared to ten days post-fertilisation in low-density static and high-density flow through systems. Positive foldchange (FC) values represent mean metabolite abundances which were higher in the flowthrough system samples and negative FC values represent those that were lower.

Metabolite	Mean relative abundance (± SD)		Log_2 FC	T-test *p*-value
	Static	Flowthrough		
Alanine	$1.60^{E+01} \pm 5.24^{E-02}$	$1.10^{E+01} \pm 6.72^{E-01}$	-0.69	0.004
Aspartic acid	$2.07^{E-02} \pm 2.93^{E-02}$	$2.93^{E-01} \pm 6.78^{E-02}$	2.33	0.014
Benzoic acid	$4.29^{E-02} \pm 2.48^{E-03}$	$1.11^{E-02} \pm 1.02^{E-02}$	-1.62	0.068
Decanoic acid	$1.30^{E-01} \pm 2.87^{E-02}$	$5.89^{E-02} \pm 3.56^{E-02}$	-0.72	0.267
Fumarate	$1.06^{E+01} \pm 3.58^{E+00}$	$3.26^{E+00} \pm 2.84^{E+00}$	-1.65	0.028
Glycine	$6.25^{E+01} \pm 3.61^{E+00}$	$8.16^{E+01} \pm 3.19^{E+00}$	0.38	0.002
Heptadecanoic acid	$2.71^{E-01} \pm 9.62^{E-02}$	$1.24^{E-01} \pm 4.35^{E-03}$	-0.89	0.064
Hexanoic acid	$1.68^{E-01} \pm 1.21^{E-01}$	$4.83^{E-02} \pm 3.54^{E-02}$	-1.84	0.126
Isoleucine	$5.53^{E-02} \pm 2.15^{E-02}$	$2.40^{E-02} \pm 9.41^{E-03}$	-1.36	0.014
Itaconic acid	$3.74^{E-02} \pm 1.67^{E-02}$	$1.92^{E-02} \pm 4.20^{E-03}$	-0.77	0.092
Lactate	$4.72^{E-01} \pm 1.19^{E-01}$	$1.71^{E-01} \pm 1.89^{E-02}$	-1.06	0.091
Leucine	$9.45^{E-02} \pm 4.29^{E-03}$	$4.14^{E-02} \pm 3.14^{E-02}$	-1.76	0.042
L-Norvaline	$1.01^{E+00} \pm 2.53^{E-01}$	$7.30^{E-01} \pm 8.91^{E-02}$	-1.05	0.099
Myristic acid	$1.27^{E-01} \pm 5.23^{E-02}$	$1.02^{E-01} \pm 1.09^{E-02}$	-0.29	0.253
Nicotinic acid	$8.91^{E-02} \pm 1.47^{E-02}$	$1.05^{E-01} \pm 2.35^{E-02}$	0.23	0.381
Octadecanoic acid	$8.38^{E-01} \pm 4.89^{E-01}$	$3.36^{E-01} \pm 6.15^{E-02}$	-1.06	0.119
Oleic acid	$8.22^{E-02} \pm 2.95^{E-02}$	$6.51^{E-02} \pm 1.57^{E-02}$	-0.17	0.564
Oxalic acid	$3.91^{E-01} \pm 1.91^{E-01}$	$1.80^{E-01} \pm 1.39^{E-01}$	-0.86	0.227
Palmitelaidic acid	$3.74^{E-02} \pm 7.38^{E-03}$	$4.24^{E-02} \pm 9.81^{E-03}$	0.01	0.979
Palmitic acid	$5.32^{E-01} \pm 1.23^{E-01}$	$2.92^{E-01} \pm 3.35^{E-02}$	-0.69	0.034
Palmitoleic acid	$3.83^{E-02} \pm 6.67^{E-03}$	$5.71^{E-02} \pm 1.05^{E-02}$	0.18	0.514
Pentadecanoic acid	$2.49^{E-02} \pm 1.64^{E-02}$	$3.03^{E-02} \pm 1.02^{E-02}$	0.66	0.244
Phenylalanine	$2.33^{E-02} \pm 1.82^{E-03}$	$2.42^{E-02} \pm 3.06^{E-03}$	-0.35	0.317
Proline	$3.30^{E-01} \pm 1.07^{E-01}$	$1.06^{E-01} \pm 3.82^{E-02}$	-1.93	0.007
p-Toluic acid	$3.80^{E-01} \pm 2.15^{E-01}$	$1.45^{E-01} \pm 6.91^{E-02}$	-1.17	0.101
Pyroglutamate	$2.21^{E-01} \pm 2.13^{E-02}$	$3.47^{E-01} \pm 3.30^{E-02}$	0.47	0.017
Succinate	$2.74^{E+00} \pm 5.59^{E-01}$	$3.10^{E-01} \pm 3.47^{E-01}$	-2.51	0.090

5.3.2 Effect of culture system

The effect of culture system on assigned metabolites is displayed in Table 5.3. Univariate analysis identified nine metabolites with significantly altered (unpaired *t* test; $p < 0.05$) expression patterns (Figure 5.2).

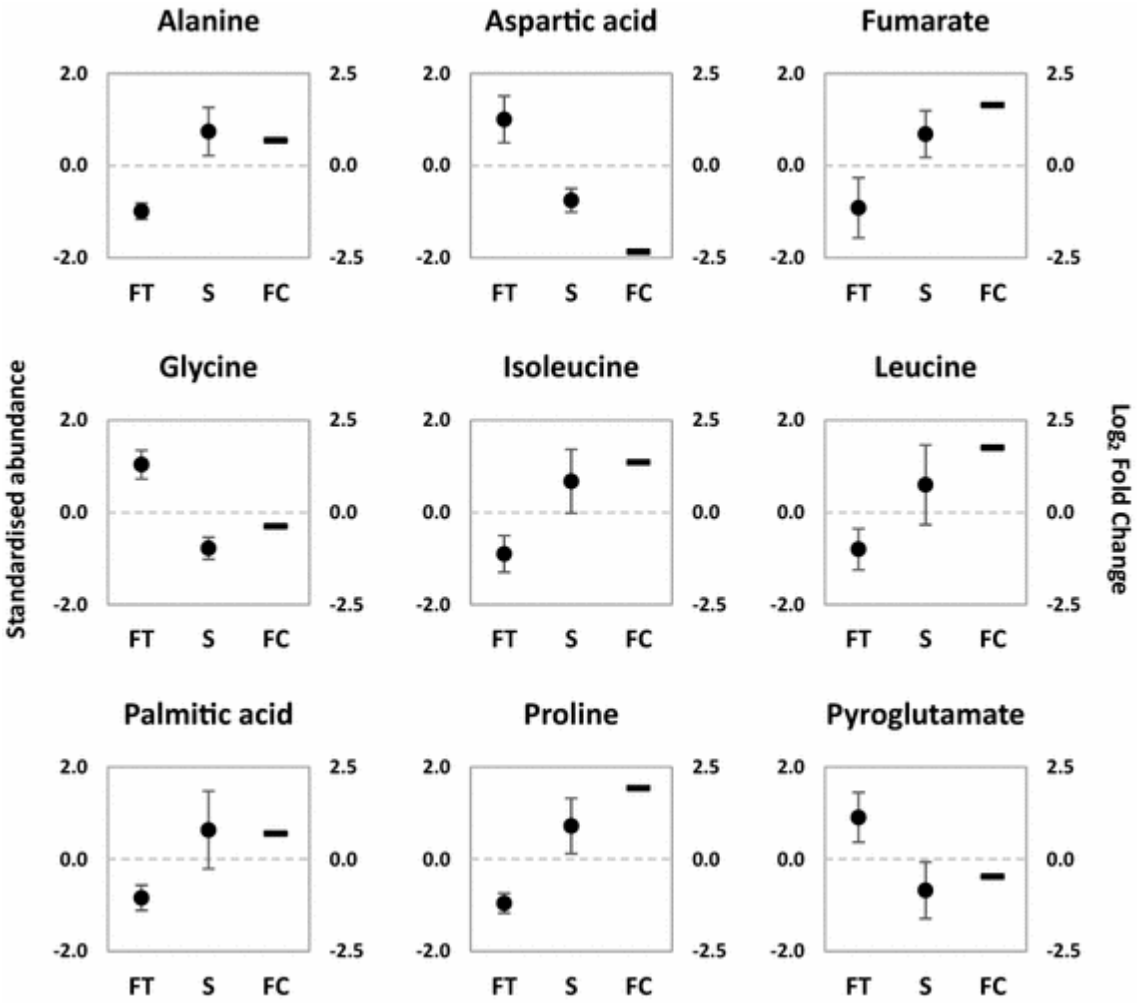

Figure 5.2. Metabolites that were identified as being statistically different (*t*-test; $p < 0.05$) between the static (S) and flow through (FT) system samples (mean ± SD). The primary *y*-axis represents a standardised (autoscaled) data measure which places abundances of different metabolites on the same scale since absolute variations in abundance are not necessarily proportional to the biological relevance of metabolites. The secondary *y*-axis represents foldchange (FC) values which indicates the relative magnitude of difference between the sample groups in the unstandardised data.

HCA analysis of all metabolites showed the intrinsic clustering of samples and revealed two main groupings, which could be attributed to the flow through and static culture systems (Figure 5.3A). In addition, there was no clear clustering of the static samples before and after the water exchange. PCA analysis revealed that the first two components explained 76.7 % of the variability in the data (Figure 5.3B). Furthermore, samples clustered distinctly into their respective treatments with non-overlapping 95 % confidence intervals, which indicated that a good level of class discrimination was possible. The scores along PC1 were primarily responsible for this split and the PC1 loadings (Figure 5.3C) revealed that variations in numerous metabolites contributed towards the separation. Since an effect of handling and water exchange was not apparent using the unsupervised HCA and PCA methods, these four samples were subsequently re-classified as 'static system samples' for the remaining multivariate analyses.

PLS-DA analysis was performed to identify and quantitatively rank the main metabolites accountable for the between-class variation. The 3D score plot revealed that the first three latent variables explained 86.7 % of the variance between the culture systems (Figure 5.3D). The performance of the model was evaluated using the first two latent variables and was assessed via LOOCV, which showed a good level of predictive ability ($Q^2 = 0.84$; $R^2 = 0.98$). Twelve metabolites with VIP scores >1.0 were considered as main features, which contributed towards the classification model (Figure 5.3E).

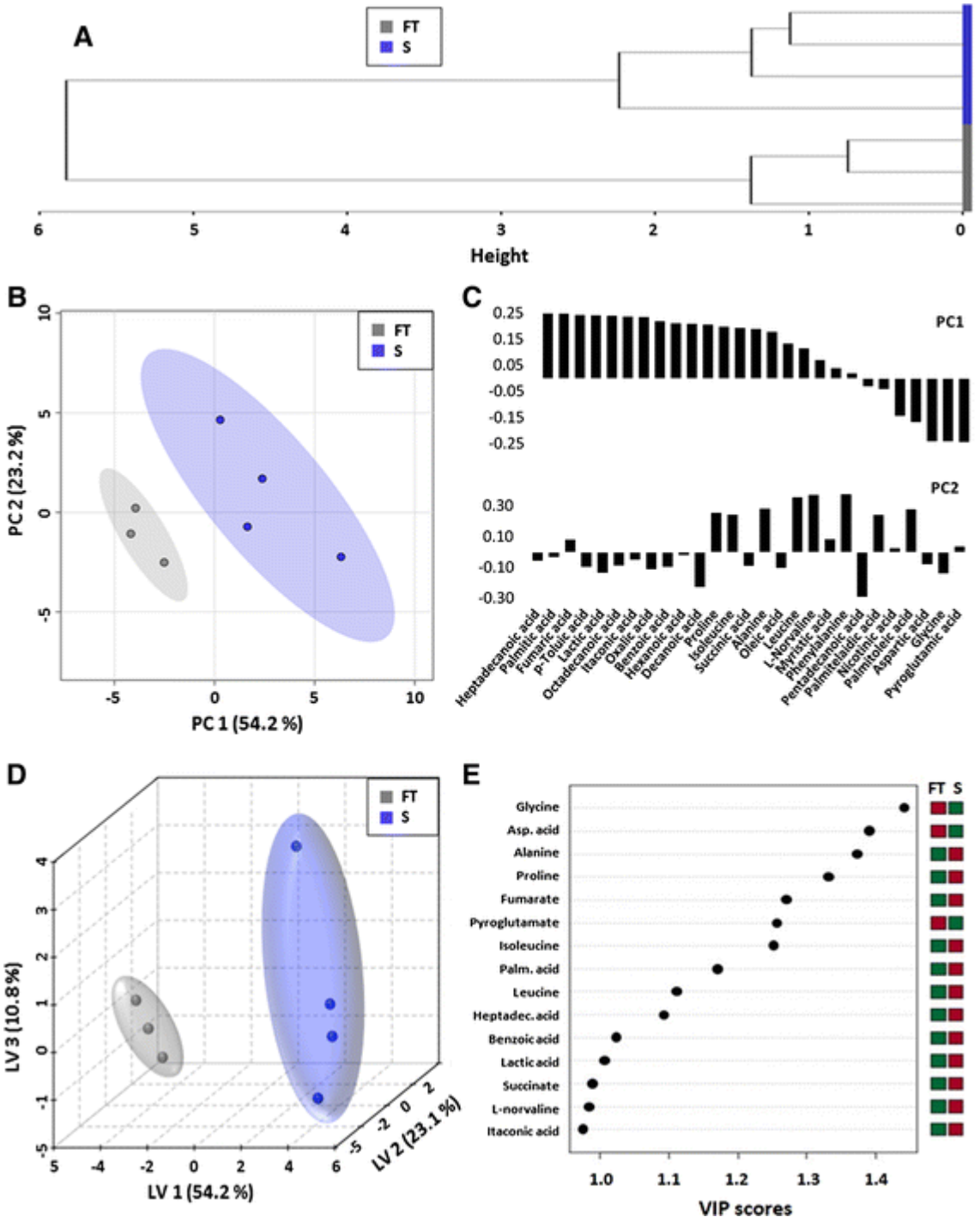

Figure 5.3. Multivariate pattern recognition of metabolic profiles in larvae reared under low-density static (S) and high-density flow through (FT) culture systems: **A)** hierarchical cluster analysis of all metabolite data; **B)** principal component analysis (PCA) 2D score plot of all metabolite data (shaded ellipses represent 95% confidence intervals); **C)** bar charts of PCA loadings for PC1 and PC2; **D)** projection to latent structures discriminant analysis (PLS-DA) 3D score plot of all metabolite data (shaded ellipsoids are arbitrarily overlaid to enhance visualisation of between-group density clusters); **E)** PLS-DA variable importance in projection (VIP) plot of metabolites which contribute most (VIP > 1.0) towards the PLS-DA classification model. The boxes on the right of the VIP plot represents relative expression of metabolites in the static and flow through systems, where red = high values, and green = lower values.

HCA combined with heatmap analysis was performed to assist visualisation of the twelve metabolites abundances between the culture system classes (Figure 5.4). Cluster analysis of the metabolites identified two distinct groups with different expression profiles. Three metabolites generally had higher relative abundances in the flow through system, whereas nine metabolites were lower when compared to the static system.

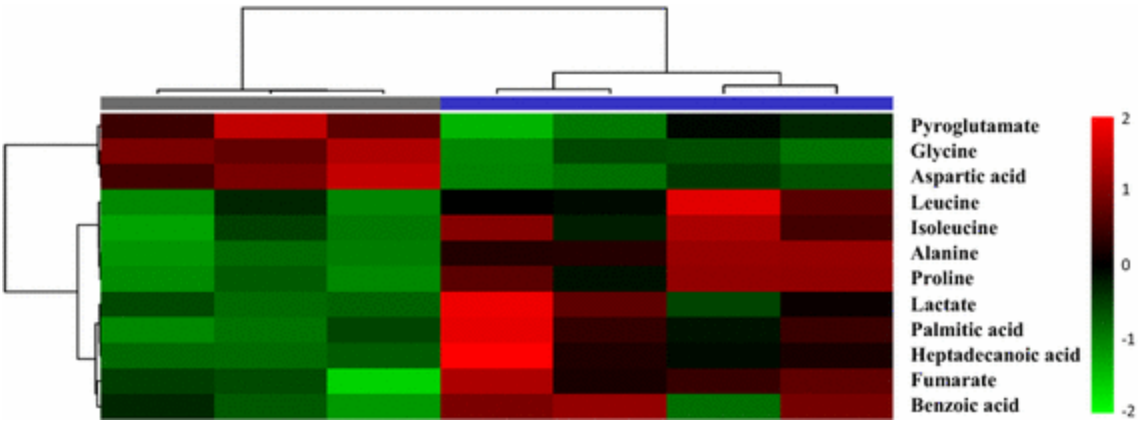

Figure 5.4. Two-way hierarchical cluster analysis combined with a heatmap to assist visualisation of between-sample and between-metabolite variations. Columns represent samples (grey = flow through, blue = static) and rows represent metabolites. The red/green colour scale represents standardised (autoscaled) abundance data, where red = higher values, and green = lower values.

5.4 Discussion

The effect of handling and water exchange on the metabolic profile of larvae resulted in alterations of succinate and alanine. Succinate is a tricarboxylic acid cycle (TCA) intermediate and is capable of donating electrons to the electron transport chain via oxidation to fumarate by succinate dehydrogenase/ respiratory complex II (SDH/CII). Decreased expression of SDH/CII and increased levels of succinate are indicative of oxidative stress in shellfish when oxygen availability is limited (Tuffnail et al. 2009; Anestis et al. 2010; Connor and Gracey 2012), and when organisms are exposed to toxins (Wu et al. 2013a; Hanana et al. 2014) or pathogens (Ji et al. 2013; Wu et al. 2013b). Elevated tissue concentrations of succinate are also indicative of poor larval quality due to lower capacity for energy production (Young et al. 2015b). Compared to the before-handled baseline level, our findings show that succinate levels were lower after the larvae were handled, which suggests increased TCA cycle activity and lack of negative impact on oxidative phosphorylation.

Alanine is a common osmolyte in marine molluscs (Deaton 2009), and the small increase in this metabolite may indicate a minor shift in osmotic balance after the larvae were screened, exposed to air and placed in fresh seawater. As osmoconformers, shellfish typically accumulate and reduce levels of intracellular osmolytes to maintain cell volume and homeostasis when exposed to higher and lower salinities, respectively. However, concentrations of other principal osmolytes, such as glycine and proline, did not vary after the water exchange. Under substantial osmotic imbalance, concomitant increases or decreases in these metabolites would also be expected (Carregosa et al. 2014; Koyama et al. 2015). These results suggest that the water exchange process has negligible lasting impacts on larval physiology and performance. In addition, subsequent multivariate analyses (HCA and PCA) of all data were supportive of a limited handling effect, since there was no clear treatment-based structure in the samples from the static system.

Conversely, the effect of culture conditions (static vs. flow through) had a more pronounced influence on the metabolic profile of larvae. After 10 days of rearing, mean levels of fumarate and succinate were both lower in the flow through system, which indicates subtle differences in central carbon metabolism, aerobic respiration, and energy production through variations in larval activity and/or nutrient assimilation and conversion. Stocking density is known to affect larval bioenergetics due to changes in animal behaviour (Liu et al. 2006), and may partially explain the variation in TCA cycle intermediates between the different rearing conditions. Free amino acids (FAAs) and free fatty acids (FFAs) were responsible for the majority of other dissimilarities between the flow through and static systems. While FAAs serve osmoregulatory functions in molluscs, they also provide the pool of subunits required for peptide and protein biosynthesis. The FFA profile represents the breakdown products of digested dietary lipids and the available building blocks from which newly required lipids can be synthesised. Thus, these variations are indicative of differences in protein and lipid metabolism. Interestingly, growth performance was similar between the static and flow through systems after 10 days of culture. If protein and lipid metabolism were differentially regulated between the two systems, the energetics involved in 'scope for growth' must have been metabolically buffered through an adaptive mechanism. The mechanisms and extent to which mussel larvae can buffer particular metabolic constraints without compromising their developmental timing and growth is unknown, but such knowledge would be highly valuable for the aquaculture industry.

Other metabolites that contributed towards the PLS-DA classification model included benzoic acid and lactate, which, while not statistically different, were both slightly higher in the static system. Fluctuations in endogenous benzoic acid are known to occur in aquatic organisms when exposed to particular pollutants. For example, Spann et al. (2011) recently used a metabolomics-based approach to investigate the effect of

sediment contamination on clams (*Corbicula fluminea*) and found that levels of benzoic acid increased in response to low-level heavy metal exposure. In another metabolic profiling study, Ralston-Hooper et al. (2011) also found similar responses in amphipods (*Hyalella azteca*) exposed to the widely used agricultural herbicide atrazine. We did not measure potential contaminants in the seawater used in the current study. However, it is possible that the water in the static and flow through systems had different chemical compositions at certain intervals during the culture period since holding ponds are re-filled daily during high tide for continuous flow through use, and 100 % water exchanges in the static system were performed every other day. Lactate in molluscs is an end product of anaerobic respiration and high levels are indicative of respiratory stress (Liu et al. 2014). On the other hand, low levels of lactate in molluscs are indicative of food limitation due to conversion to glucose during gluconeogenesis (Roznere et al. 2014). Although there is not yet have a reference value for lactate production in mussel larvae, the variability within and between each culture system suggests reasonably similar performance in terms of oxygen and food availability, which is in accordance with the employed parameters.

Metabolite profiling was used in Chapter 4 to identify four candidate biomarkers for assessing the quality of hatchery-reared larvae based on intra-cohort variations in growth. The biomarkers involve metabolite ratios of succinate to alanine, glycine, myristic acid and pyroglutamate. In the current study, analysis of these values showed no significant differences between larvae from the flow through and static systems (Table 5.4). This finding agrees with the observed similarities in shell length and further substantiates the use of these biomarkers for evaluating proxies for larval quality. The fact that metabolic profiling can be used to successfully detect variable physiological characteristics in hatchery-reared larvae which have both observable and non-observable

morphological traits highlights the unique value that metabolomics-based approaches can deliver.

Table 5.4. Statistical analyses of metabolite ratio biomarkers previously identified in Chapter 4 for assessing intra-cohort growth variation as a proxy for larval quality in larvae reared under low-density static and high-density flow through culture systems.

Candidate biomarker	Log_2 Ratio ± SD		T-test p-value
	Flowthrough	Static	
Pyroglutamate/Succinate	1.42 ± 2.82	-2.48 ± 1.44	0.123
Glycine/Succinate	9.30 ± 2.95	5.50 ± 1.27	0.143
Alanine/Succinate	6.41 ± 2.88	3.67 ± 1.40	0.235
Myristic acid/Succinate	-0.35 ± 3.03	-3.52 ± 1.21	0.205

5.5 Conclusion

In conclusion, the current results demonstrate that the highly sensitive and dynamic larval metabolome can be used to construct classification models to identify the culture conditions under which the organisms were reared, and assist novel hypothesis generation for future studies. In addition, these results also support the continued use of high-density culture practices and indicate that short-term restrictions on the duration of screening are unnecessary. The combination of metabolic profiling with multivariate pattern recognition techniques is a powerful approach, which will undoubtedly be applied more widely in the future of aquaculture-related research to assist optimisation and fine-scale tuning of larviculture parameters. With demonstrated applications of metabolomics in marine mollusc development and aquaculture research (Chapters 4 & 5), the following case study (Chapter 6) attempts to expand on these applications to investigate aspects of larval health and immunology using a different model organism, the Pacific oyster (*Crassostrea gigas*), in response to viral infections.

5.6 References

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Host-virus interactions in oyster larvae: Metabolic responses to OsHV-1 µVar

Note: A modified version of this chapter has published with the following citation:

Young T., Kesarcodi-Watson A., Alfaro A.C., Merien F., Nguyen T.V., Mae H., Le D.V. & Villas-Bôas S.G. 2017. Differential expression of novel metabolic and immunological biomarkers in oysters challenged with a virulent strain of OsHV-1. *Comparative Development & Immunology*, 73: 229–245.

Abstract

The Pacific oyster, *Crassostrea gigas*, is highly susceptible to infection by OsHV-1 µVar. A number of recent virus outbreaks are thought to be responsible for increased incidences of mass mortalities around the globe, resulting in significant losses for the oyster aquaculture sector. Early life-stages are particularly vulnerable to the virus, but little information exists regarding metabolic or pathophysiological responses of larval hosts. Using a metabolomics approach and a variety of statistical and bioinformatics techniques, a range of metabolic and immunological responses in oyster larvae exposed to OsHV-1 µVar were identified; some of which have not previously been reported in molluscs, nor associated with mechanisms of innate immunity. These responses included a coordinated disruption of the TCA cycle in accordance with mammalian macrophage stimulation via activation of immunoresponsive gene 1 and production of itaconic acid, induction of a Warburg-like effect, and production of free fatty acids for virion assembly, among others. Unsupervised and supervised multivariate analyses of entire metabolite profiles were clearly able to separate infected from non-infected control larvae. Correlation analysis revealed the presence of major perturbations in the underlying biochemical networks in exposed organisms. In addition, secondary pathway analysis of functionally-related metabolites identified a number of prospective pathways differentially regulated in virus-exposed larvae, including alterations to cysteine–methionine transulphuration, glycolysis/gluconeogenesis, nitrogen metabolism, and lipid metabolism. These initial results provide new insights into the pathogenic mechanisms of OsHV-1 infection in oyster larvae, which may be applied to develop disease mitigation strategies and/or as new phenotypic information for selective breeding programmes aiming to enhance viral resistance.

6.1 Introduction

With an estimated value of $4.17 billion USD (FAO 2016), oysters are one of the most commercially important groups of aquatic organisms in the world. In 2014, global aquaculture harvests reached 5.2 million tonnes, representing one third of all cultivated marine molluscs. Although total production volume remains high, growth of the industry has been severely hampered in recent years by extreme disease outbreaks during warmer summer months. Ostreid Herpesvirus (OsHV-1) is a new and emerging viral disease of several molluscan taxa, including oysters (Batista et al. 2015; Sanmartín et al. 2016), scallops (Arzul et al. 2001; Ren et al. 2013), mussels (Burge et al. 2011), and clams (Xia et al. 2015a). Over the past couple of decades, OsHV-1 has been widely associated with mass mortalities of farmed oysters around the globe. A growing number of epidemiology studies and experimental trials suggest that the virus is a causal factor in these events (Friedman et al. 2005; Burge et al. 2007; Segarra et al. 2010; Garcia et al. 2011; Schikorski et al. 2011a,b; Dégremont et al. 2015a,b). With stock losses of up to 100%, economic and social consequences due to the spread of the disease have been devastating in countries such as France, Ireland, USA, China, Australia and New Zealand where oyster aquaculture is a vital primary industry (Burge et al. 2006; Lewis et al. 2012; Castinel et al. 2015). From the perspectives of many scientists, farmers and stakeholders alike, OsHV-1 has been articulated to represent the biggest individual threat to oyster production that the sector has ever faced (Lewis et al. 2012; Castinel et al. 2015).

First evidences for the presence of herpesvirus genetic material in bivalves was obtained in 1976 from samples of *Ostrea edulis* in the UK (Davison et al. 2005). However, widespread detection of herpesviruses and associations with mass mortalities of shellfish were not apparent until the early 1990's (Renault et al. 1995). During the following decade, many occurrences of viral infections were documented around the world, and by 2005 molecular characterisations had led to the designation of the pathogen

as the OsHV-1 reference genotype (GenBank accession no. AY509253.2) (Renault & Arzul 2001; Davison et al. 2005). More recently, there has been an emergence of numerous OsHV-1 variants affiliated with mortalities in different bivalve species displaying different epidemiological characteristics, and it appears that OsHV-1 is undergoing rapid evolution (Grijalva-Chon et al. 2013; Renault et al. 2014; Bai et al. 2015; Martenot et al. 2015). In 2008, the detection of a highly virulent new strain, OsHV-1 µVar (GenBank accession no. HQ842610.1), was described in association with massive losses of oyster spat in France, Ireland and the UK (Segarra et al. 2010). By 2010, this new variant had reached the coasts of Australia and New Zealand, killing huge numbers of oyster stock within days and leading to sector collapses in certain regions over the following few years (Jenkins 2013; Keeling et al. 2014). Between 2011 and 2013, genetic analysis of cultured oysters from China, Korea and Japan revealed widespread herpesvirus infections from numerous genotypes across the East Asiatic region (Shimahara et al. 2012; Hwang et al. 2013; Jee et al. 2013; Bai et al. 2015 & 2016). High mortalities associated with OsHV-1 µVar were observed in Swedish and Norwegian hatcheries towards the end of 2014 (Mortensen et al. 2016). More recently, a new outbreak in Tasmania in 2016 has crippled the Australian oyster aquaculture sector and its selective breeding program (Davis 2016; Milne 2016; Whittington et al. 2016). Thus, it is clear that the extent of this new variant's geographical reach is indeed a major global concern.

Due to the widespread prevalence and substantial socioeconomic consequences of OsHV-1 µVar, it is vital that knowledge of the interactions between the virus and its hosts are obtained to better understand pathogenesis of the disease, develop mitigation strategies, and guide management decisions. To provide such knowledge, a series of focused research themes relating to the spread of the virus and its mechanisms of infection have been conducted in recent years including genotyping and phylogenetics (Renault et

al. 2012; Martenot et al. 2015; Mineur et al. 2015; Burioli et al. 2016), development of experimental infection models (Paul-Pont et al. 2015), modes of transmission (Burge & Friedman 2012; Lionel et al. 2013; Petton et al. 2013; Evans et al. 2016), viral replication and virulence processes (Segarra et al. 2014a & 2016; Green et al. 2015; Martenot et al. 2016), antiviral features of immunity and host responses at transcriptomic and proteomic levels (Renault et al. 2011; Corporeau et al. 2014; Green et al. 2014a,b; Normand et al. 2014; Segarra et al. 2014a,b; He et al. 2015) and identification of virus-resistant traits for selective breeding trials (Dégremont 2013; Dégremont et al. 2015a,b). Most of these studies have focused on post-metamorphic life stages. However, size and age are significant factors in viral susceptibility and pre-metamorphic larval forms appear to be more vulnerable than their juvenile or adult counterparts (Oden et al. 2011; Dégremont 2013; Paul-Pont et al. 2013; Azéma et al. 2016; Dégremont et al. 2016).

Many oyster farms rely on large-scale hatchery production of larvae to supply spat for growout, with increasing demand and stakeholder interests to enhance larval production capacities (Barnard 2014). Thus, it is essential that we extend our knowledge to characterise the pathophysiology of the disease during early ontogeny. Furthermore, the impacts of OsHV-1 µVar on the health of wild populations and their connectivity through larval mortalities, altered larval dispersal potentials, and reduced spat-falls are almost wholly unknown, but are likely to be substantial (Dégremont et al. 2016). In order to assess the ecological consequences of the disease and understand natural vectors and boundaries which may influence its spread, it is important to focus research across all developmental stages. In addition, the identification of specific genotypic and phenotypic traits in larvae which reflect disease susceptibility/resistance would be highly beneficial for monitoring early outcomes of selective breeding programs. Detailed physiological analysis of the host-virus interaction via use of –omics technologies (e.g., transcriptomics, proteomics and metabolomics) may provide fruitful for discovering such traits (Gómez-

Chiarri et al. 2015). There are very few studies which have focused on the highly susceptible pre-metamorphic life-stage and, to our knowledge, none which have utilised metabolomic-based approaches to better understand the physiological effect of OsHV-1 infection on homeostatic control mechanisms of metabolism and immunity.

Metabolomics is a newly developing and rapidly advancing field under the –omics banner which aims to provide global snapshots of alterations in the metabolite, or small molecule (<1 KDa), cellular component (Holmes et al. 2008). Metabolites are the ultimate end-products of gene expression and are strongly influenced by endogenous regulatory mechanisms, as well as by external elements (Fiehn 2002). As intermediates of metabolism, metabolites comprise the available biochemical depot of macromolecular precursors and energy transfer molecules required for optimal organismal growth and functioning. Thus, the composition of the metabolite pool and their flux dynamics provide a closer representation of an organism's phenotype than molecular features at other levels of biological organisation, such as gene transcripts, which may display considerable temporal variations in expression compared to the final phenotypic response, or be entirely decoupled from downstream metabolic processes (Cascante & Marin 2008; Winter & Krömer 2013; Feussner & Polle 2015). With many recent applications across the life sciences (e.g., functional genomics [Sévin et al. 2015], selective breeding [Hill et al. 2015; Hong et al. 2016], aquaculture-related research [Young et al. 2015, 2016; Alfaro & Young 2016], toxicology [Bouhifd et al. 2013; Størseth & Hammer 2014; Chen et al. 2016a] and disease diagnostics, monitoring and prevention [Pallares-Méndez et al. 2016; Wishart 2016]), metabolomics is proving extremely valuable as a highly efficient approach for generating new hypotheses and deciphering complex metabolic and gene regulatory networks of vertebrate and invertebrate models.

By scanning broad sets of metabolic features in whole organisms, tissues or biological fluids in response to environmental influences, such as bacterial or viral

infections, metabolomics-based approaches can provide novel information to gain insights into the mechanisms of disease progression, resistance and remediation in aquatic organisms (reviewed in Chapters 2 & 3). For example, metabolomics has recently been successfully applied to identify biomarkers for *Vibrio* spp. infections in mussels and crabs (Wu et al. 2013; Ellis et al. 2014; Su et al. 2014; Ye et al. 2016), to gain detailed metabolic information on tissue-specific host responses of shrimp and crayfish to white spot syndrome virus (Liu et al. 2015; Chen et al. 2016b; Fan et al. 2016), and to develop practical treatment methods for streptococcal disease in fish (Ma et al. 2015; Zhao et al. 2015). Although limitedly applied to the investigation of marine invertebrate early life stages thus far, metabolomics has great potential to provide new insights into the interactions between OsHV-1 µVar and its oyster larval hosts. Thus, we have conducted the first metabolomics study to assess gross compositional alterations within the oyster larval metabolome in response to OsHV-1 infection.

6.2 Methods

6.2.1 OsHV-1 µVar preparation

OsHV-1 µVar inoculum was prepared from oysters that had been stored at −80°C and previously tested positive by qPCR (primers: GTCGCATCTTTGGATTTAACAA [BF] and ACTGGGATCCGACTGACAAC [B4], after Martenot et al. [2010]). Briefly, whole tissues from OsHV-1 µVar infected moribund oysters were homogenised, centrifuged (3000*g* for 10 min at 4°C) and filtered (serially to 0.22 µm) to remove particles and bacteria. A 0.1 mL aliquot of the filtrate was injected into the adductor muscle of naïve oysters to multiply the virus and create a fresh supply. After 3–4 days, newly infected oysters were shucked, homogenised, and filtered as previously described. Cryoprotectant (10% glycerol and 10% fetal calf serum) was added and the solution aliquoted to 0.5 ml to provide a Master Stock (MS) solution which was stored at −80°C.

To assess virus levels in the MS, qPCR was conducted using BF and B4 primers in a SYBR Green assay with 10 µL reactions. Reactions contained 5 µL of PCR Enhancer Cocktail 2 (DNA Polymerase Technology: St Louis, MO, USA), 1 µL of 10x Taq Mutant Reaction Buffer (DNA Polymerase Technology), 0.2 µL of each primer (5 µM), 0.08 µL of 10 µM dNTP (Invitrogen – Thermo Fisher Scientific: Auckland, New Zealand), 0.4 µL of 100x Sybr Green (Invitrogen), 0.1 µL of Omni KlenTaq (DNA Polymerase Technology), 1.52 µL of ultrapure water, and 1.5 µL of DNA template. Template DNA was extracted using *prep*GEM (ZyGEM: Hamilton, New Zealand) according to the manufacturer's instructions. The thermal cycling profile started at 95°C for 2 min to activate the enzyme, followed by 40 cycles of denaturing at 95°C for 40 sec, annealing at 58°C for 40 sec and extension at 68°C for 60 sec. A melt curve analysis was incorporated in the run at the end of the PCR, starting with 72°C for 90 sec and increasing to 90°C by holding for 5 sec at each 1°C interval.

6.2.2 Larval production

Mature oysters from Cawthron Institute's selective breeding programme were obtained from oyster farm repositories in the North Island, New Zealand (AFL-Moana: Whangaroa and Te Matuku Bay Oysters: Waiheke Island), and were conditioned within a dedicated biosecure system on pond grown and monocultured algae at the Cawthron Aquaculture Park (CAP; Nelson, New Zealand) for a period of up to 10 weeks prior to being used. Gametes were acquired via strip spawning; oysters were opened and gametes were collected by lacerating the gonad wall with the tip of a pipette. Oocytes were gently scraped into 1 L glass beakers filled with 1 µm filtered UV treated seawater (FSW). Sperm was collected into 70 mL plastic containers (LabServ – Thermo Fisher: Auckland, NZ) with a small amount of FSW. Oocytes were enumerated in BD Falcon™ 24-well Tissue Culture Plates (BD Biosciences: NSW, Australia) via microscopy (Olympus: model CK-X31). Sperm counts were performed on serial dilutions using a Neubauer counting chamber after immobilising the gametes with 10% dilute Lugol's solution.

Gametes were mixed and triploidy was induced using a proprietary method developed by the Cawthron Institute. Triploid embryos were then transferred to a conical plastic incubation tank containing 170 L of continuously aerated 1 µm filtered (Arkal system) UV treated (30–50 mJ/cm^2) seawater that had been aged with 4 uM EDTA and pH adjusted to 8.4. Embryos (ca. 225 embryos mL^{-1}) were incubated at 23.0–23.5°C for 24 hrs (until the D-larval stage) under static conditions. D-larvae were then reared for a further 15 days (to first signs of developmental transition from late-stage veliger to pediveliger larvae) in a similar tank but under flow through conditions (ca. 3 L min^{-1}) at a temperature of 23.0–24.0°C. Larvae were continuously fed, via pneumatic pump into the header tank, a mixed microalgal diet consisting of *Chaetoceros calcitrans*, *Isochrysis galbana*, and *Pavlova lutherii* using optimised dietary ratios previously developed by CAP depending on development stage and growth rate. Microalgal concentrations were

routinely measured via chlorophyll analysis using a handheld fluorometer as described by Ragg et al. (2010), and rations were attuned accordingly to provide *ad libitum* access to food. Larval densities were adjusted every 1–2 days throughout the rearing period to provide ideal culture conditions (i.e., starting at around 150 larvae mL^{-1} and progressing down to around 30 larvae mL^{-1} by time of sample collection). At 16 days post-fertilisation, approximately 150,000 larvae were transferred in FSW within a 20 L plastic container to a PC2 laboratory in accordance with CAP biosecurity requirements.

6.2.3 Viral challenge and larval sampling

Immediately prior to viral exposures, larvae were evenly distributed among 12 × 2L glass beakers, each containing 1.8 L of sterile synthetic seawater (Instant Ocean®, made to the manufacturers specifications) to provide equal densities of around 7 larvae mL^{-1}. OsHV-1 µVar MS solution was serially diluted (1:500) in FSW to achieve a dose/dilution level which had previously been determined to cause mortality (personal communication, A. Kesarcodi-Watson; Cawthron Institute, 2015 [unpublished data]). A 12 µL aliquot of OsHV-1 µVar inoculum was pipetted into six of the beakers, with the remaining beakers serving as negative controls. Beakers were individually aerated, maintained at 22°C (air temperature) in the dark. Larvae were not fed during the experiment, following Dégremont et al. (2016). General observations of larval behaviour were made on subsamples (ca. 100 larvae) every 12 hrs until signs of obvious differences between treatments and controls were discerned in the majority of the population (i.e., changes in swimming speeds, trajectories and distributions within the water column, after Burge & Friedman [2012]). Larvae were sampled after 48 hours. The contents of each beaker were filtered through a 150 µm mesh screen and larvae were carefully transferred via disposable pipette to a BioStor™ 2.0 mL cryovial (National Scientific Supply Company: CA, USA). Excess water was aspirated and samples were immediately snap-frozen in liquid nitrogen to rapidly quench metabolic processes. Care was taken to ensure that the

time to recover larvae and the handling processes used were consistent for every sample. Samples were stored at −80°C until metabolite extraction.

6.2.4 Metabolite extraction & GC-MS analysis

Metabolite extractions were performed as previously outlined in Chapter 4. Derivatisations were similarly conducted, but with minor modifications for high biomass larval samples to enhance metabolite coverage. Briefly, the same fixed ratios of 1 M sodium hydroxide, pyrimidine and methanol were used but with higher volumes (500 µL NaOH; 85 µL pyrimidine; 418 µL methanol). Metabolite extracts were also analysed by a different GC-MS platform (Thermo Trace GC Ultra system coupled to an ISQ mass selective detector [EI] operated at 70 eV [Thermo Fisher Scientific Inc.]). Samples were injected using a CTC PAL autosampler into a Siltek™ 2 mm ID straight unpacked inlet liner under pulsed splitless mode. Instrumental parameters were the same as in Chapter 4, with the exception of the mass spectrometer. The quadrupole temperature was set to 230°C, and the MS was operated in scan mode starting after 5.5 min, with mass range of 38–550 amu, and a scan time of 0.1 sec. A derivatised sample blank containing the internal standard, a derivatised standard amino acid mix, a non-derivatised standard alkane mix, and a sample of pure chloroform solvent were analysed along with metabolite extracts for QC purposes.

6.2.5 Spectral processing and metabolite identification

Deconvolution of raw spectral data and metabolite identifications were conducted using the same bioinformatics procedures described in Chapter 4. A Microsoft® Excel file containing peak height data for each metabolite was generated and manually checked for the presence of contaminants. Aberrant records were removed and the resulting QC-filtered data was correctly formatted and saved as a .csv file for subsequent statistical analyses.

6.2.6 Statistics

The data matrix of peak intensities was pre-processed for QC purposes and to meet the distributional requirements prior to statistical analysis using MetaboAnalyst 3.0 (Xia et al. 2015b). Data were normalised against the internal standard and by sample-specific biomass. The resulting dataset was then autoscaled to alleviate the dependency of the variance on the metabolite concentrations due to their large dynamic ranges and provide a Gaussian distribution.

6.2.6.1 Univariate analysis

Standard univariate student's *t*-test and fold-change analyses were conducted to identify metabolites which were statistically different ($p < 0.05$) between control and treatment samples. To provide more robust statistical estimates of metabolite alterations, Significant Analysis of Microarrays/Metabolites (SAM) and Empirical Bayes Analysis of Microarrays/Metabolites (EBAM) were additionally applied to account for multiple hypothesis testing on high dimensional data, avoid assumptions of metabolite independence by accounting for correlations via permutation-based analysis, and provide False Discovery Rate (FDR) values. For SAM, a delta value of 1.3 was selected after analysis of FDR versus the number of significant features as a function of various delta values. An acceptable biomarker selection criteria was considered when the FDR *q*-value < 0.05. For EBAM, the default delta value of 0.9 was used.

6.2.6.2 Unsupervised multivariate cluster analysis

Three unsupervised multivariate cluster analysis methods were subsequently performed to gain a better understanding of the global metabolite variations among larval samples. Agglomerative Hierarchical Cluster Analysis (HCA) was initially used as an unbiased method to identify intrinsic sample groupings based on the underlying structure of the data, and provide data visualisation in the form of a dendrogram. Euclidian distance and Wards criterion were selected as the measure of distance and aggregation, respectively.

Non-hierarchical *k*-Means Clustering (*k*-MC) was similarly applied to discover natural groups in the data, where *k* clusters were defined by the number of sample class labels (i.e., $k = 2$). Principal Component Analysis (PCA) was further performed to reduce dimensionality, assess inter-sample variation, provide a measure of confidence for sample groupings, and to give information about which features contribute most towards the variance explained by the model.

6.2.6.3 Supervised multivariate classification analysis

Two supervised classification modelling and feature selection approaches were then employed to assess whether the metabolite profiles could reliably be used to discriminate larvae which had been exposed to OsHV-1 µVar from control samples, and, if so, to identify important classifiers/biomarkers for larval health condition. Projection to Latent Structures Discriminant Analysis (PLS-DA) was performed to detect changes in metabolic profiles associated with OsHV-1 µVar infection, and thus, identify the differential metabolites that account for the separation between groups. PLS-DA essentially aims to rotate PCA components using *a priori* knowledge of the sample classes such that further separation between treatment groups are obtained, and to understand and quantify which metabolites and directions in the multivariate space carries the class separating information. The PLS-DA model was validated using the leave-one-out cross-validation (LOOCV) method. The performance and predictive capability of the model was evaluated by R^2, the goodness-of-fit parameter, and Q^2, the goodness-of-prediction parameter. Generally, when these two parameters are close to 1.0 they represent an excellent model. The significance of metabolites in the PLS-DA model was evaluated using the Variable Importance in Projection (VIP) method. Metabolites were considered to be important classifiers if their VIP scores ≥ 1.0.

Random Forest (RF) analysis, a more recently developed classification algorithm increasingly being used in metabolomics studies (Braundmeier-Fleming et al. 2016;

Cameron et al. 2016; Kalantari et al. 2016), was then applied using bootstrap sampling to identify and rank metabolites predictive of the OsHV-1 µVar diseased phenotype. RF is a powerful non-parametric machine learning technique based on an ensemble of randomly generated classification trees which are combined to vote for a decision (Touw et al. 2012). Within MetaboAnalyst's 'Biomarker Analysis' module, multivariate RF Receiver Operator Characteristic (ROC) curves were generated by Monte-Carlo Cross Validation (MCCV) using balanced subsampling. In each MCCV, two thirds of the samples were used to evaluate the feature importance. The top 5, 10, 15, ...100 important features were then used to build classification models which were validated on the remaining third of the samples that were left out. This procedure was repeated multiple times to calculate the performance (Area Under Curve [AUC]) and confidence intervals of each model.

6.2.6.4 Functional biochemical pathway analysis

To assist with interpretation of the data beyond investigation of single metabolite variations, established biochemical pathways were examined via secondary bioinformatics procedures under the general banner of Metabolite Set Enrichment Analysis (MSEA) techniques to probe functional relationships among the annotated metabolites. Using MetaboAnalyst's 'Pathway Analysis' module, various sets of metabolites involved in the same biochemical pathway were extracted from the metabolomics dataset and analysed together to determine the likelihood of entire pathways being impacted as a pathophysiological response to OsHV-1 µVar exposure. All reliably annotated metabolites were mapped into metabolic pathways using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa & Goto 2000), and 81 pathways associated with the *Danio rerio* animal model were selected for further analysis. Two different types of pathway analyses were performed; Quantitative Enrichment Analysis (QEA) and Network Topology Analysis (NTA).

QEA is a novel way to identify biologically meaningful patterns in metabolite concentration changes for quantitative or semi-quantitative metabolomic studies (Xia & Wishart 2011). The approach has the potential to identify subtle but coordinated virus-induced changes among a group of functionally related compounds within a pre-defined set of metabolites, or a biochemical pathway, which may go undetected with conventional statistical methods. QEA is a modified version of the popular Gene Set Enrichment Analysis (Tilford & Siemers 2009) which utilises the Geoman's Global Test algorithm and allows different-sized sets of metabolites to be compared. The test avoids problems associated with multiple hypothesis testing, and provides a single *p*-value with an accompanying FDR for the group, rather than individual significance values for each metabolite.

In addition to considering the variations in metabolite abundances within the pre-defined metabolite sets, we also examined the structure of the metabolic networks themselves. NTA assesses the local positioning of significantly altered metabolites within the pathways annotated by KEGG (Nikiforova & Willmitzer 2007). A 'Pathway Impact' (PI) index is estimated using a measure of the centrality (Relative Betweenness Centrality) of detected metabolites in relation to all compounds within a given metabolic network. Centrality is a local quantitative measure of the position of a metabolite relative to the other metabolites by calculating the number of shortest paths going through the target compound, which is used to estimate a metabolite's relative importance or role in network organisation (Xia & Wishart 2010). The overall pathway impact is the cumulative value of the importance measures of the detected/matched metabolites normalised by the sum of the importance measures of all the metabolites within a particular pathway. The results of QEA and NTA can be graphically displayed together in a scatterplot; where the *y*-axis represents QEA-derived pathway enrichment *p*-values, and the *x*-axis represents the NTA-derived PI scores. Biochemical pathways involving

two or more annotated metabolites with simultaneous QEA *p*-values < 0.05, QEA FDRs < 0.1, and with NTA PI scores > 0.1 were considered as potential primary target pathways of interest.

6.2.6.5 Correlation analysis

To further investigate interactions among metabolites beyond routine statistical data analysis and *a priori* assumptions of functional associations, correlation analyses were performed as a discovery approach to reveal potentially unknown relationships. Pairwise metabolite–metabolite Pearson correlation matrices for each treatment group were constructed, displayed as separate heatmaps, and cross-referenced with one another. Correlations with R^2 coefficients > 0.7 or < −0.7 were selected and compared to determine particular occurrences of major differences in slopes (i.e., positive vs. negative relationships). Finally, Correlation Network Analysis (CNA) was performed to provide enhanced visualisation of metabolite relationships (correlations and sub-network clusters or modules) within control and virus-exposed larvae; thus exposing any major perturbations which may be present within the underlying metabolic networks that are representative of the pathophysiological conditions. For network construction, metabolomics data matrices were imported into Cytoscape 3.0 software (Shannon et al. 2003), and processed using the ExpressionCorrelation plugin (Karnovsky et al. 2012). High and low cut-offs of the Pearson correlation coefficient of 0.9 and −0.9, respectively, were conservatively selected and the networks for each treatment group were visualised using Cytoscape. The relative positioning of metabolite nodes within the networks were arranged using default settings.

6.3 Results

General observations of larval behaviour were made every 12 hrs during the trial until first signs of differences between virus-exposed larvae and controls were discerned, i.e., changes in swimming speeds, trajectories and distributions within the water column. After 48 hrs, organisms that had been challenged with OsHV-1 µVar tended to be aggregated in the lower 30–50% of the water columns compared to control larvae which were more evenly distributed. When examined under the microscope, virus-exposed larvae also displayed slower motility and abnormal swimming patterns (i.e., horizontal planar circular motions rather than random) characteristic of OsHV-1 infections (Renault 2008; Burge & Friedman 2012; OIE 2016; personal communication, A Kesarcodi-Watson, Cawthron Institute, 2015). However, larval coloration (a commonly used crude assessment which can indicate severe poor health status [personal communication, N. Hawes, SPATnz, 2014]) generally appeared to be visually similar between treatments. Mortality assessments revealed that 100% of oyster larvae in all beakers were alive at the time of sampling for metabolomics, making direct comparisons between sample classes acceptable.

6.3.1 Univariate analysis

GC-MS analysis of larval extracts detected a total of 105 unique metabolites after QC filtering of the data. Of these, 75 were attributed specific chemical identities by matching chromatographic and mass spectral information against our in-house metabolite library. The remaining 30 features are currently listed as ‘unknowns’ since no matches were found. Univariate statistical analyses showed a number of differences in the metabolite profiles between control and virus-infected larvae (Figure 6.1). SAM identified 30 metabolites as being differentially ($p < 0.05$) expressed between larvae exposed to OsHV-1 µVar and control larvae with an FDR of 3.1% (Figure 6.1A), whereas EBAM identified 28 metabolites as being differentially expressed with an FDR of 4.7% (Figure 6.1B). The

summarised results of SAM and EBAM are displayed in Figure 6.1C, along with their relative fold changes. Taking the results of these analyses together, the abundances of nine metabolites were likely under expressed in virus-infected larvae compared to the metabolic baseline of control organisms, and 20 metabolites were likely over expressed.

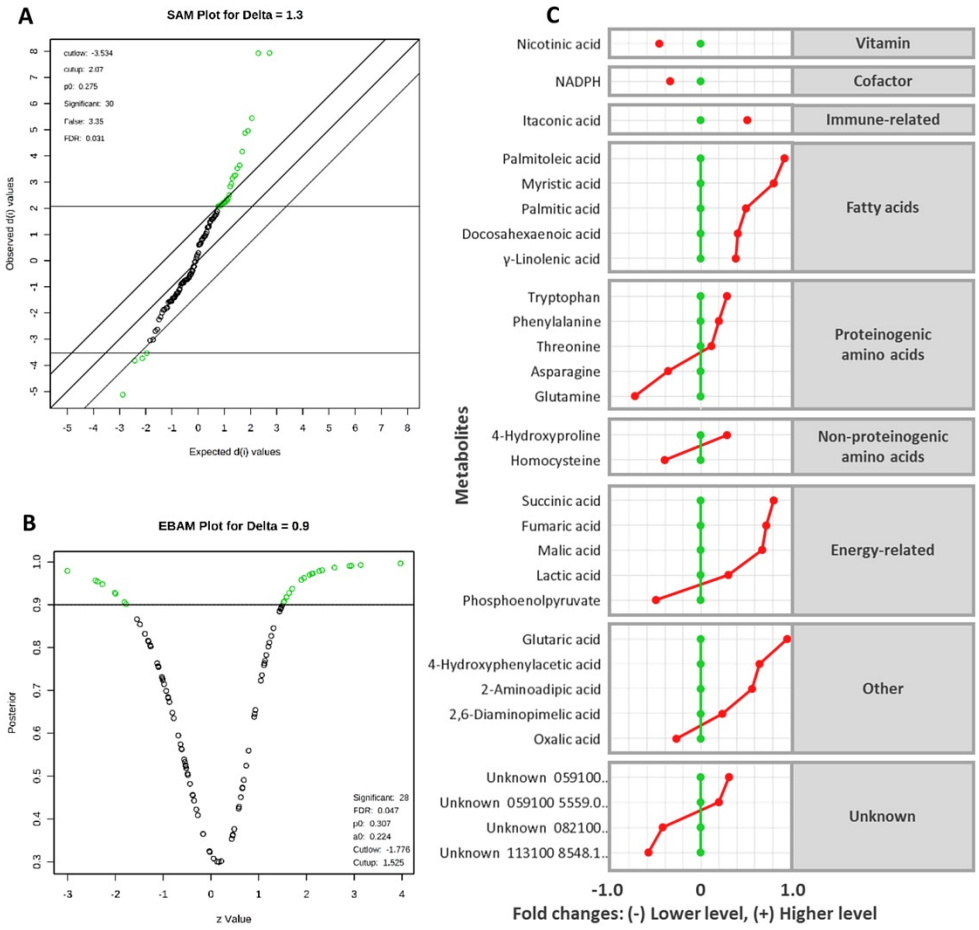
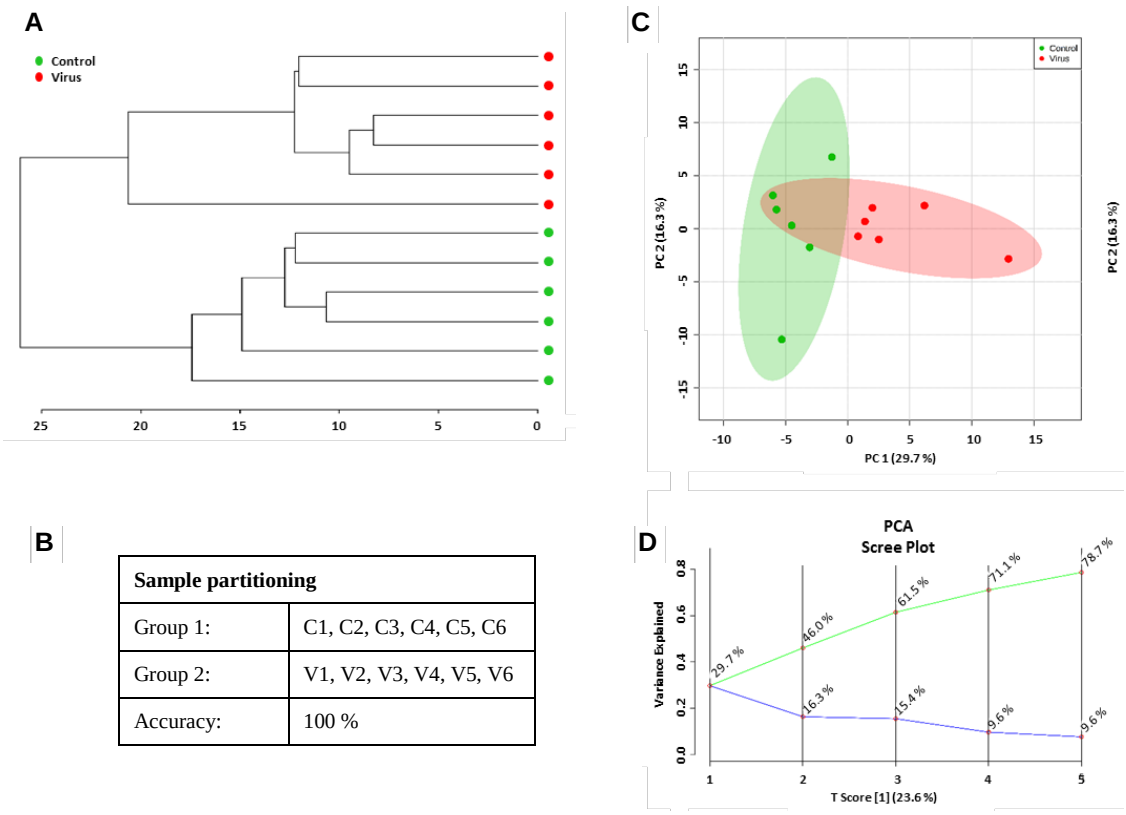


Figure 6.1. Metabolites detected as being significantly different ($p < 0.05$) between control and OsHV-1 µVar-infected larvae: **A)** Significant Analysis of Metabolites (SAM) plot; **B)** Empirical Bayes Analysis of Metabolites (EBAM) plot; **C)** Summary of statistically different metabolite levels between treatment groups with their respective Log_2 fold change values (virus-infected [red circles] / control [green circles] larvae), and assembled into general biochemical classes.

6.3.2 Unsupervised multivariate cluster analysis

Unsupervised multivariate analyses of entire metabolite profiles revealed that good separation between control and virus-infected larvae could be obtained based on the underlying structure of the data (Figure 6.2). HCA correctly positioned samples into two main groups (group 1, controls $n = 6$; group 2, treatment $n = 6$) (Figure 6.2A), indicating that the within-class variation was considerably lower than the between-class variation.

*k*MC corroborated this by also correctly assigning larval samples into groups based on the treatment that they received (Figure 6.2B; inserted table). PCA produced a 2-D score plot containing two distinct clusters of samples which appropriately reflected their class labels and with no indication of sample outliers (Figure 6.2C). The two clusters are separated along PC1 with the relative abundances of around 40 metabolites explaining much of the divide. Although the calculated 95% confidence interval ellipses overlapped, the accumulative variation among all samples explained by PC1 and PC2 was only 46.0%. It is therefore possible that the OsHV-1 µVar-infected larval samples may be separated from control samples along other PC vectors not discernible in the 2-D score plot which might be revealed via supervised multivariate techniques.



Sample partitioning	
Group 1:	C1, C2, C3, C4, C5, C6
Group 2:	V1, V2, V3, V4, V5, V6
Accuracy:	100 %

Figure 6.2. Unsupervised multivariate cluster analyses of metabolite profiles from larvae infected with OsHV-1 µVar vs. control larvae: **A)** Hierarchical Cluster Analysis (Euclidian distance; Ward's method); **B)** Table of results from *k*-Means cluster analysis where *k* clusters = 2 (C*n* = control sample *n*; V*n* = virus-infected sample *n*); **C)** Principal Component Analysis (PCA) score plot; **D)** PCA scree plot showing variation explained by *n* PC (blue line), and the cumulative variance explained in *n* PCs (green line).

Supervised multivariate classification analysis

Supervised multivariate classification analysis was clearly able to discriminate larval samples based on the treatment they received (Figure 6.3). Compared to PCA, the 2-D PLS-DA score plot better separated virus-infected from control larval samples along the *x*-axis (Figure 6.3A), with good cross-validated model performance using the first two latent variables (Accuracy = 100%; R^2 = 96.9%; Q^2 = 79.6%) (Figure 6.3B). PLS-DA additionally informed upon which metabolites were most important for the classification model via their VIP scores (Figure 6.3C).

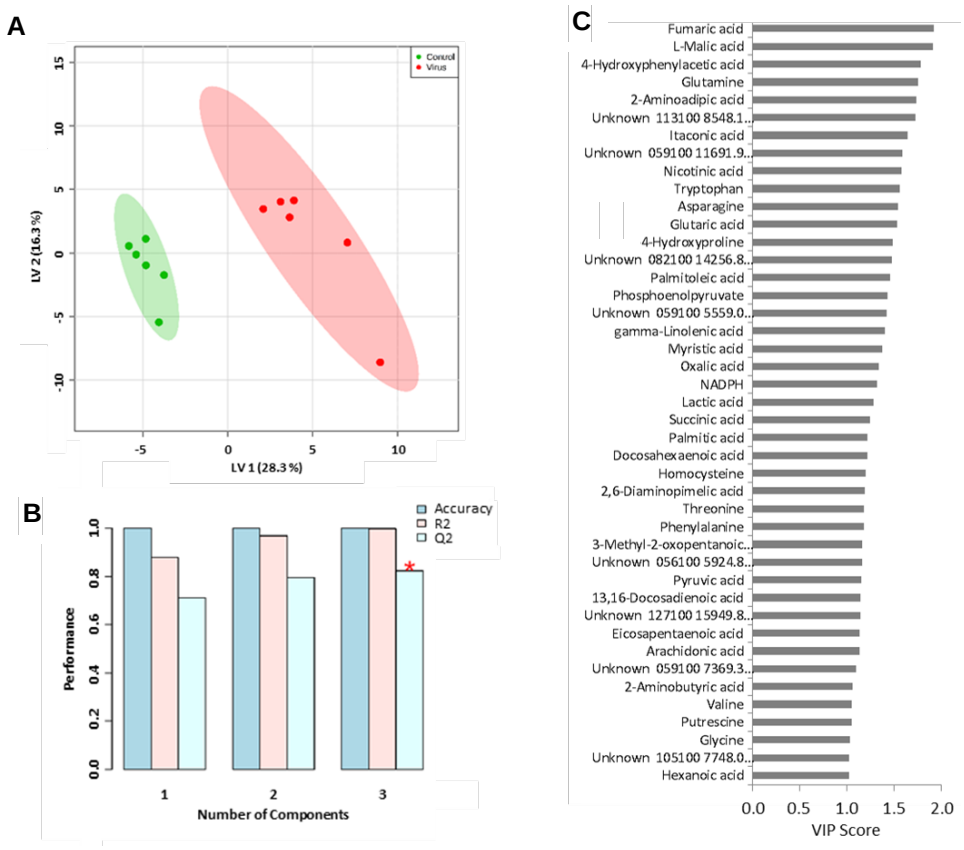


Figure 6.3. Supervised multivariate classification analyses of metabolite profiles from larvae infected with OsHV-1 µVar vs. control larvae: **A)** Projection to Latent Structure Discriminant Analysis (PLS-DA) score plot with accuracy of 100%, multiple correlation coefficient (R^2) of 96.9%, and cross-validated R^2 (Q^2) of 79.6%; **B)** PLS-DA model performances with *n* components; **C)** Variable Importance in Projection (VIP) scores for the PLS-DA model.

Significant classifiers for the separation between virus-infected and control groups were ranked, yielding 43 metabolites (35 annotated and 8 unannotated) with VIP scores > 1.0 (Figure 6.3C). In addition to the 30 differing metabolite abundances identified via SAM and/or EBAM (Figure 1C), PLS-DA also recognised 2-aminobutyric acid, glycine, hexanoic acid, homocysteine, putrescine, valine, and four additional unannotated metabolites as being important classifiers.

The RF machine learning algorithm was further employed as a complimentary feature selection method to similarly rank the most salient metabolite features responsible for class separation via a different statistical approach more resistant to over fitting than PLS-DA (Figure 6.4). A default RF classification model was first constructed using ten features (i.e., ~ $\sqrt{n}$) and 500 permutations, which correctly classified all samples. A series of ROC curve analyses were then performed to generate various *n*-feature classification models which were validated using MCCV sub-sampling to assess predictive accuracies (Figure 6.4A). The predictive accuracies of the 5-, 10-, and 15-feature RF models were 94.5, 98.0, and 100%, respectively, with AUC's of 0.985, 1.0, and 1.0, respectively (Figure 6.4B). ROC curve analysis of the 5-feature model with corresponding confidence intervals is shown in Figure 6.4C, and the predicted class probabilities of the model is shown in Figure 6.4D. The average importance and selected frequencies of metabolites in the 5-feature RF model are shown in Figures 6.4E and 6.4F, respectively. Most metabolites identified as potential biomarker candidates via SAM, EBAM and PLS-DA were also selected to some degree by RF which further corroborates their significance as key classifiers of larval health condition. The most frequently selected compounds (> 20%) with high measures of average importance (> 1.0) were fumaric acid, 4-hydroxyphenylacetic acid, glutamine, glutaric acid, myristic acid, 2-aminoadipic acid, and two unannotated metabolites. As indicated by RF, a low error of classification could be obtained with few compounds.

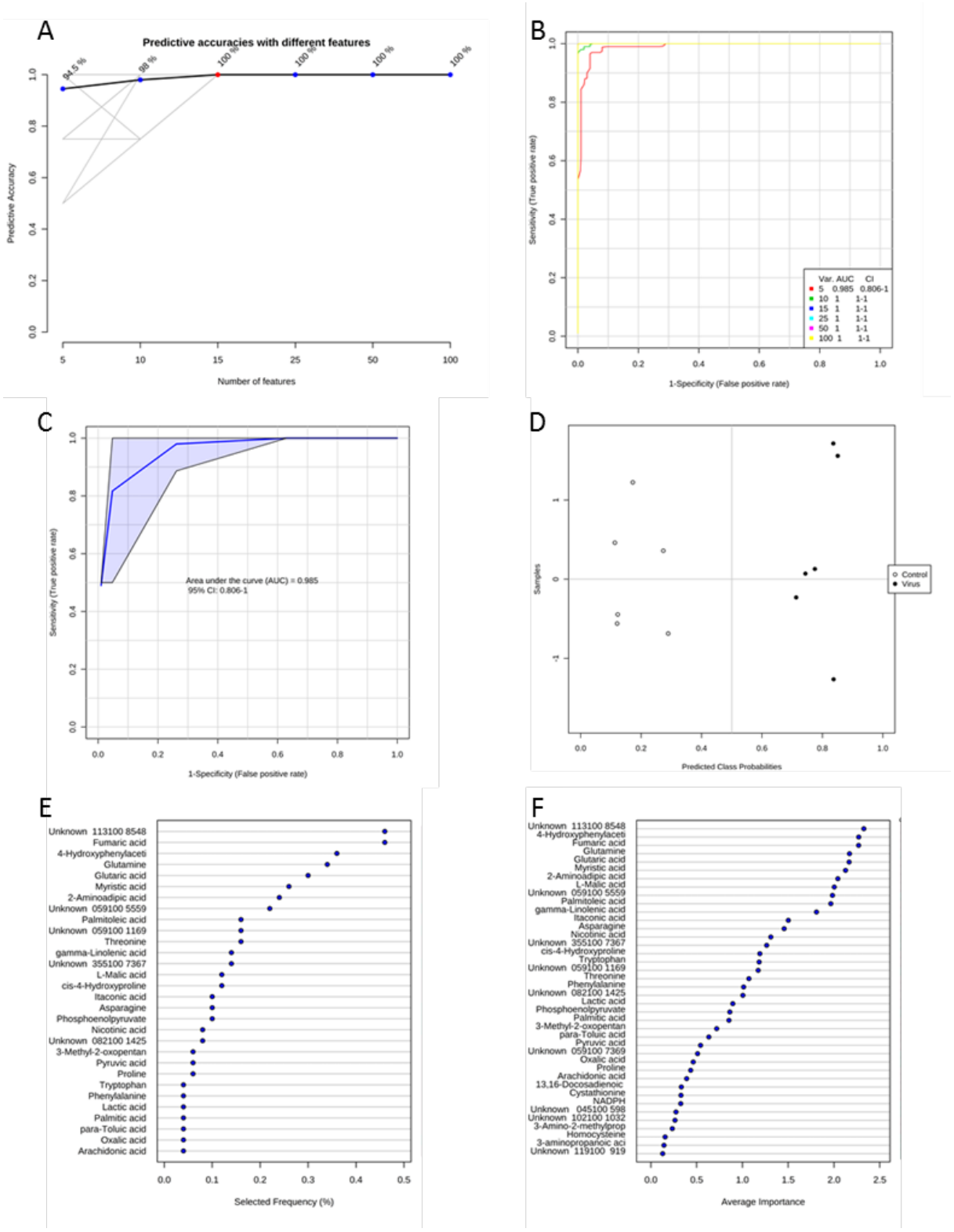


Figure 6.4. Multivariate machine learning and predictive modelling of larval sample classes via Random Forest (RF) analysis with Monte-Carlo Cross Validation (MCCV): **A)** Predictive accuracies of RF models with different *n* features; **B)** Area Under Curve (AUC) generated from Receiver Operating Characteristic (ROC) curve analysis of RF models with 5, 10, 15, 25, 50 and 100 features; **C)** AUC of the 5-feature RF model; **D)** Predicted class probabilities (average of the MCCV) for each sample using the best classifiers (based on AUC) of the 5-feature RF model; **E)** The average importance of metabolites in the 5-feature RF model based on ROC curve analysis, with the most discriminating feature in descending order of importance; **F)** The selected frequencies of metabolites in the 5-feature RF model based on ROC curve analysis.

6.3.3 Functional biochemical pathway analysis

Based on the profiles of annotated metabolites, metabolic pathway analyses were performed to reveal the most relevant pathways related to the pathophysiology of oyster larvae exposed to OsHV-1 µVar (Figure 6.5; Table 6.1). A total of 43 biochemical pathways were recognised from within the KEGG database which contained one or more of the annotated metabolites detected. Pathways involving two or more detected metabolites and with simultaneous QEA *p*-values < 0.05, QEA FDR values < 0.1, and NTA Pathway Impact (PI) values > 0.1 were screened as potential primary target pathways of interest relating to the treatment effect. According to these selection criteria, 12 biochemical pathways were identified with evidence of metabolic disturbances in virus-exposed larvae (Figure 6.5A), comprising of: glycolysis/gluconeogenesis; pyruvate metabolism; tricarboxylic acid cycle; glyoxylate and dicarboxylate metabolism; aminoacyl-tRNA biosynthesis; tyrosine metabolism; alanine, aspartate and glutamate metabolism; arginine and proline metabolism; glycine, serine and threonine metabolism; cysteine and methionine metabolism; D-glutamine and D-glutamate metabolism; butanoate metabolism; and nicotinate and nicotinamide metabolism. Nine further pathways that were identified statistically via QEA ($p < 0.05$ with FDRs < 0.1) but did not meet our ideal NTA impact assessment criteria (i.e., PI values < 0.1) were screened as potential secondary target pathways of interest, comprising of: purine metabolism; pyrimidine metabolism; lysine degradation; nitrogen metabolism; fatty acid biosynthesis; fatty acid elongation in mitochondria; and fatty acid metabolism.

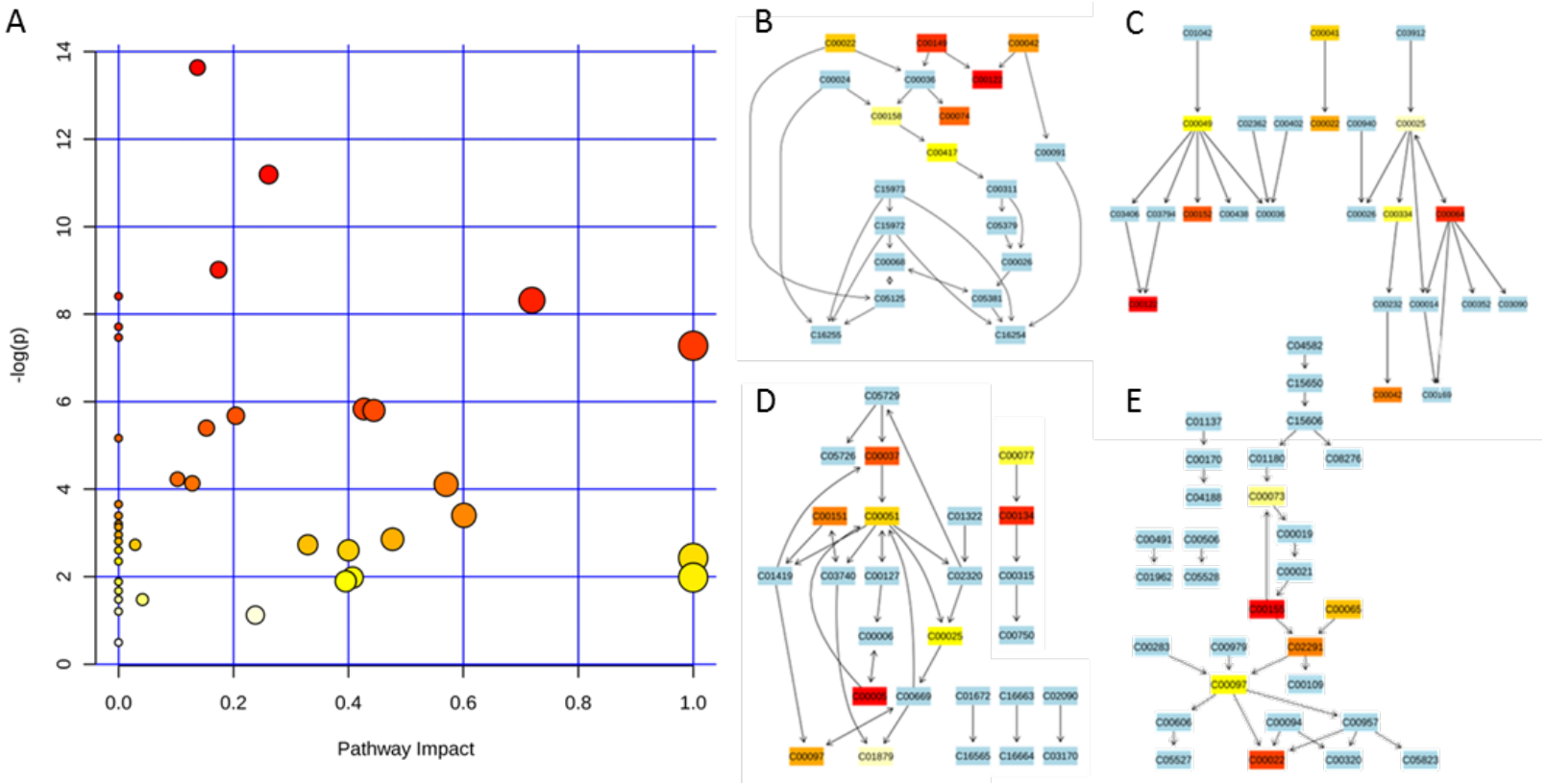


Figure 6.5. Secondary bioinformatics of annotated metabolites: **A)** Topology-based pathway analysis showing metabolic networks in oyster larvae potentially affected by OsHV-1 µVar. The most impacted metabolic pathways are specified by the volume and the colour of the spheres (yellow = least relevant; red = most relevant) according to their statistical relevance and pathway impact (PI) values resulting from Quantitative Enrichment Analysis (QTA) and Network Topology Analysis (NTA), respectively; **B–E)** Examples of four pathways containing relatively high metabolite coverages: **B)** Tricarboxylic acid cycle ($p < 0.001$, FDR < 0.000, PI = 0.26); **C)** Alanine, aspartate and glutamate metabolism ($p < 0.001$, FDR = 0.002, PI = 0.72); **D)** Glutathione metabolism ($p = 0.057$, FDR = 0.107, PI = 0.48); **E)** Cysteine and methionine metabolism ($p = 0.033$, FDR = 0.076, PI = 0.60). Boxes which vary from yellow to red represent metabolites (KEGG ID codes) that were detected and annotated with our methods. Their colour indicates the level of significance (light yellow: $p > 0.05$, light orange to red: $p < 0.05$) from unpaired *t*-tests (control vs. treatment). Light blue boxes/compounds in the pathways were not detected, but were used as background information for QEA to calculate the proportion of identified compounds within each pathway, and in NTA to determine the position (relative-betweenness centrality) and importance of each metabolite.

Table 6.1 List of altered metabolic pathways in larval hosts during viral (OsHV-1 µVar) infection.

Pathways		Match Status	Raw p	-LOG (p)	FDR	Impact (PI)
Tyrosine metabolism	**PRIMARY INTEREST**	3/44	0.001	13.630	0.000	0.137
Citrate cycle (TCA cycle)		7/20	0.001	11.190	0.000	0.261
Pyruvate metabolism		4/22	0.001	9.011	0.002	0.174
Alanine, aspartate and glutamate metabolism		9/24	0.001	8.318	0.002	0.719
D-Glutamine and D-glutamate metabolism		2/5	0.001	7.275	0.004	1.000
Arginine and proline metabolism		8/43	0.003	5.831	0.013	0.427
Glyoxylate and dicarboxylate metabolism		4/18	0.003	5.799	0.013	0.444
Glycolysis/Gluconeogenesis		3/26	0.003	5.676	0.013	0.204
Aminoacyl-tRNA biosynthesis		19/67	0.015	4.229	0.044	0.103
Nicotinate and nicotinamide metabolism		3/14	0.016	4.135	0.044	0.128
Glycine, serine and threonine metabolism		7/31	0.016	4.111	0.044	0.570
Cysteine and methionine metabolism		6/29	0.033	3.401	0.076	0.601
Fatty acid metabolism	**SECONDARY INTEREST**	1/38	0.041	3.203	0.087	0.000
Fatty acid biosynthesis		2/38	0.026	3.655	0.065	0.000
Biosynthesis of unsaturated fatty acids		7/42	0.034	3.393	0.076	0.000
Fatty acid elongation in mitochondria		1/27	0.044	3.135	0.089	0.000
Tryptophan metabolism		1/39	0.005	5.393	0.016	0.153
Pyrimidine metabolism		3/41	0.006	5.165	0.019	0.000
Nitrogen metabolism		5/9	0.001	8.414	0.002	0.000
Purine metabolism		1/66	0.001	7.714	0.003	0.000
Lysine degradation		1/18	0.001	7.468	0.004	0.000
Glutathione metabolism	**NON-SIGNIFICANT**	9/26	0.057	2.863	0.107	0.477
Glycerolipid metabolism		1/18	0.052	2.957	0.102	0.000
Butanoate metabolism		5/22	0.065	2.727	0.108	0.029
Propanoate metabolism		2/20	0.060	2.811	0.108	0.000
Arachidonic acid metabolism		1/31	0.065	2.727	0.108	0.329
Methane metabolism		2/9	0.074	2.609	0.113	0.400
Cyanoamino acid metabolism		2/6	0.074	2.609	0.113	0.000
Valine, leucine and isoleucine biosynthesis		5/13	0.088	2.425	0.131	1.000
Pantothenate and CoA biosynthesis		3/15	0.095	2.351	0.137	0.000
Phenylalanine, tyrosine and tryptophan biosynthesis		2/4	0.138	1.980	0.181	1.000
Phenylalanine metabolism		2/11	0.138	1.980	0.181	0.407
beta-Alanine metabolism		2/16	0.150	1.897	0.181	0.395
Porphyrin and chlorophyll metabolism		2/27	0.151	1.889	0.181	0.000
Sphingolipid metabolism		1/21	0.151	1.887	0.181	0.000
Biotin metabolism		1/5	0.152	1.886	0.181	0.000
Selenoamino acid metabolism		1/17	0.186	1.681	0.216	0.000
Sulfur metabolism		1/9	0.229	1.475	0.246	0.042
Taurine and hypotaurine metabolism		1/7	0.229	1.475	0.246	0.000
Thiamine metabolism		1/7	0.229	1.475	0.246	0.000
Valine, leucine and isoleucine degradation		3/38	0.300	1.203	0.315	0.000
Histidine metabolism		3/14	0.326	1.120	0.334	0.238
Ubiquinone and other terpenoid-quinone biosynthesis		1/3	0.609	0.497	0.609	0.000

6.3.4 Correlation analysis

Pairwise metabolite–metabolite correlation matrices of Pearson coefficients for each treatment group were separately constructed and displayed at heatmaps (Figure 6.6). In general, substantial treatment-induced differences in the relationships between metabolites were exposed, as demonstrated by the many contrasting colours of same cells between the two heatmaps. From these totals of 5565 pairwise comparisons within each dataset, 167 strong linear correlations (R^2 values > 0.7 or < −0.7) were found to be highly differentially expressed (i.e., positive vs. negative relationships) between larvae infected with OshV-1 µVar and baseline controls. CNA with selection criteria of R^2 > 0.9 or < −0.9 were then separately performed on control and virus-exposed larval datasets to summarise and reveal the major correlation differences in the metabolic networks (Figure 6.7).

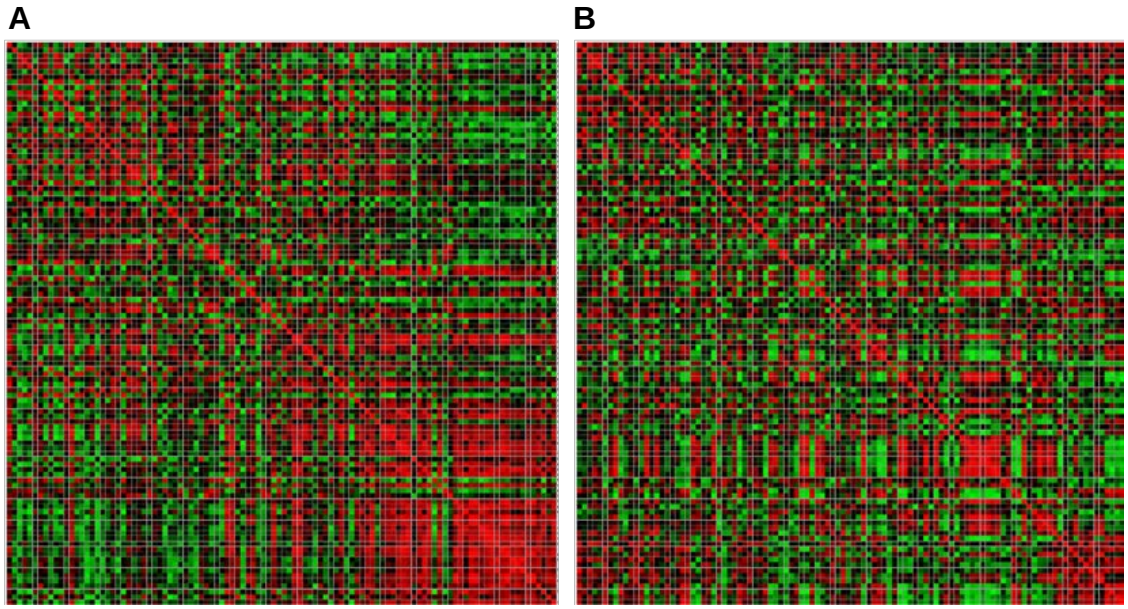


Figure 6.6. Metabolite–metabolite Pearson correlation heatmaps of healthy control larvae **(A)** vs. unhealthy virus-infected larvae **(B)**. The order of metabolites are the same for each of the heatmaps so direct comparisons can be made for particular regions.

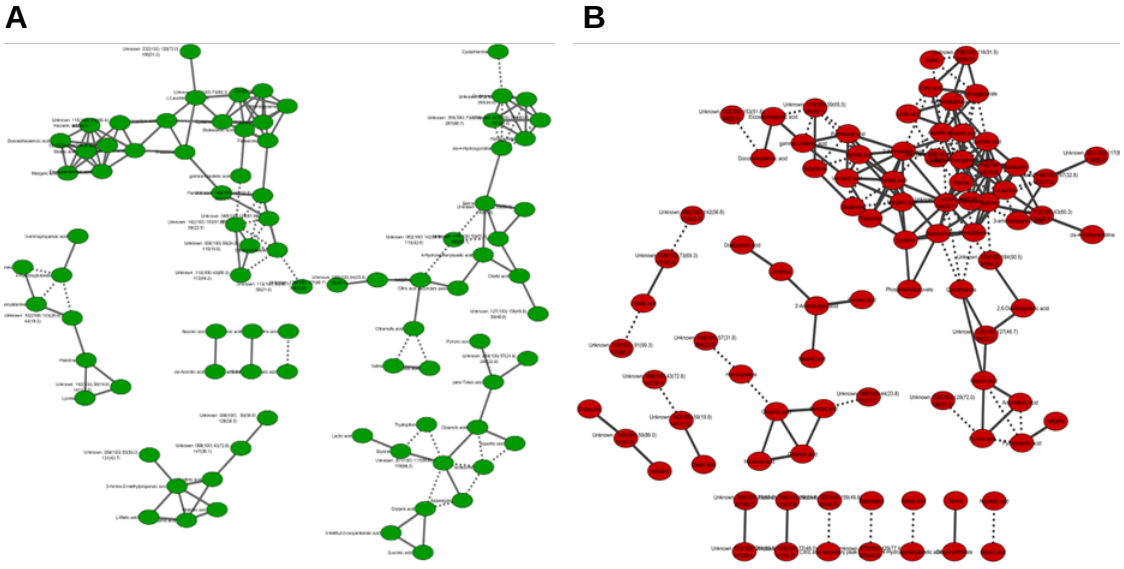


Figure 6.7. Correlation Network Analysis of control **(A)** vs. virus-infected larvae **(B)**. Metabolite–metabolite Pearson correlations > 0.9 are represented by grey solid lines, whereas those that are < −0.9 are represented by dashed grey lines.

6.4 Discussion

The aim of this study was to evaluate changes in the *C. gigas* oyster larval metabolome induced by ostreid herpesvirus and determine whether metabolomics-based approaches can deliver novel mechanistic insights into immunological defense systems of early life-stage marine invertebrates. Thus, a comprehensive determination of metabolic alterations in oyster larvae exposed to the newly emerging and highly virulent OsHV-1 µVar genotype was performed via GC/MS-based metabolomics. Our findings revealed that viral exposure had a strong effect on many metabolites involved in central carbon metabolism, across broad chemical classes with various functional roles. These virus-induced changes in the metabolite profiles enabled us to discriminate healthy from unhealthy larvae via multivariate clustering and classification techniques, discern relationships among metabolites, identify entire biochemical pathways evidenced of being altered, and further focused our attention towards specific mechanisms of immunity characteristic of the pathophysiological condition. We identified coordinated changes in TCA cycle-related metabolites in virus-infected larvae indicative of abnormal energy metabolism and biosynthesis of antimicrobial products, and also detected subtle signs of potential oxidative stress, transformation or degradation of extracellular matrix scaffolding, and disruption of normal lipid metabolism suggestive of requirements for viral appropriation of host-cell biomaterial (summarised in Figure 6.8).

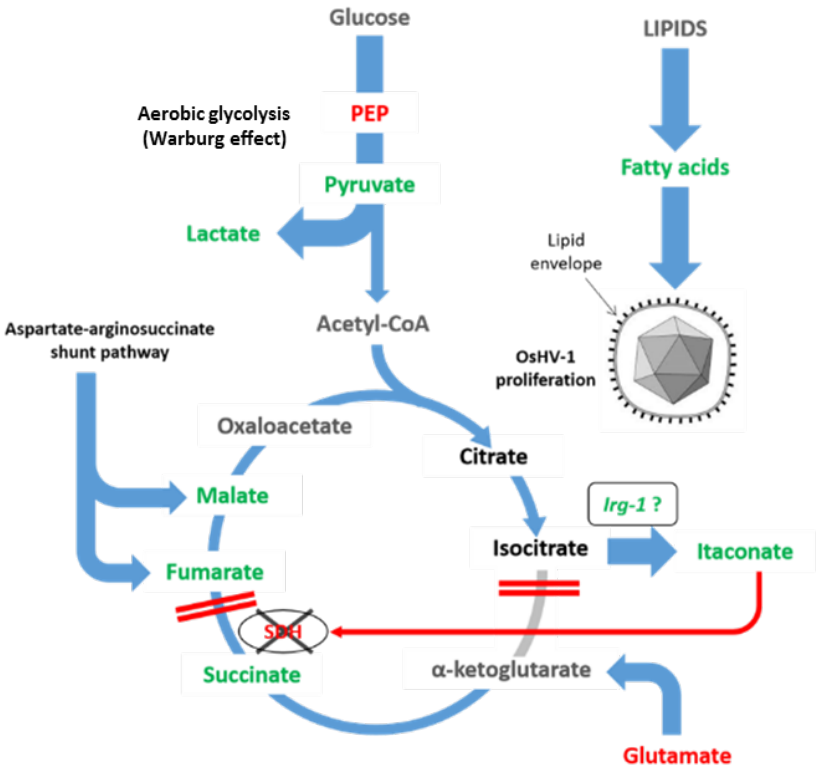


Figure 6.8. Proposed alterations in carbon metabolism during OsHV-1 µVar infection. Green metabolites = upregulated in virus exposed larvae (including hypothetical upregulation of *Irg-1* gene), red metabolites = down regulated, black metabolites = no change, grey metabolites = not detected; Red double lines = TCA cycle breakpoints.

6.4.1 Lipid metabolism

Enveloped viruses, such as those from the herpesviridae family, are known to physically and metabolically remodel host cells during infection to create optimal environments for their replication by manipulating lipid signalling and metabolism (Chukkapalli et al. 2012; Rosenwasser et al. 2016). Such viruses instructively alter host metabolism in order to supply the high quantities of fatty acids which are required as vital lipid envelope components during virion assembly (Koyuncu et al. 2013). Although the precise induction mechanisms have not yet been elucidated, enrichment of host fatty acid (FA) production is a common response of different organisms to infection by various enveloped viruses (Mazzon & Mercer 2014; Hsieh et al. 2015; Sanchez & Lagunoff

2015), including herpes-type viruses such as human cytomegalovirus (HMCV) (Spencer et al. 2011; Seo et al. 2013; Purdy et al. 2015) and Kaposi's sarcoma-associated herpesvirus (Bhatt et al. 2012). An emerging theme is that these lipid-modifying pathways are linked to innate antiviral responses which can be modulated to inhibit viral replication (Chukkapalli et al. 2012). For example, HCMV stimulates free fatty acid (FFA) production to enable and enhance assembly of infectious virions by activating expression of *ACC1* host mRNA, the gene encoding for the rate-limiting enzyme acetyl-CoA carboxylase (ACC) involved in the initial commitment stage of *de novo* FA synthesis (Spencer et al. 2011); whereas pharmacological inhibition of host ACC substantially limits the ability of HCMV to replicate (Munger et al. 2008). More recently, Koyuncu et al. (2013) reported that siRNA-induced knockdown of a suite of other enzymes involved in FA synthesis (fatty acyl-CoA synthetases and elongases) inhibited herpesvirus replications, whereas knockdown of proteins responsible for FA catabolism (the peroxisomal β-oxidation enzyme acetyl-CoA acyl-transferase 1) and the first step of triglyceride synthesis (1-acylglycerol-3-phosphate O-acyltransferase 9) enhanced viral replication by elevating the available FFA pool.

Thus, the FA synthesis pathway is currently gaining considerable attention as a prime target for the development of innovative therapeutics that are not dependent on mechanisms of adaptive immunity, and therefore resilient to emerging virus variants which have become resistant to anti-viral therapies (Goodwin et al. 2015). Although significant inroads have recently been made to better-understand FA synthesis/modification mechanisms in oyster larvae (da Costa et al. 2015), our knowledge is still highly fragmentary. Unfortunately, it has not yet been established whether larvae even have the ability to synthesise FAs *de novo* from a single precursory acetyl-CoA subunit, let alone determined whether larval transcripts or the activities of enzymes involved in pathway initiation or early FA elongation can be regulated by OsHV-1.

Nevertheless, whether through a similar process or otherwise, OsHV-1 does appear to alter host lipid metabolism to enhance production, if not *de novo* synthesis, of FFAs.

Looking at the global metabolic changes in larvae induced by OsHV-1 µVar exposure, there was a strong signature consisting of FFAs, presumably involving either a change in the relative rates of production and/or breakdown. These variation patterns contributed towards earmarking FA pathways (FA metabolism, FA β-oxidation and FA elongation in mitochondria) as being candidate targets of interest in our study via secondary bioinformatics techniques, and also were key metabolites causative to the major perturbations observed within the differential metabolic correlation networks. Under the starvation conditions we employed during the viral challenge, an effect on basal lipolysis would be the most obvious potential mechanism for the FFA changes observed here. Compared to non-infected control larvae, the general increase in medium and long chain FFAs (C16:0, C18:3n-6, C20:4n-6, C20:5n-3, C22:2n6, C22:6n-3) and microalgal-derived dietary FFAs (C14:0, C16:1n-7) in virus-infected larvae are indicative of enhanced catabolism of endogenous triacylglycerol lipid supplies. This pre-metamorphic host-response appears to be somewhat similar to that of post-metamorphic life stages. Proteomic-based analyses of adult Pacific oysters experimentally infected with OsHV-1 µVar recently identified that a key enzyme involved in the first step of lipid hydrolysis, triacylglycerol lipase (TGL), was over-accumulated in virus-exposed animals which likely reflects enhanced lipolysis during initial stages of infection (Corporeau et al. 2014). In support of these findings, transcriptomic-based analyses revealed over-expression of genes encoding for TGL and phospholipase A2 (an enzyme that releases FAs from the second carbon group of glycerol in phospholipids) in OsHV-1 µVar-infected oysters (He et al. 2015), and several other studies also report triglyceride levels being substantially decreased in juvenile and adult oyster hosts exposed to the virus (Pernet et al. 2010, 2014; Tamayo et al. 2014).

Such reductions in energetic reserves during pathogen infections could be due in part to the re-allocation of stored energy to sustain immune, antioxidant and cryoprotection processes (Genard et al. 2013). Under high energetic demands, cytosolic FFAs stemming from lipid hydrolysis can undergo β-oxidation in the mitochondrial matrix in a series of steps to supply the TCA cycle with acetyl-CoA, and the electron transport chain with NADH and $FADH_2$ coenzymes. FAs generally yield more ATP per unit mass than carbohydrates, and can be used to augment energy supply. In adult oysters, FFA accumulations do not appear to coincide with the reduced lipid contents following OsHV-1 µVar infection likely due to them being transitory intermediates (Tamayo et al. 2014), for which simultaneously enhanced rates of β-oxidation could explain. However, infected adult oysters display a down-accumulation in fatty acid-binding protein (FABP) (Corporeau et al. 2014), a chaperone involved in trafficking FFAs across the mitochondrial membrane, and, at the height of the viral load, decreased *Fabp* transcription and expression of a gene encoding the alpha subunit of FA oxidation complex (He et al. 2015), all of which would limit β-oxidation rather than promote it. Thus, aside from being used for host energy metabolism, the FFAs produced during virus-induced lipolysis in oysters may be used as precursor synthesis molecules for constructing the lipid envelope during virus assembly and proliferation; as previously reported for HCMV infections.

Pre-metamorphic oyster larvae infected with pathogenic marine bacteria (*Vibrio corallilyticus*) similarly exhibit increased levels of FFAs compared to healthy larvae, with concomitant reductions in triglyceride levels (Genard et al. 2013). However, analysis of enzymes involved in fatty acid β-oxidation (i.e., acyl-CoA synthetase, acyl-CoA dehydrogenase, and enoyl-CoA hydratase) revealed they are over-expressed during the infection. These authors hypothesise that activation or enrichment of β-oxidation is a characteristic response of oyster larvae to pathogenic bacteria to provide cells with the energy required to mount an immunological defence strategy. The observed FFA

accumulations reported by Genard et al (2013) were likely due to the increased rates of β-oxidation being overshadowed by substantially enhanced rates of lipolysis. This appears to be a different mechanism from viral infections in which the cellular machinery is hijacked in order to provide FFAs for virion propagation by suppressing β-oxidation and simultaneously enhancing *de novo* FA synthesis and/or lipolysis.

Although FFA levels at a particular time reflect the complex metabolic balance between lipolysis, β-oxidation, and any other FA production (e.g., *de novo* synthesis) or consuming processes (e.g., triglyceride synthesis and utilisation for virion assembly), the FFA accumulations we observed are consistent with the general findings of other studies which have investigated various models of herpes-type infections. Perhaps a key point of difference in host-virus interactions between OsHV-1 and vertebrate-infecting herpesviruses could be the primary source from which the FAs are derived from (i.e., lipolysis vs. *de novo* synthesis). We recommend that targeted analyses of these pathways are additionally conducted at transcriptional and translational levels, in combination with metabolite profiling, in order to tease out the mechanistic intricacies of OsHV-1 µVar-induced modulation of host lipid metabolism in oyster larvae. With FAs being necessary components required for OsHV-1 replication and proliferation, establishing the precise viral targets of host lipid metabolism could assist in the development of antiviral therapeutics, and/or identification of unique disease resistant genomic or metabolic traits for selective breeding purposes.

Indeed, the pathophysiological information we uncovered via a metabolomics-based analysis of larval hosts, combined with molecular data at other levels of biological organisation in adult hosts (Corporeau et al. 2014; He et al. 2015), provide some intriguing lines of inquiry when synthesised with our current knowledge of disease resistant traits in *C. gigas*. Summer mortality, or Pacific Oyster Mortality Syndrome (POMS), is well-known to be associated with incidences of OsHV-1 infections (Segarra

et al. 2010; Jenkins et al. 2013; Keeling et al. 2014; Paul-Pont et al. 2014), and although causal factors are likely due to a myriad of influences with complex interactions (Petton et al. 2013, 2015; Green et al. 2014b; Lassudrie et al. 2015; Solomieu et al. 2015), a number of experimental field- and laboratory-based trials over multiple generations have identified families which display various levels of resistance to POMS and/or OsHV-1 (Sauvage et al. 2010; Dégremont 2011; Fleury & Huvet 2012; Dégremont et al. 2010, 2015b, 2016). This ongoing research is being conducted through number of dedicated programs (e.g., the MOREST Program and SCORE Project) aiming to selectively breed for disease resistance by leading organisations or consortiums, including Cawthron Institute, Australian Seafood Industry (ASI), Oysters Australia, French Research Institute for Exploration of the Sea (IFREMER), and the French Poultry and Aquaculture Breeders Association (SYSAAF) (Puyo et al. 2014; Allison & Destremau 2015; Dégremont et al. 2015a; Davis 2016). The successes of these programmes are thought to be crucial for the oyster aquaculture sector to survive and thrive over the coming years.

With lipid metabolism being identified as a key area of focus in the virus-host interaction, more specific research efforts on these FA production pathways may provide valuable insights into OsHV-1 resistant traits. Interestingly, oysters which have been selectively bred for field-resistance to POMS have 2-fold lower transcript expressions of a gene encoding lipase, compared to their disease-susceptible counterparts (Fleury & Huvet 2012). This indicates that resistant animals have a lower capacity for FFA production through lipolytic processes which would potentially impede viral replication by limiting the amount of material available for envelope assembly. Furthermore, resistance to POMS is also associated with higher levels of acylcarnitine carrier protein (ACP) in resilient oysters, compared to susceptible animals (Fleury et al. 2010). ACP mediates the transport of acylcarnitines of different length across the mitochondrial inner membrane from the cytosol to the mitochondrial matrix for their catabolism by the FA β-

oxidation pathway (Kerner & Hoppel 2000). Thus, disease-susceptible oysters have reduced capacities for FA catabolism, which would potentially promote viral replication by enhancing the amount of material available for envelope assembly, as well as limit the production of lipid-derived energy for mounting immunological defense mechanisms and maintaining crucial cellular basal metabolic processes. In line with reduced capacities for energy production in POMS-susceptible oysters, significant impacts on substrate level and oxidative phosphorylation were also detected in virus-exposed larvae.

6.4.2 TCA cycle and immunoresponsive gene 1

We identified a remarkably strong and specific signature of TCA cycle-related metabolite changes indicative of immunoresponsive gene 1 (*Irg1*) activation, which directly affects carbon flux through the cycle and modifies energy metabolism. *Irg1* is commonly and highly expressed in vertebrate macrophages during inflammation and infection by a variety of pathogens (Preusse et al. 2013). Although the gene itself was identified as an innate immune-related transcript over two decades ago (Lee et al. 1995), its functional role, downstream products, and regulators have remained almost wholly elusive until only very recently (Tallam et al. 2016). *Irg1* encodes immune-responsive gene 1 protein/ *cis*-aconitic acid decarboxylase (IRG1/CAD) which links cellular metabolism with immune defence by catalysing the decarboxylation of *cis*-aconitic acid (the citrate → isocitrate isomerisation intermediate in the TCA cycle) to itaconic acid (ITA) (Michelucci et al. 2013; Vuoristo et al. 2015).

ITA is a metabolite with potent antimicrobial properties (Naujoks et al. 2016), and was identified in our study as being over-accumulated in virus-exposed oyster larvae. ITA being discovered as the gene product of *Irg1* is arguably one of the most important biological insights made in recent times (Sévin et al. 2015), and was only revealed through taking a non-hypothesis driven metabolomics profiling approach as we have in

the current study. ITA is now newly recognised as a crucial regulatory metabolite involved in posttranscriptional mechanisms of reprogramming mitochondrial metabolism through modulation of substrate level phosphorylation, TCA cycle flux and succinic acid signalling (Mills & O'Neill 2016; Cordes et al. 2016; Németh et al. 2016), production of inflammatory cytokines (Lampropoulou et al. 2016) and its ability to alter cellular redox balance (Tretter et al. 2016).

Upregulation of *Irg1* transcription leads to a characteristic metabolic signature of a "broken TCA cycle" in stimulated macrophages (O'Neill 2015; O'Neill & Pearce 2016; O'Neill et al. 2016). ITA accumulation represents the first of two distinctive break-points in the pathway due to decreased transcription of isocitrate dehydrogenase (IDH; catalyses isocitrate → α-ketoglutarate), and the redirection of *cis*-aconitic acid metabolism via enriched *Irg1*-encoded IRG1/CAD expression (Jha et al. 2015; Yanamoto et al. 2015). The increased production of ITA decreases citric acid oxidation through the cycle. To compensate for the reduced flux under such conditions, Meiser et al. (2016) showed that glutamine uptake is co-enhanced with *Irg1* expression, serving to replenish the pathway with α-ketoglutaric acid through glutaminolysis, downstream of citric acid. In agreement, the reduction in free glutamine content that we observed in OsHV-1 µVar-exposed larvae is consistent with enhanced glutamine uptake into the TCA cycle, via conversion to α-ketoglutaric acid, as an anaplerotic mechanism. Herpes-infected human cells can switch substrate utilisation from glucose to glutamine to accommodate the biosynthetic and energetic needs of the viral infection, and allow glucose to alternatively be used biosynthetically (Chambers et al. 2010). Virus-induced reprogramming of glutamine metabolism and anaplerosis of the TCA cycle at this particular point appears to be critical for successful replication of herpes-type viruses, as well as maintenance of cellular viability during latent infections (Sanchez et al. 2015; Thai et al. 2015).

The second characteristic break-point in the TCA cycle occurs at succinate dehydrogenase/ respiratory Complex II (SDH/CII), the enzyme which catalyses the oxidation of succinate → fumarate, and also crucially regulates respiration in the electron transport chain (Mills & O'Neill 2016). ITA is a competitive inhibitor of SDH/CII (Cordes et al. 2016), and thus, when ITA levels increase, enzyme activity is attenuated leading to an accumulation of succinic acid and a concomitant decrease in oxidative phosphorylation (OxPhos) (Lampropoulou et al. 2016). Directly in line with this second TCA cycle break-point feature, oyster larvae infected with OsHV-1 µVar exhibited elevated levels of succinic acid. The functional purpose of reprogramming host cell metabolism to accumulate succinic acid in response to pathogen infections appears to stem in part from its ability to mediate inflammatory responses. Aside from having a fundamental role in the TCA cycle, succinic acid can act as a regulatory signal, via succinate receptor 1 (GPR91/SUCNR1), to induce production of pro-inflammatory cytokines (TNF-α, IL-1β) which can enhance immune-stimulatory capacity, but also can exasperate disease when produced in excess (Rubic et al. 2008; Tannahill et al. 2013; Mills & O'Neill 2014; Littlewood-Evans et al. 2016). GPR91/SUCNR1 is therefore involved in sensing the immunological danger exposed by *Irg1*/ITA-induced succinic acid accumulations, thus further establishing direct links between immunity and cellular respiration. Interestingly, our observations relate to the danger theory proposed in the 1990s by Matzinger (1994) and later (Matzinger 2007, 2012) when an immune response occurs due to the perception of 'danger' or 'alarm' signals somewhere. This theory seems to be well supported in the domain of innate immunity in invertebrates (Pradeu & Cooper 2012). The list of signals triggering an immune response is increasing with time and with damage signals (e.g., IL-1, extracellular matrix components generated after cellular injury and high concentrations of ATP) recently described by other investigators (Pradeu & Cooper 2012).

Rather than downstream TCA cycle intermediates being depleted as a consequence of this second break at SDH/CII, the metabolic response involves enrichment of the aspartate-arginosuccinate shunt pathway which provides a compensatory mechanism to replenish the system (Jha et al. 2015), thus leading to significant increases in levels of fumaric and malic acids regardless of SDH/CII inhibition (Lampropoulou et al. 2016). In agreement, both of these TCA metabolites were over-accumulated in virus-exposed larvae. Thus, our metabolite data suggest that larval oyster cells have a comparable host response to OsHV-1 µVar as mammalian macrophages when stimulated or infected with other viruses, including herpes-type. To the best of our knowledge, this is the first report of such metabolic reprogramming of the TCA cycle in an invertebrate with the specific metabolite signature of pathogen-induced *Irg1* transcription directly in accordance with vertebrate cell models. How OsHV-1 might stimulate genomic components leading to activation of *Irg1* transcription in oysters is not known, but would likely share some parallels with mechanisms of higher taxa.

In human and murine cell lines, *Irg1* expression is regulated by the transcription factor Interferon Regulatory Factor 1 (IRF1) (Tallam et al. 2016). IRF1 modulates the production of various interferons which are non-specific inhibitors of mRNA transcription that can reduce rapid replication of viruses and switch on genes of the innate immunity pathway, such as *Irg1* to eliminate pathogens (Owens & Malham 2015; Naujoks et al. 2016). The roles of cytokines of the interferon (IFN) family have been studied extensively in vertebrate models, but their functional presence is less clear in invertebrates. Nevertheless, a number of studies implicate IFN involvements in marine mollusc innate immunity (Miyazaki et al. 2000; Canesi et al. 2003; Nehyba et al. 2009; Bettencourt et al. 2010; Huang et al. 2013; Zhang et al. 2015, 2016), and, although there are uncertainties and gaps regarding the pathogen-dependant activation of IRF1 in molluscs, the identification of IRF1- and IFN-like sequences indicate the presence of

interferon-sensitive response elements in bivalve genomes (Green & Montagnani 2013; Wang et al. 2013a; Rosani et al. 2015; Gerdol & Venier 2015; He et al. 2015; Ertl et al. 2016; Green et al. 2016). For example, recent identification and functional analysis of an IFN-like protein (*Cg*IFNLP) in *C. gigas* not only confirmed the existence of IFN in molluscs, but also established that some invertebrates, such as oysters, have IFN-based antiviral responses (Zhang et al. 2015), contrary to previous expectations of the IFN system being a much later-evolved component of immunity in vertebrate-only lineages (Langevin et al. 2013).

Only two cases of *Irg1* involvement in marine mollusc immune responses have thus far been reported. Martín-Gómez et al. (2012) detected an up-regulation of *Irg1* transcription in the flat oyster, *Ostrea edulis*, exposed to Bonamiosis disease under light and heavy infection scenarios, which suggest that *Irg1* could play a role at early infection stages with prolonged expression at later stages. Furthermore, although not stated nor discussed in their manuscript, He et al. (2015 [supplementary material]) identified via untargeted gene expression profiling that the *C. gigas Irg1* transcript was over-expressed 9-fold in adult oysters exposed to OsHV-1 at the height of the viral replication process. In combination with our findings of a classic metabolic signature for *Irg1* over-expression and enhanced aconitase activity in virus-exposed larvae, these data are strongly supportive of an active role of *Irg1* and its metabolic product, ITA, in the innate immunity of oysters, and further provide the first reports of such associated pathophysiological mechanisms of disease in marine invertebrates. Moreover, these data also suggest that this particular metabolic reprogramming mechanism develops very early in the oyster lifecycle, and is a conserved feature of immunity across the metamorphic boundary. These findings provide fresh insights into the early evolution of innate immunity. We suggest that a detailed characterisation of this system, including endogenous regulatory networks and exogenous effectors, be conducted through ontogeny which may provide

useful information for identifying disease resistant traits. Investigation of other mechanisms associated with altered host energy metabolism, such as the Warburg effect, may also deliver important insights into the pathophysiology of the disease.

6.4.3 Warburg effect

The Warburg effect is an abnormal metabolic shift that was first discovered in proliferating cancer cells (Ferreira 2010). It has since been detected in vertebrate cells infected by viruses (Delgado et al. 2010, 2012; Darekar et al. 2012; Thai et al. 2014), and was recently implicated as an actuated pathway during viral infections in shrimp and oysters (Corporeau et al. 2014; Su et al. 2014; Hsieh et al. 2015; Fan et al. 2016; Li et al. 2016). Herpes-type viruses are known to activate oncogenes (genes responsible for causing cancer), thus providing a mechanistic link with cancerous cell phenotypes (Mesri et al. 2014). The Warburg effect is distinguished by a high rate of glycolytic flux and unusual aerobic fermentation of glucose to lactic acid even though there is enough oxygen available for OxPhos to proceed (Kelly & O'Neill 2015). It is often accompanied by the activation or enrichment of other metabolic pathways that provide energy and direct the flow of carbon and nitrogen, such as the pentose phosphate pathway, nucleotide biosynthesis, lipolysis, and glutaminolysis (Zaidi et al. 2013; Tannahill et al. 2013; Su et al. 2014; Sanchez & Lagunoff 2015; Li et al. 2016), and also with mechanisms of innate immunity such as *Irg1* activation/ ITA over-accumulation (Kelly & O'Neill 2015).

Metabolic alterations characteristic of the Warburg effect involves increased glycolysis, elevated levels of lactic acid, and changes in rates of nicotinamide adenine dinucleotide phosphate (NADPH) production/utilisation. These effects result from the diversion of glucose metabolism, glutamine oxidation, and requirements of reducing equivalents for FA biosynthesis and for mounting anti-oxidant responses to Reactive Oxygen Species (ROS) via re-oxidisation of glutathione (vander Heiden et al. 2009;

Weljie & Jirik 2011; Senyilmaz & Teleman 2015). Although the precise initiating mechanism/s responsible for reprogramming the glycolytic and gluconeogenic pathways that result in these metabolite changes are not yet completely understood (Vijayakumar et al. 2015), succinic acid accumulations act as an innate immunity regulatory signal to trigger a switch in core metabolism from OxPhos to glycolysis. Succinic acid stabilises the alpha subunit of hypoxia inducible factor 1 (HIF-1α) thereby activating transcription of genes which downregulates OxPhos (e.g., via indirect inhibition of pyruvate kinase to reduce TCA cycle flux), enhances glycolysis (e.g., via increased production of hexokinase and glucose transporters), and promotes lactic acid production (e.g., via regulation of lactate dehydrogenase and monocarboxylate transporter 4) (Ben-Shlomo et al. 1997; Selak et al. 2005; Semenza 2010; Palsson-McDemott & O'Neill 2013; Tannahill et al. 2013; Mills & O'Neill 2014). Thus, with ITA-induced inhibition of SDH/CII, succinic acid may be an important metabolite linking *Irg1* activation with the Warburg effect in virus infected cells.

Compared to baseline control larvae, lactic acid was over-accumulated in OsHV-1 µVar-infected larvae, whereas NADPH levels were lower. Secondary bioinformatics analysis of the metabolomics data also recognised glycolysis/gluconeogenesis and nucleotide metabolism as being differentially modulated as a larval host response to the virus, which could reflect an active Warburg-like effect. Our findings align with those of Corporeau et al. (2014) who utilised a proteomic-based approach to assess global protein changes in adult oysters infected with OsHV-1 µVar. Altered host protein expressions included changes in mitochondrial membrane permeability (accumulation of voltage-dependant anion channels [VDAC]), and enhanced glycolysis via an increase in the glycolytic enzyme Triose phosphate isomerase and decreases in the gluconeogenic enzymes Fructose 1,6-biphosphatase and Malate dehydrogenase (MDH); signatures which resemble induction of the Warburg effect (Chen et al. 2011; Maldonado &

Lemasters 2012; Corporeau et al. 2014). Supporting the findings of Corporeau et al. (2014), increased and decreased expressions of genes encoding VDAC and MDH, respectively, were detected in adult oysters exposed to the virus (Renault et al. 2011; He et al. 2015). Taken together, these characteristic evidences at various levels of organisation (i.e., gene, protein and metabolite) suggest an involvement of the Warburg effect as a pathophysiological feature of OsHV-1 µVar infection.

It is thought that the Warburg effect in cancer cells is adapted to facilitate the uptake and incorporation of nutrients into the biomass needed to produce new cells during proliferation at the expense of efficient, albeit slow, ATP production via OxPhos (vander Heiden et al. 2009; Zhang et al. 2012). The functional purpose for selection of energy inefficient lactic acid fermentation over OxPhos in virus-infected oysters is less clear. However, it is possible that the Warburg effect is 'strategically' induced by OsHV-1 as a metabolic reprogramming mechanism beneficial to the pathogen. With the catabolism of glucose exceeding the bioenergetics needs of cells during Warburg activation (Thomas 2014), the high yields of intermediates created through enriched glycolysis and a truncated TCA cycle could be used for production of purine and pyrimidine nucleotides and other components required for viral DNA synthesis and envelope assembly. Aerobic fermentation would also provide energy for these processes more swiftly than through OxPhos and with less risk of constraining glycolytic flux via ATP-induced negative feedback inhibition (Zhang et al. 2012; Sanchez & Lagunoff 2015), thus facilitating rapid and persistent viral replication.

6.4.4 Oxidative stress

It was hypothesised that significant changes in the abundances of metabolites reflective of oxidative stress would be represented in OsHV-1 Var-exposed oyster larvae. Exposure to invading pathogens initially triggers robust innate immune responses, and a rapid

release of reactive oxygen species (ROS) called an oxidative burst is usually registered soon afterwards (Torres et al. 2006). Some of the reactive species generated include hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radical (OH), hypochlorite (OCl^-), and peroxynitrite ($OONO^-$) (Pisoschi & Pop 2015). As strong oxidants, productions of ROS are beneficial since they can facilitate degradation of invading pathogen biomaterial. ROS also are involved in antiviral defense mechanisms by acting as signalling molecules to potentiate other immune responses, such as activation of IRF's/IFN's (Chiang et al. 2006). However, when produced in excess, they can cause irreparable damage to crucial host cells through degradation of macromolecular cellular components, including lipids, proteins, and DNA (Pisoschi & Pop 2015). During viral infections, this can actually promote virus proliferation by enhancing dispersion from lysed or apoptotic cells (Stehbens 2004). Thus, oxidative bursts should ideally be reduced before attaining critical levels, and can be achieved through an intricate balance of co-regulated antioxidant processes. These include production of the antioxidant metabolite glutathione (GSH), and a number of enzymes (e.g., GSH reductase, GSH peroxidase, GSH-S-transferase, catalase, superoxide dismutase) which regulate GSH turnover, directly recycles ROS, or are involved in repairing ROS-induced damage (Knight 2000; Apel & Hirt 2004). Adult and juvenile oysters exposed to OsHV-1, or showing variable susceptibilities to POMS, display differential expression of these enzymes, and/or the genes which encode them (Fleury et al. 2010; Fleury & Huvet 2012; Schmitt et al. 2013; Normand et al. 2014; Corporeau et al. 2014; He et al. 2015). This indicates a change in ROS balance and induction of oxidative stress as a response to the infection, and also suggests that the ROS-regulatory system is an important feature which underpins disease resistance.

We detected a relatively high coverage of metabolites within the glutathione metabolism pathway. However, subtle variations of metabolites central to network topology, such as glutathione itself, were not differentially expressed resulting in the

entire pathway being only marginally affected (p = 0.057). On the other hand, the transulphuration pathway (cysteine and methionine metabolism) which is responsible for supplying precursor metabolites for glutathione synthesis under low-mid stress conditions was altered, which indicates a mild oxidative stress response. The subtle signs of oxidative stress and perturbed redox balance in virus-exposed larvae indicate that the homeostatic control mechanisms responsible for governing the production and detoxification of ROS were functioning at optimal capacities and well within acceptable boundaries. These findings suggest that OsHV-1 either does not induce major oxidative stress in oyster larvae beyond the adaptive ability of the ROS-regulatory system, or that the level or stage of infection in our study was low or early, respectively. These results also may highlight a potential limitation in the exclusive use of metabolomic-based approaches to recognise changes in metabolic activity under circumstances where enzymatic regulation tightly constrains metabolite levels within the range of normal baseline variations. Indeed, cellular metabolism, and glutathione turnover/ ROS regulation in particular, is extremely well-adapted to achieve this feat. Thus, to better define the influence of OsHV-1 on oxidative stress parameters, further analysis of enzymes associated with glutathione recycling and ROS regulation would be required.

6.4.5 Other signatures

A number of other metabolites were considered to be important features responsible for larval health class discrimination in PCA, PLS-DA and RF models. These included elevated levels of 4-hydroxyphenylacetic acid, 4-hydroxyproline, and 2-aminoadipic acid, and a reduction in nicotinic acid contents. Four unannotated metabolites were also important in the multivariate models. Future efforts to identify these molecules may further complement our interpretations or provide new insights into the virus-host interaction.

4-hydroxyphenylacetic acid (4-HPA) is a tyrosine-derived metabolite with antioxidant activity that can scavenge reactive oxygen and nitrogen species *in vitro* and *in vivo* (Biskup et al. 2013), and also has an ability to reduce excessive release of proinflammatory cytokines which protects against inflammation and disease (Liu et al. 2014; Ford et al. 2016). Reports of 4-HPA in invertebrates are limited. 4-HPA has been detected in the neuroendocrine system of larval stage locusts where it is thought to be derived from tyrosine via tyramine intermediate (Tanaka & Takeda 1997), and also has been extracted from marine sponges (Yang et al. 2012). Increased levels of 4-HPA are associated with various mammalian disease pathologies and inborn errors in metabolism, including phenylketonuria and tyrosinemia disorders (Xiong et al. 2015), pancreatic and lung cancers (Nishiumi et al. 2010; Hori et al. 2011), chronic renal failure (Kikuchi et al. 2010), lysinuric protein intolerance (Kurko et al. 2016) and liver disease (Manna et al. 2010, 2015). An accumulation of this metabolite during such disease onsets has been attributed to differential catabolic pathways of tyrosine (Xiong et al. 2015). In our study, tyrosine metabolism was identified as a pathway with signs of being differentially regulated. It was recently demonstrated that the mechanism by which 4-HPA reduces proinflammatory cytokine production involves suppression of their transcription via promotion of HIF-1α protein degradation (Liu et al. 2014). Thus, with a functional role in downregulating HIF-1 activity, 4-HPA could directly compete with *Irg1*/ITA/succinic acid-induced HIF-1α stabilisation. As a result, HIF-1 induced enrichment of pathways responsible for redirecting carbon and nitrogen metabolism in trajectories which support OsHV-1 proliferation might be moderated, whereas the negative host consequences associated with co-induced respiratory dysfunction and excessive inflammation may partially be alleviated.

4-hydroxyproline (4-HP) is produced via the posttranslational hydroxylation of proline and is formed in proteins only after peptide linkage (Cooper et al. 2008). 4-HP is

predominantly found in collagen, a major structural component of the extracellular matrix (ECM) scaffold in marine invertebrate embryos and larvae (Spiegel et al. 1989; Phang et al. 2010). Thus, accumulation of free 4-HP is a specific biomarker of collagen degradation, and indicator of cell structure damage through compositional transformation of the ECM (Karna & Palka 2002; Phang et al. 2008). The production of free 4-HP resulting from ECM degradation is thought to play a role in initiating the apoptotic cascade (programmed cell death) via activation of the caspase-9 protease (Cooper et al. 2008), as well as promoting HIF-1 activity by inhibiting the degradation of HIF-1α (Surazynski et al. 2008). Matrix metalloproteinases (MMPs), such as collagenase, are responsible for degrading the ECM. MMPs play crucial roles during normal embryonic and larval development, such as in cell growth and differentiation, tissue remodelling, and mechanisms of immunological defense (Mannello et al. 2003, 2005; Mok et al. 2009). However, MMPs can be excessively produced in pathological situations such as inflammation, cancer progression and infections (Itoh et al. 2006; Phang et al. 2008). Physical stress, oncogenic transformation, ROS and cytokines are all inducible factors (Mancini & Battista 2006; Reuter et al. 2010). MMPs and their importance in restructuring the ECM as a response to pathogens have previously been implicated in OsHV-1 infections and disease resistance vs. susceptibility traits of oysters (McDowell et al. 2014; Nikapitiya et al. 2014; Rosani et al. 2015). The elevated levels of free 4-HP in OsHV-1 µVar-exposed larvae indicates that collagen degradation in the ECM was enhanced, although further investigation will be required to determine whether the 4-HP accumulations represent negative consequences for the host due to significant cell structure damage.

2-aminoadipic acid (2-AAA) is a component of the lysine metabolism pathway and is recognised as a small-molecule biomarker of oxidative stress (Sell et al. 2007; Zeitoun-Ghandour et al. 2011). Its presence has been linked with diabetes risk and regulation of

glucose homeostasis (Yuan et al. 2011; Wang et al. 2013b), and elevated levels have been reported as a putative biosignature of respiration chain disorders (Smuts et al. 2013). Production of 2-AAA in fish is associated with low oxygen transport capacity (Allen et al. 2015), and can be induced in shellfish by exposure to physiological stressors (Chen et al. 2015; Koyama et al. 2015). Accumulations of 2-AAA are also associated with oncogene activation and carcinogenesis, leading to its recent candidacy as a potential new clinical biomarker for various cancers (Hori et al. 2011; Bellance et al. 2012; Jung et al. 2013; Rosi et al. 2015; Ren et al. 2016). Production of 2-AAA correlates with the bioenergetic signature characteristic of a switch in cellular respiration modes from OxPhos to aerobic glucose fermentation (Hori et al. 2011; Aa et al. 2012; Bellance et al. 2012). Thus, the accumulation of 2-AAA in virus-exposed larvae is consistent with the global changes we detected in organic acid metabolism reflective of TCA cycle reprogramming, reduced mitochondrial respiration and ATP production, activation of the Warburg effect, and subtle signs of oxidative stress.

Nicotinic acid (NA) plays an important role in redox reactions and can be converted to nicotinamide (NAM) *in vivo*. In invertebrates and some fish, NA and NAM, collectively known as niacin or vitamin B3, are important precursors for synthesis of the pyrimidine nucleotide coenzymes NAD^+ and $NADP^+$ which participate in many hydrogen transfer processes, such as fatty acid synthesis, lipolysis and glycolysis (Ng et al. 1997; Sauve 2008; Houtkooper et al. 2009; Cantó et al. 2015; Yuasa & Ball 2015; Yuasa et al. 2015). NAD^+ is also a substrate and signalling metabolite required for regulation of transcription, proteasomal function, and posttranslational protein modifications involved in DNA replication, recombination, repair mechanisms and maintenance of genomic stability (Bürkle 2001; Surjana et al. 2010; Vyas et al. 2013; Fouquerel & Sobol 2014; Cantó et al. 2015). Unlike most metabolic redox reactions which reversibly oxidise or reduce pyrimidine nucleotides to maintain constant levels of NAD^+/$NADP^+$, substrate

utilisation and NAD^+-dependant signalling processes are highly consumptive, and regeneration from niacin precursors is required when such mechanisms are activated (Lin 2007; Chiarugi et al. 2012). The reduction of free NA in virus-exposed larvae is consistent with its role in these processes which are upregulated during herpes-type viral infections (Grady et al. 2012; Li et al. 2012). Herpes-induced consumption of NAD^+ as a substrate for enzymes involved in host DNA modifications is likely a response to DNA damage pathways being activated by replication of the viral genome (Grady et al. 2012). However, efficient virus replication itself and synthesis of viral proteins are also reliant on NAD^+ substrate supply (Li et al. 2012). Thus, the importance of NA and NAD^+/$NADP^+$ metabolism in host-pathogen interactions is gaining considerable attention as targets for the treatment of infectious diseases in humans (Mesquita et al. 2016). Interestingly, activation of the Warburg effect involves the unusual overproduction of NAD^+ via enhanced fermentation of glucose (i.e., pyruvic acid + NADH → lactic acid + NAD^+) (Chiarugi et al. 2012), and may serve/function as a replenishing mechanism in response to NAD^+ depletion to complement *de novo* synthesis from its niacin precursors.

6.5 Conclusion

In summary, we identified and measured the metabolic responses of oyster larvae during exposure to the virulent ostreid herpesvirus microvariant which has recently been responsible for mass mortalities of shellfish around the globe. Viruses can reshape their host's metabolism to create a unique metabolic state that supports their specific requirements. Indeed, profiling of larval metabolites revealed virus-induced reprogramming of host-encoded metabolic networks, including alterations to the glycolytic pathway, the TCA cycle, and lipid metabolism. Intriguingly, we observed metabolic response parallels with a number of innate immune system mechanisms previously characterised in mammalian cell models, such as induction of the Warburg

effect and downstream metabolic consequences of immunoresponsive gene 1 activation. The functional genomes of OsHV-1 and its variants are mostly unknown at present, but it is likely that virus-encoded auxiliary genes also provide infected host cells with novel metabolic capabilities, and the outcomes of their transcription may be manifested within our results. These findings provide the first comprehensive insights into early ontogenic host physiology and susceptibility of oysters towards OsHV-1 µVar. Characterisation of host-virus interactions can provide knowledge to enable development of therapeutic agents and identify traits for improving the outcome of selective breeding programmes. Our study also highlights the value of metabolomics-based approaches in elucidating host-virus interactions and the metabolic networks which characterise and underpin the pathophysiological state, and further supports its application for investigating pathogenesis of disease in early life stage marine invertebrate models.

6.6 References

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Chapter 7 Mollusc larval toxicology

Metabolic regulation of Cu^{2+} toxicity during marine mussel embryogenesis

Note: This chapter is being prepared for publication:

Young T., Gale S.L., Alfaro A.C., Ragg N.L.C., Le D.V., Sander S.G., Burritt D.J., Benedict B., Villas-Bôas S.G. Assessing health of early life stage marine molluscs: Application of a multiresolution, multidisciplinary biomarker toolkit. *In prep*

Young T., Gale S.L., Alfaro A.C., Ragg N.L.C., Le D.V., Sander S.G., Burritt D.J., Benedict B., Villas-Bôas S.G. Metabolic regulation of copper toxicity during marine mussel embryogenesis. *In Prep*

Part A

Untargeted metabolite profiling

Abstract

The development of new tools for assessing the health of cultured shellfish larvae is crucial for the New Zealand aquaculture industry, which is seeking to develop and refine hatchery methodologies. To this end, a large scale ecotoxicology/ health-stressor trial was established by exposing mussel (*Perna canaliculus*) embryos to copper. GC/MS-based metabolomics was applied to identify potential biomarkers for monitoring embryo/larval health, and to also characterise mechanisms of copper toxicity in these organisms for the first time. Cellular viability, developmental abnormalities, larval behaviour, and mortality were simultaneously evaluated to provide a complementary framework for interpretative purposes, and authenticate the biochemical data.

Sublethal levels of bioavailable copper resulted in coordinated metabolite profile changes which were dependant on development stage, treatment level, and exposure duration; and also had negative impacts on developmental timing and larval behaviour. Metabolic trajectory analysis indicated that larvae were successfully employing various endogenous mechanisms involving biosynthesis of antioxidants and a restructuring of energy-related metabolism in an attempt to alleviate the toxic effects on cells and developing tissues. These results suggest that regulation of trace metal-induced toxicity is tightly linked with metabolism during early ontogenic development of marine mussels. Lethal-level bioavailable copper induced severe metabolic dysregulation after 3 hrs exposure which worsened with time, substantially delayed embryonic development, initiated the apoptotic pathway, provided many evidences for the occurrence of oxidative stress, and resulted in cell/organism death shortly after 18 hrs exposure.

In summary, this case study provides strong support for the application of metabolomics to assess the health status of bivalve embryos and larvae, detect early warning biomarkers for trace metal contamination, and identify novel regulatory mechanisms of copper-induced toxicity.

7.1 Introduction

Copper is an essential trace element involved in many crucial biological processes, and is required for the survival of all living organisms (Uriu-Adams & Keen 2005). Cells use copper as a structural element in regulatory proteins, and due to its unique redox potential, copper serves as a cofactor for several enzymes which carry out fundamental functions in embryonic development (Lee et al. 2001), mitochondrial respiration (Madsen & Gitlin 2007; Horn & Barrientos 2008), iron metabolism (Nose et al. 2006), antioxidant defense (Klotz et al. 2003), and synthesis of neurotransmitters and neuropeptides (Scheiber et al. 2014). However, when intracellular concentrations exceed the capacity of cells to sequester the ion, free copper is extremely cytotoxic. Redox cycling between Cu^{2+} and Cu^{+} can catalyse the production of highly toxic hydroxyl radicals, known as reactive oxygen species (ROS) (Harrison et al. 2000). Although ROS themselves are essential for many biological processes at low concentrations (including embryonic development [Dennery 2007]), when generated in excess they can severely damage various cellular components via oxidative attack of lipids, proteins, DNA, and other biomolecules (Temple et al. 2005). Thus, to ensure that metal availability is in accordance with physiological needs, it is crucial that all organisms have mechanisms in place to detect and regulate intracellular copper levels through a controlled balance of uptake, efflux, and sequestration (Puig & Thiele 2002; Nose et al. 2006; Kim et al. 2008; Rubino & Franz 2012).

Bivalve molluscs have high capacities to bioaccumulate dissolved and particulate-bound metals from seawater, and are routinely used for trace metal contamination biomonitoring purposes around the world (e.g., the long-running US National Oceanic and Atmospheric Administration's 'Mussel Watch' program [reviewed by Schöne & Krause 2016; Farington et al. 2016]). However, many bivalves are also particularly sensitive to copper (Arnold et al. 2009), and are absent from some coastal zones where

human activities are responsible for high loadings of the metal into marine environments (Funes et al. 2006). Dissolved copper concentrations in oceanic and coastal waters typically range from 0.05–1.7 μgL^{-1} (Neff 2002). However, concentrations as high as 20 μgL^{-1} have been detected in New Zealand harbour waters (Gadd & Cameron 2012). This value far exceeds the Australian and New Zealand Environment and Conservation Council 95% protection trigger value of 1.3 μgL^{-1} (ANZECC & ARMCANZ 2000), and the EC50 embryotoxicity values reported for numerous marine invertebrate species (e.g., mussels [Martin et al. 1981; Rosen et al. 2005; Nadella et al. 2009; Bosse et al. 2014], oysters [Martin et al. 1981; Worboys et al. 2002] and sea urchins [King & Riddle 2001]). Contributing sources of copper in New Zealand waters include stormwater drainage, leachates from antifouling paints, and run-off from hard-stand activities, such as boat-washing, scraping and repainting (Gadd & Cameron 2012). For the Auckland region alone, it is estimated that up to 22,000 kg of copper are leached from boat hulls every year (Beca Infrastructure 2012), with about half of the quantity coming from boats berthed in marinas (Gadd et al. 2011). Toxic levels of marine pollutants such as copper have the potential to adversely affect wild populations of endemic New Zealand marine molluscs (as already has been case for tin [Smith 1996]), and cultured species with high commercial value.

Shellfish farming is a lucrative primary industry for New Zealand, and is currently being targeted for expansion (reviewed in Chapter 1). In particular, cultivation of the endemic Greenshell^{TM} mussel (*Perna canaliculus*) is a sector which is realising considerable investment for development. For example, recent construction of a commercial-scale mussel hatchery is expected to provide farms with high quality spat in the very near future (reviewed in Chapter 1). However, this species is one of New Zealand's most sensitive endemic bivalves to heavy metal contamination (Chandurvelan et al. 2012), and a number of knowledge gaps exist regarding the toxicity thresholds of

certain metals, such as copper, and their toxicological mechanisms in different life history stages (i.e., embryos/larvae/adult). In addition, identification of new tools for assessing early life-stage health would be highly beneficial for the developing mussel aquaculture sector. Currently, hatchery practices rely on highly skilled personnel with significant experience in identifying visual signs of poor health through microscopic observations (e.g., larval coloration, behaviour), and general biological parameters (e.g., growth rate, food consumption, mortality) which may lack informative resolution (e.g., temporal precision of detecting poor health, causation, and predictive capacity).

Metabolomics-based approaches (reviewed in Chapter 3) have recently been applied within the field of aquatic ecotoxicology to assess health status, identify biomarkers of pollution exposure, and investigate mechanisms of toxicity for a variety of environmental contaminants in marine molluscs, such as abalone (Zhou et al. 2015), oysters (Chen et al. 2016; Ji et al. 2015a, 2016a), clams (Liu et al. 2011a,b; Wu et al. 2013a,b; Hanana 2014; Campillo et al. 2015; Ji et al. 2013a, 2015b,c,d), and mussels (Cappello et al. 2013; Ji et al. 2013b, 2016b; Qiu et al. 2016; Song et al. 2016a,b; Yu et al. 2016). The results of such studies are demonstrating that the effects of toxin exposure on the profile of metabolites in various aquatic organisms, and their influence on particular metabolic pathways, are toxin-specific (Song et al. 2016a,b), species-specific (Liu et al. 2011a), pedigree-specific (Liu et al 2011b), sex-specific (Ji et al. 2013b, 2014, 2016b), size-specific (Spann et al. 2011), tissue-specific (Wu & Wang 2011; Li et al. 2014), and environment-specific (Wu et al. 2013a,b). All of the toxicological investigations on molluscs conducted thus far which contain a metabolomics-based component have focused solely on the metabolic responses of the adult life-stage. However, it is well-recognised that embryonic and larval stages of molluscs are far more susceptible to toxins than their juvenile or adult counterparts (Mohammed 2013). Indeed, their great sensitivity is one of the principal rationales as to why mollusc embryos are

routinely used as animal models for standard aquatic ecotoxicity testing around the globe, such as the 'Bivalve Acute Toxicity Test (Embryo-Larval)' method (EPA 1996; His et al. 1997; Rosen et al. 2005; Nadella et al. 2009; Fabbri et al. 2014). The bioassay uses first appearance of the characteristic D-shaped larval shell as a marker for normal development, and is usually performed with oyster or mussel embryos as test organisms.

Although the application of metabolomics in mollusc embryo ecotoxicology and health assessment has not thus far been realised, investigations using embryos of other animal models (e.g., humans, foul and fish) demonstrate that such approaches can successfully be applied to provide rich sets of biochemical information. For example, metabolomics-based approaches have been used to detect general metabolic responses of embryos and embryonic stem cells to toxin exposures (Viant et al. 2005; Van Scoy et al. 2012; Mattsson et al. 2015), characterise mechanisms of toxicity and tolerance (Viant et al. 2006a,b; Akhtar et al. 2016; Chai et al. 2016; Wang et al. 2016), identify biomarkers reflective of toxin-induced stress with predictive capacities (West et al. 2010; Kleinstreuer et al. 2011), and to establish biomarker assays for developmental toxicity screening (Palmer et al. 2013). Thus, it is expected that the transfer of these high resolution technologies and methodologies to the study of marine invertebrate embryonic models will substantially expand our available 'toolkit' of capabilities for characterising embryonic baseline metabolic processes, detecting endogenous responses to environmental influences such as toxin and pathogen exposures, and identifying sensitive and robust biomarkers reflective of health status. To test this novel application, GC/MS-based metabolomics is used to assess the effects of copper on the embryonic and larval metabolomes of the commercially important New Zealand Greenshell™ mussel.

7.2 Methods

7.2.1 Experimental design summary

Fertilised mussel embryos (1 hr post-fertilisation) were exposed to various concentrations of copper in the presence of EDTA during a large scale hatchery trial at the Cawthron Institute Aquaculture Park (CAP), Nelson, New Zealand. Approximately 150 million embryos were evenly distributed among 15 × 170 L conical plastic tanks containing natural seawater and 4 µM EDTA. The addition of EDTA into the embryonic incubation seawater is current standard commercial practice for the first 48 hrs of development [personal communication, N. Hawes; SPATnz, 2015], and is important for successful development of *P. canaliculus* embryos [Buchanan 1999; Gale et al. 2016]). Volumes of a copper sulphate stock solution were added to the relevant tanks to provide four nominal levels of total copper (0.00, 0.10, 0.20, 0.30 mgL^{-1}), with three tank replicates per treatment level. Samples of embryos/larvae were taken recurrently from each tank over a period of 72 hrs for assessments of cell viability and mortality, development rate, structural abnormalities, swimming behaviour, and metabolite profiling. A comprehensive compositional analysis of the source seawater used in the experiment was conducted through a variety of standard analytical methods. Due to the complexities of incorporating EDTA into the experimental design, analysis of copper speciation during the trial was essential, and thus determined via diffusive gradients in thin films, mass spectrometry, and a newly-established metal speciation modelling approach. The specific aquatic toxicology context of this chapter also prescribes that a higher level of methodological detail be provided to describe the seawater chemistry and laboratory protocols used than in previous chapters.

7.2.2 Chemicals and seawater preparation

Unless otherwise stated, all reagents were obtained from Sigma-Aldrich Pty Ltd (Sydney, Australia). Stock solutions of $CuSO_4$ (1000 mgL^{-1} dissolved in Milli-Q^{TM} H_2O; Quantum

R Tex cartridge with Biopack polisher) and EDTA disodium salt (5 mM molecular grade [Scharlau: Chemie, Spain] dissolved in Milli-Q H_2O, and buffered to pH 8.0 with 5M NaOH) were prepared using acid washed glassware and stored at 4°C three days prior to use. Twenty four hours before the start of the experiment, embryo incubation tanks were filled with 154.8 L of 16°C seawater. This seawater had been pumped from the Tasman Bay (Nelson) and filtered to 40 µm (Arkal Filtration Systems CS Ltd: Jordan, Israel); stored in 25,000 L tanks; pumped sequentially through three in-line filters 25 µm, 5 µm and 1 µm; foam fractionated (PPS4 Foam Fractionator – Aquasonic: NSW, Australia); temperature mixed (achieved by drawing from a 10,000 L storage tank maintained at the target temperature via an external loop passing through a titanium plate heat exchanger); and end-of-line 1 µm cartridge filtered (model E1PP10-FG – Filterpure: Auckland, New Zealand).

Once all tanks were filled with seawater, 132 mL of stock EDTA solution was added to each tank (based on prior optimisation trials at CAP; unpublished data) to provide final EDTA concentrations of 4 µM, and gentle aeration was provided (500–1000 mL min^{-1}). At the same time, 15 × 15 L fertilisation tanks were filled with 10.2 L of similarly treated seawater, and 12 mL of stock EDTA was added to each tank. The bulk seawater and EDTA in all tanks were given 24 hours to mix and become thermally stable at room temperature (17°C). Two hours prior to adding the fertilised mussel embryos, aliquots of the $CuSO_4$ stock solution were administered to the relevant embryo incubation tanks in order to give the systems time to equilibrate/stabilise (i.e., mixtures of dissolved organic matter, inorganic compounds, EDTA-metal complexes and various other Cu-species).

7.2.3 Broodstock collection and spawning

Mussel (*Perna canaliculus*) broodstock (~300 individuals) were collected 24 hrs prior to spawning from suspended rope culture in Admiralty Bay (Marlborough Sounds, Nelson, New Zealand), and transported to CAP. Mussels were biosecurity treated according to CAP protocols (i.e., gently scrubbed clean, induced to close with a freshwater dip, and then treated with 0.4% [v/v] sodium hypochlorite in freshwater for 2–5 min), briefly rehydrated, and then maintained dry overnight under a damp cloth ~16°C ready for spawning the next day. According to the protocols described in Chapter 4, spawning was induced via thermal cycling and gametes from 50 female and 50 male mussels were collected, pooled by sex, and enumerated.

7.2.4 Fertilisation and tank incubation

Approximately 10.2 million oocytes were added to each of the 15 × 15 L fertilisation tanks containing 10.2 L of 24 hr aged seawater incorporating 4 µM EDTA. The sperm pool was diluted with FSW and aliquoted into each tank to provide a ratio of 500 sperm per oocyte. After 30 min, successful fertilisations were confirmed by the presence of polar bodies, and the total contents of each fertilisation tank were transferred into one of the incubation tanks (providing a total incubation seawater volume of 165 L). Ambient air temperature, and therefore tank seawater temperature, was maintained at 17 ± 1°C throughout the trial (routinely validated by spot thermometer checks of the water in the incubation tanks).

7.2.5 Embryo and larval sampling

A range of embryo and larval samples were taken from each of the incubation tanks at 3, 18, 42 and 72 post Cu-exposure for various microscopical and biochemical analyses. To first determine embryo/larval densities, triplicate 2 mL samples were taken from each tank via pipette (whilst homogenising the seawater with a large perforated plunger),

transferred to individual wells of BD Falcon™ 24-well Tissue Culture Plates (BD Biosciences: NSW, Australia), and enumerated under a microscope. To assess cell viability of embryos and larval survival, similar 2 mL samples were taken and characterised using a vital stain (neutral red) in accordance with protocols described by Young et al. (2015). To evaluate temporal developmental parameters, 2 mL samples were taken from each tank (while homogenising tank contents), pooled for each treatment level, gently filtered through a 15 µm mesh sieve, transferred to 1.5 mL Eppendorf tubes, and fixed in Davidson's solution (according to Howard & Smith 1983) for subsequent microscopy.

To obtain high biomass samples for metabolomic-based analyses (ca. 200,000 individuals per sample), organisms were sampled by opening valves at the bottom of the conical tanks (whilst homogenising) and draining a specific volume (calculated from the embryo/larval densities) into a 15 µm sieve immersed in seawater. Organisms were gently rinsed in FSW to remove debris/biofloc and treatment solutions, concentrated, transferred to 15 mL Falcon™ centrifuge tubes using 3 mL disposable plastic Pasteur pipettes (LabServ – Thermo Fisher: Auckland , New Zealand), and then made up to 14 mL with FSW. Samples were centrifuged at 4*g* for 30 sec and 6 mL of seawater was removed before being re-homogenised. 1.5 mL aliquots were transferred to a series of BioStor™ 2 mL RNase/DNase-free cryovials (National Scientific Supply: Claremont, CA, USA). Cryovials were centrifuged at 25*g* for 30 sec, excess seawater removed, then immediately snap-frozen in liquid nitrogen and stored at −80°C until metabolite extraction. Towards the end of the experiment (66 hrs post-treatment), samples of D-stage larvae (containing approximately 50 individuals) were directly taken via pipette from each incubation tank to assess swimming behaviour, and conduct in situ determinations of development stage, and presence/absence of structural abnormalities (e.g., shell deformation) by optical microscopy (Olympus model CK-X31).

7.2.6 Seawater chemistry

To characterise the composition of the bulk seawater used for incubations during the trial and evaluate Cu speciation differences among the treatment conditions, an array of analyses were performed by an IANZ accredited laboratory (R J Hill Laboratories Ltd, Hamilton, New Zealand), and the Centre for Trace Element Analysis (CTEA), University of Otago, New Zealand. These laboratories were selected for logistical purposes since fast analysis (< 24 h) of the seawater was imperative to prevent sample degradation and analyte loss, and also for the excellent quality control/assurance procedures that they employ. For total metals, alkalinity and selected nutrients testing, seawater samples were collected prior to EDTA additions from one of the 170 L control tanks which had been equilibrated for 24 h after filling. Samples were chilled to 4°C in insulated polystyrene boxes containing ice packs, and immediately sent for analysis to the respective laboratories.

7.2.6.1 Bulk seawater composition

The composition of bulk seawater prior to EDTA additions was characterised using standard analytical methods, according to APHA (2005) and included: total alkalinity (APHA 2520 B) total organic nitrogen (APHA 4500-N_{org} D), total ammonical nitrogen (APHA 4500-NH_3 F), nitrate and nitrite (APHA 4500-NO^{3-} I), dissolved reactive phosphorus (APHA 4500-P G), reactive silica (APHA 4500-SiO_2 F: modified from flow injection analysis), chloride (APHA 4500 Cl^- E: modified from continuous flow analysis), bromide (APHA 4110 B), total organic carbon (APHA 5310 B), and concentrations of up to 32 metals, including total dissolved Cu via ICP-MS (APHA 3125 B). However, in seawater some of those 32 elements are below detection limit. pH was monitored in situ using a calomel glass electrode, and salinity was calculated from the total chloride concentration, where: salinity (ppt) = $1.8066E^{-3}$ x [Cl^-] (mgL^{-1}). Temperature and pH readings (recorded with both bench top Mettler Toledo and mini handheld ISFET pH

meters [Shindengen model KS701]) were also performed during every seawater collection, every embryo/larval sampling, and also during the deployment and retrieval of Diffusive Gradients in Thin Film (DGT) passive samplers.

7.2.6.2 Cu speciation analysis

In order to assess actual vs. nominal total dissolved Cu concentrations, and to evaluate potential rates of Cu loss/gain through bio-uptake and/or adhesion to the tank material and/or evaporation, 500 mL of seawater was sampled from every incubation tank 2 hrs after embryos had been added, and also at the end of the trial (72 hrs later). Trace metal clean plastic containers (Nalgene, LabServe – Thermo Fisher: Auckland New Zealand) were used during seawater sampling, and gloves were worn at all times to avoid contamination. Samples were obtained by immersing a clean 15 µm sieve into the tanks and collecting the embryo-free seawater inside. These samples were immediately chilled to 4°C and dispatched for analysis to CTEA within 12 hrs of collection. Total dissolved Cu concentrations were measured by ICP-MS using an Agilent 7500ce system with an ESI PFA100 micro-concentric nebuliser, following the manufacturer's recommendations for robust conditions. Calibration was performed with commercial multi-element solutions (Claritas CLMS-2) with In and Sc as reference elements.

DGT passive samplers were also installed for the duration of the experiment to estimate the bioavailable Cu fraction. DGTs are a relatively new approach for in situ determinations of labile metal species in aquatic systems (reviewed by Zhang & Davison 2015). The devices house a binding gel, diffusive gel, and a membrane filter, and passively accumulate labile species from solution while deployed in situ. Labile species are assimilated by the binding gel in a predictable rate-controlled manner. After recovery of the DGT samplers, the gel layers are leached in acid and analysed by ICP-MS which allows the estimation of labile copper. Duplicate DGTs were randomly deployed into one

incubation tank for every treatment level, two hours after embryo additions. DGTs were placed adjacent to the air flow to promote movement of seawater across the devices, and care was taken not to contaminate the membrane filters. At the end of the experiment, DGTs were carefully recovered, packaged at 4°C and dispatched to CTEA for ICP-MS analysis.

The DGT-labile metal concentration was calculated according to Warnken et al. (2006), Davison & Zhang (2012) and Stewart et al. (2016). The bioavailable Cu concentration is assumed to be the labile fraction of dissolved Cu. Other Cu speciation was modelled using the Visual MINTEQ (v 3.0) program (Gustafsson 2011). While the inorganic-bound Cu speciation is defined by the bulk seawater composition, EDTA and DOC at the concentration levels quantified in the samples were used to estimate the free Cu concentration. A fixed humic acid to fulvic acid ratio of 1:9, as commonly assumed, was used to estimate the humic-bound Cu.

7.2.7 Metabolite analysis

Metabolites were co-extracted with an internal standard, derivatised, and analysed via GC-MS using the same protocols previously described in Chapter 6. Deconvolution of raw spectral data and primary bioinformatics were conducted in accordance with the protocols described in Chapter 4. An Excel file containing peak height data for each metabolite was generated and manually checked for the presence of contaminants. Aberrant records were removed and the resulting QC-filtered data was correctly formatted and saved as a .csv file for subsequent statistical analyses.

7.2.8 Statistical analysis and data visualisation

Statistical analysis of survival data was conducted using SPSS v22.0 statistical software (IBM Corp: Armonk, NY, USA). One-way Analysis of Variance (ANOVA) with Tukey's Post-Hoc Tests was performed separately for each of the three sampling times (18, 42,

and 72 post Cu-exposure) across the treatment levels. Statistical analysis of the development and behavioural data were not conducted since its structure violated the required assumptions, and clear trends in the data were visually apparent. Pre-processing and statistical analysis of the metabolite data was conducted using MetaboAnalyst v3.0 (Xia et al. 2015). After uploading the .csv files containing the formatted peak intensity tables, data were pre-processed using the same protocols outlined in Chapter 5 for QC purposes and to meet the distributional requirements for subsequent data analyses.

Lethal and sublethal effects of Cu on the embryonic/larval metabolic profiles were assessed separately due to the occurrence of high mortalities between 18 and 42 hrs post-exposure of embryos to 50.3 μgL^{-1} bioavailable Cu. To first investigate the underlying structure of the data, Hierarchical Cluster Analysis (HCA) was performed as an unsupervised classification method to identify inherent sample groupings. Euclidian distance and Ward's criterion were selected as the measure of distance and aggregation, respectively. Heatmap analysis combined with HCA of all metabolite features identified in the embryonic samples was performed to provide a simple visual summary of the data, and cluster metabolites with similar expression profiles across the samples.

For each exposure duration (3 and 18 hrs), supervised Projection to Latent Structures Discriminant Analysis (PLS-DA) was performed to construct sample classification models, and to identify metabolites which contributed most towards group partitioning. Metabolites contributing most towards the classification model were identified based on the Variable Importance in Projection (VIP) scores. Metabolites with VIP scores ≥ 1.0 were considered as being important to the model. The quality of the models were evaluated via the R^2 and Q^2 values of the first two latent variables (LVs), which indicates the total variation explained in the data and the cross-validated predicted variation, respectively, using the leave-one-out cross validation (LOOCV) approach.

To identify the metabolite features which were significantly altered by Cu treatment within each timeframe of exposure, Significance Analysis of Microarrays/Metabolites (SAM) and fold-change analyses were performed at 3 and 18 hrs post-treatment as univariate measures to discriminate the toxicological effects.

To assist interpretation of the data beyond investigation of single metabolite variations and examine the metabolomics profiles more comprehensively, secondary bioinformatics procedures were performed to extract various sets of functionally-related metabolites involved in the same biochemical pathways. These sets were then analysed together to determine the likelihood of entire pathways being differentially regulated as a response to Cu treatment. All reliably annotated metabolites were mapped into metabolic pathways using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database, and assessed via Quantitative Enrichment Analysis (QEA) and Network Topology Analysis (NTA) as described in Chapter 5. QEA and NTA were conducted on the control vs. treatment data for each exposure duration. In order to assess the effect of Cu exposure duration on the differential regulation of the enriched biochemical pathways, the results of the QEA and NTA analyses for each timeframe were extracted and re-plotted together (i.e., overlaid) so that any shifts in the significance of particular metabolic pathways could more easily be visualised and discerned.

To investigate the effects of sublethal Cu exposures, PLS-DA analysis of all metabolomics data across development stages and treatments was first performed. Since some data were missing for particular development stages (due to metabolite levels being zero or below the detection limits), these were replaced using the *k*-Nearest Neighbour approach as recommended by Armitage et al. (2015). Mean LV1 and LV2 values (± SE) were plotted on a 2-D score plot to summarise general temporal trends in the developing embryonic/larval mussel metabolome, and expose the toxicological effect on metabolism.

As the embryos developed, large phenotypic effects were clearly apparent in the baseline metabolic trajectory. Thus, all data were phenotypically normalised and subsequently re-analysed. The normalisation procedure involved division of each metabolite peak height by the respective mean control value for each development stage. This process essentially resulted in control sample values being centred on around 1.0, and treatment data being expressed as a relative fold-change compared to controls for each of the four developmental phenotypes. The produced plot revealed new information that was not discernible in the original plot. To provide a visual summary of the metabolite abundances which were responsible for the positioning of sample groups and the metabolic trajectories in the phenotype-normalised PLS-DA plot, Heatmap analysis with combined HCA of metabolites was performed. For each development stage/ exposure duration, mean $\log_2$ foldchange values relative to control samples were calculated, imported into PermutMatrix (v 1.9.3) software (Caraux & Pinloche 2005), then clustered using Euclidian distance and Ward's criterion as measures distance and aggregation, respectively.

7.3 Results

7.3.1 Seawater chemistry

The composition of the bulk seawater used during the experiment is presented in Table 7.1. The salinity and pH were 34.3 ppt and 8.1, respectively. Analysis of the DGT devices and the modelled Cu speciation data exposed large differences in the between-treatment composition of the Cu fractions throughout the experiment (Table 7.2).

Table 7.1. Composition of the bulk seawater used during the experiment prior to $CuSO_4$ and EDTA additions. See the methods section for details how the Cu-containing fractions were determined.

Bulk seawater composition					
Copper species/fraction	**Concentration**	**Element**	**Concentration**	**Element**	**Concentration**
Total dissolved Cu	2.90 μgL^{-1}	Aluminium	<13.0 μgL^{-1}	Magnesium	1.49 gL^{-1}
Humic-bound Cu	2.76 μgL^{-1}	Arsenic	<4.2 μgL^{-1}	Manganese	<1.1 μgL^{-1}
Free Cu^{2+}	0.01 μgL^{-1}	Barium	5.9 μgL^{-1}	Mercury	<80.0 ngL^{-1}
Inorganic-bound Cu	0.123 μgL^{-1}	Beryllium	<0.63 μgL^{-1}	Molybdenum	11.1 μgL^{-1}
Bioavailable Cu	0.133 μgL^{-1}	Boron	4.0 mgL^{-1}	Nickel	<6.3 μgL^{-1}
Nutrient profile	**Concentration**	Bromine	82.0 mgL^{-1}	Phosphorus	22.0 μgL^{-1}
Total ammoniacal-N	<10.0 μgL^{-1}	Cadmium	<0.21 μgL^{-1}	Potassium	0.4 gL^{-1}
Nitrite-N	<2.0 μgL^{-1}	Caesium	<1.9 μgL^{-1}	Rubidium	0.12 mgL^{-1}
Nitrate-N	4.0 μgL^{-1}	Calcium	0.42 gL^{-1}	Selenium	<4.2 μgL^{-1}
Nitrite-N + Nitrate-N	4.0 μgL^{-1}	Carbon	24.0 mgL^{-1}	Silver	<0.43 μgL^{-1}
Dissolved reactive P	8. 0 μgL^{-1}	Chloride	19.0 gL^{-1}	Sodium	11.2 gL^{-1}
Other	**Concentration**	Chromium	<1.1 μgL^{-1}	Strontium	8.7 mgL^{-1}
Alkalinity ($CaCO_3$)	116.0 mgL^{-1}	Cobalt	<0.63 μgL^{-1}	Thallium	<0.21 μgL^{-1}
Total Kjeldahl Nitrogen	<0.2 mgL^{-1}	Fluoride	1.6 mgL^{-1}	Tin	<1.7 μgL^{-1}
Reactive silica (SiO_2)	0.29 mgL^{-1}	Iron	5.4 μgL^{-1}	Uranium	3.3 μgL^{-1}
Dissolved non-purgeable organic C	1.1 mgL^{-1}	Lead	<1.1 μgL^{-1}	Vanadium	1.9 μgL^{-1}
		Lithium	0.19 mgL^{-1}	Zinc	6.3 μgL^{-1}

Table 7.2. Concentrations of various Cu species, presented as combined fractions, in the incubation tanks after addition of EDTA and the three levels of Cu treatment (T1–3). Note the differences in concentration units.

Copper species/fraction	**Experimental copper concentrations**			
	Control	**T1**	**T2**	**T3**
Target total dissolved Cu	0.0 μgL^{-1}	100 μgL^{-1}	200 μgL^{-1}	300 μgL^{-1}
Measured total dissolved Cu	2.6 μgL^{-1}	130 μgL^{-1}	250 μgL^{-1}	370 μgL^{-1}
Humic-bound Cu	26.0 ngL^{-1}	1.6 μgL^{-1}	9.0 μgL^{-1}	57.0 μgL^{-1}
EDTA-bound Cu	2.6 μgL^{-1}	130 μgL^{-1}	240 μgL^{-1}	260 μgL^{-1}
Free Cu^{2+}	0.06 ngL^{-1}	3.2 ngL^{-1}	83.0 ngL^{-1}	4.3 μgL^{-1}
Inorganic-bound Cu	4.0 ngL^{-1}	39.0 ngL^{-1}	0.98 μgL^{-1}	51.0 μgL^{-1}
Non-bioavailable Cu	2.6 μgL^{-1}	130 μgL^{-1}	249 μgL^{-1}	310 μgL^{-1}
Bioavailable Cu	0.47 ngL^{-1}	0.04 μgL^{-1}	1.1 μgL^{-1}	50.3 μgL^{-1}

The bioavailable Cu which was previously present in the source seawater was almost entirely chelated (99.6%) by the addition of EDTA in the control tanks. The presence of the chelator also made substantial amounts of Cu biologically unavailable in the treatment tanks. The ability of 4 µM EDTA to complex high levels of toxic Cu species became limiting when the measured total dissolved Cu was between 250 μgL^{-1} (96% EDTA-bound) and 370 μgL^{-1} (70% EDTA-bound). Final treatment levels of bioavailable Cu were determined to be 0.04, 1.10 and 50.30 μgL^{-1}. The pH and water temperatures in the incubation tanks did not change significantly throughout the experiment.

7.3.2 Survival and Development

At sublethal bioavailable Cu levels (0.04 and 1.10 μgL^{-1}), the proportion of surviving embryos and larvae at each sampling time (18, 42 and 72 hrs post-treatment) were comparable to those surviving in the control tanks (*t*-tests; $p > 0.05$) (Figure 7.1A).

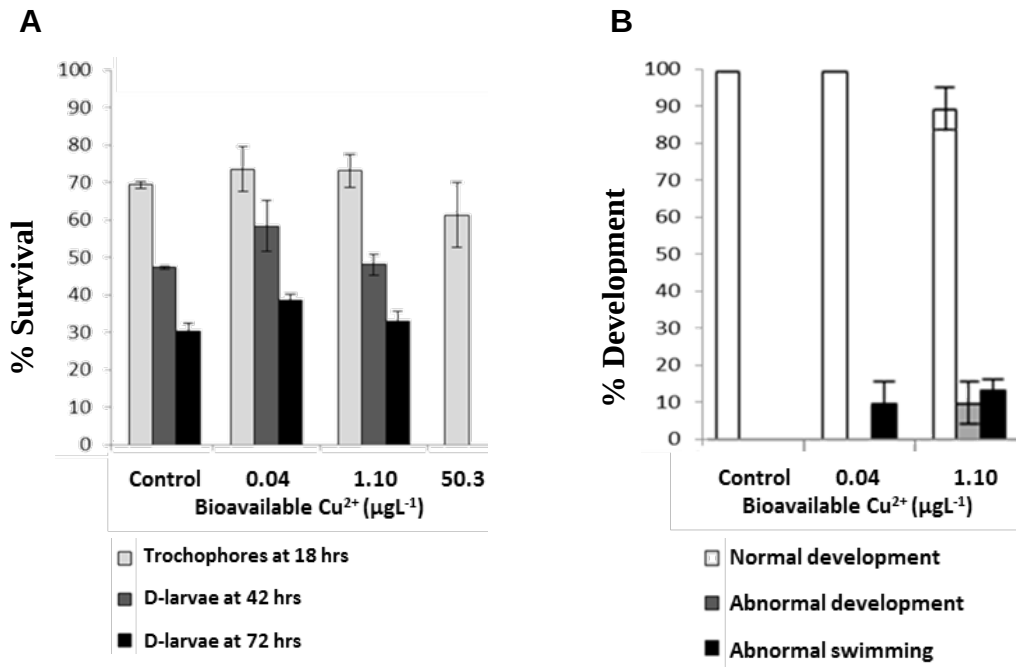

Figure 7.1. Effects of copper exposure on embryo/larval survival, larval development and larval swimming behaviour: **A)** Percent of organisms surviving at 18, 42 and 72 hrs post exposure to sublethal and lethal Cu levels, compared to controls; **B)** Percent of normally developed D-larvae, abnormally developed D-larvae (very small and/or with shell structure deformities), and abnormal swimming behaviour (static or slowed movement) after 66 hrs exposure to sublethal Cu levels, compared to controls.

Survival data were derived from the embryo/larval densities within each incubation tank and represent the proportion of organisms present in relation to the number of embryos each tank was initially stocked with. All organisms sampled during the density enumerations were confirmed to be alive via neutral red uptake cell viability assays. The percent difference which represents mortality is assumed to be due to disintegration of biological material very soon after the organisms died.

Although exposure of embryos to 50.3 μgL^{-1} of bioavailable Cu resulted in high survival for the first 18 hrs post-treatment, it is likely that they perished soon thereafter since there were no visible indications of biological material remaining after a 36 hr post-treatment spot-check. A visual assessment during the cell viability assay after 18 hrs of high Cu exposure revealed that those embryos were also substantially delayed in their development. These comprised mostly of 16 to 32-cell blastulas, some gastrulas and a few non-swimming trochophores with evidence of abnormal cell differentiation in treatments vs. normally-developed and highly mobile trochophore-stage embryos in control tanks. In addition, although cell membrane integrity appeared to be mostly maintained in embryos exposed for 18 hrs to the high Cu treatment (due to the active transport of neutral red across the membrane and permanent incorporation into lysosomes), there were some evidences of changes in cell morphology and size (mostly through oncosis, but with some shrinkage also observed), cellular injury and blebbing, and a commencing loss in cellular adhesion through signs of cellular dissociation. These observations are strongly suggestive that the initiation of Cu-induced mechanisms of cellular apoptosis and/or necrosis was starting to occur in these samples.

Of the surviving organisms towards the end of the experiment (66 hrs post-treatment), prolonged exposure of embryos to the sublethal Cu levels resulted in significant proportions of D-larvae displaying signs of reduced swimming velocity and abnormal trajectories (Figure 7.1B). In addition, approximately 10% of the D-larvae

which had been exposed to 1.10 μgL^{-1} bioavailable Cu showed clear indications of developmental abnormalities, such as retarded growth and deformities in shell structure.

7.3.3 Metabolomics

7.3.3.1 Lethal exposure effects

Exposure of embryos to 50.3 μgL^{-1} bioavailable Cu caused extensive metabolic alterations prior to cell death. Hierarchical cluster analysis (HCA) (Figure 7.2) of the 3 and 18 hr Cu-exposed groups and their respective controls revealed that all samples were correctly clustered based on the underlying structure of the metabolomics data.

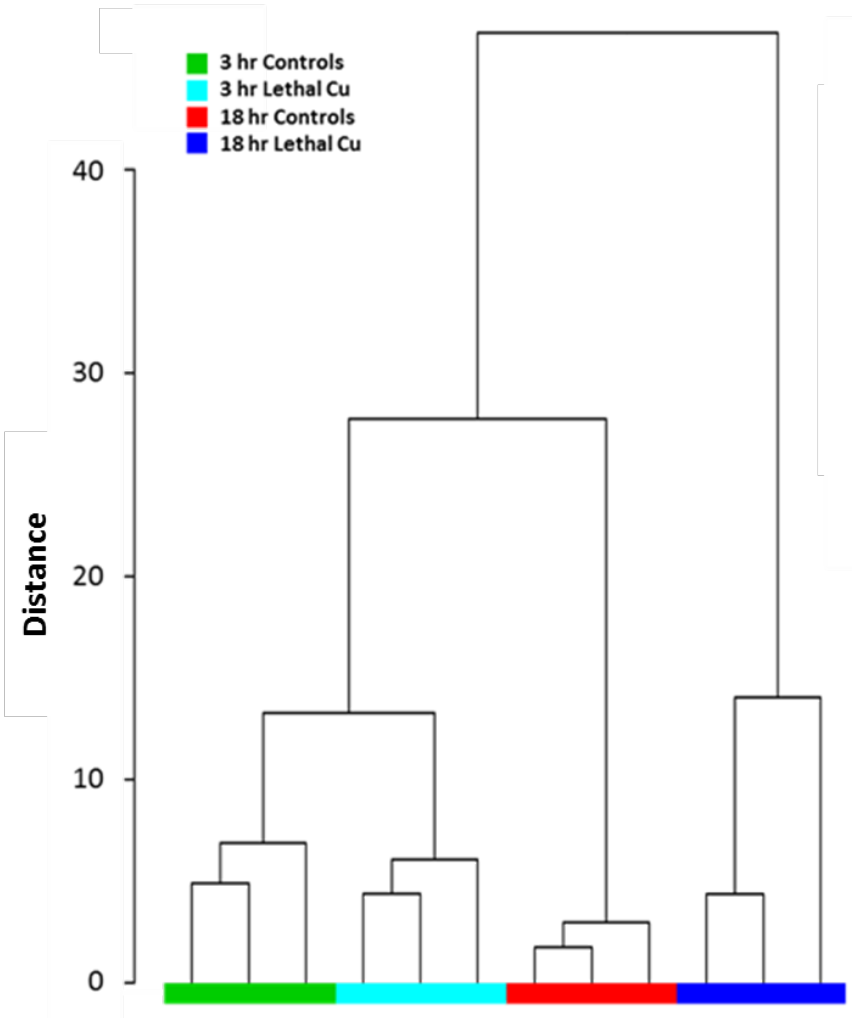

Figure 7.2. Hierarchical Cluster Analysis (Euclidian distance; Ward's criterion) of embryo samples (branches) based on global compositional changes in metabolites as a response to lethal-level Cu exposure.

Heatmap analysis (Figure 7.3A) of the 83 metabolites detected provides a very clear visualisation of the global differences/similarities in the metabolite expression profiles which were responsible for the distinct HCA clustering of the embryonic samples. Although variations exist between sample replicates for some metabolites, these are mostly quite low considering the entire dataset, and general metabolite expression patterns are easily discerned. Due to the large influence of the baseline developmental phenotype on the embryonic metabolomes (i.e., 3 vs. 18 hr control samples), and the differential metabolic responses to lethal Cu levels between these groups, separate PLS-DA models were constructed for each development stage (Figures 7.3B & 7.3C).

For the 3 hr Cu-exposed embryos (Figure 7.3B), LV1 accounted for 30.5% of the explained variation between sample groups in the PLS-DA model (R^2 = 0.99; Q^2 = 0.61). Analysis of the VIP scores (Figure 7.3D) revealed that the abundances of 35 features contributed most (i.e., VIP ≥ 1.0) towards sample classification, including metabolites associated with energy production (succinic, fumaric and malic acids), lipid metabolism (free fatty acids), protein metabolism and osmotic regulation (free amino acids), among others. Univariate SAM analysis (Figure 7.4A) identified 17 metabolites that were statistically different between control and treatment groups after 3 hrs exposure, with varying directions of expression (+ve vs. –ve foldchanges).

After 18 hrs exposure, the effect of lethal level Cu on the embryonic metabolome was far more pronounced. PLS-DA analysis of the 18 hr Cu-exposed embryos (Figure 7.3C) resulted in LV1 accounting for 76.3% of the explained variation between groups in the classification model (R^2 > 0.99; Q^2 > 0.99). Of the total number of metabolites detected, a substantial proportion (61%) contributed highly (VIP ≥ 1.0) towards the PLS-DA model (Figure 7.3E). After 18 hrs exposure, SAM analysis (Figure 7.4B) identified 62 metabolites, including 18 unannotated metabolites/ features, with abundances that were statistically different between control and treatment groups.

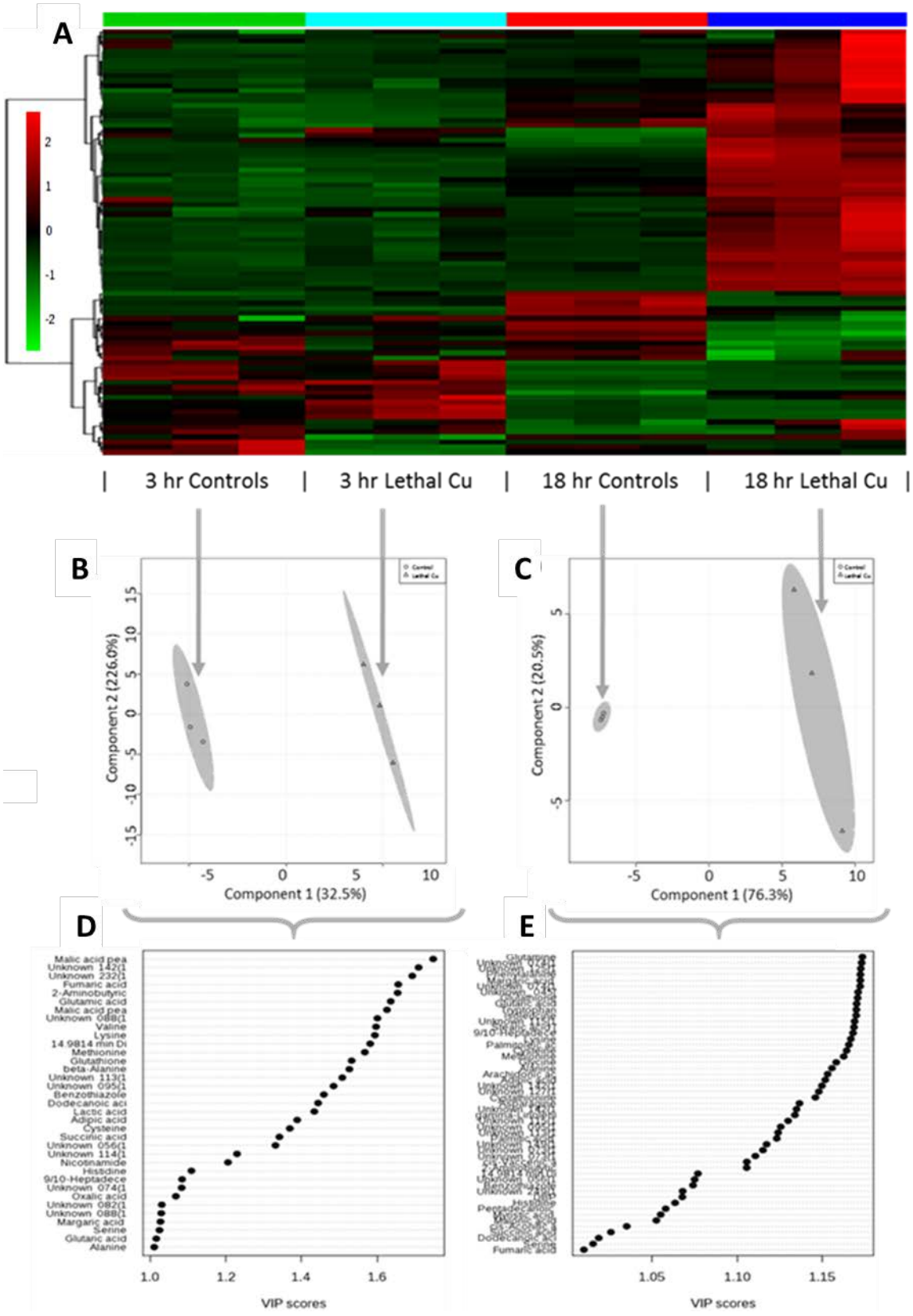

Figure 7.3. Toxicological effects of lethal level Cu on the embryonic metabolome after 3 and 18 hrs exposure: **A)** Heatmap of metabolite levels (red > green) with combined HCA of all metabolite features (rows where n = 90); **B–C)** Projection to Latent Structures Discriminate Analysis (PLS-DA) plots of 3 and 18 hr exposed embryos, respectively, compared to their control groups; **D–E)** Variable of Importance (VIP) scores for each of the PLS-DA models

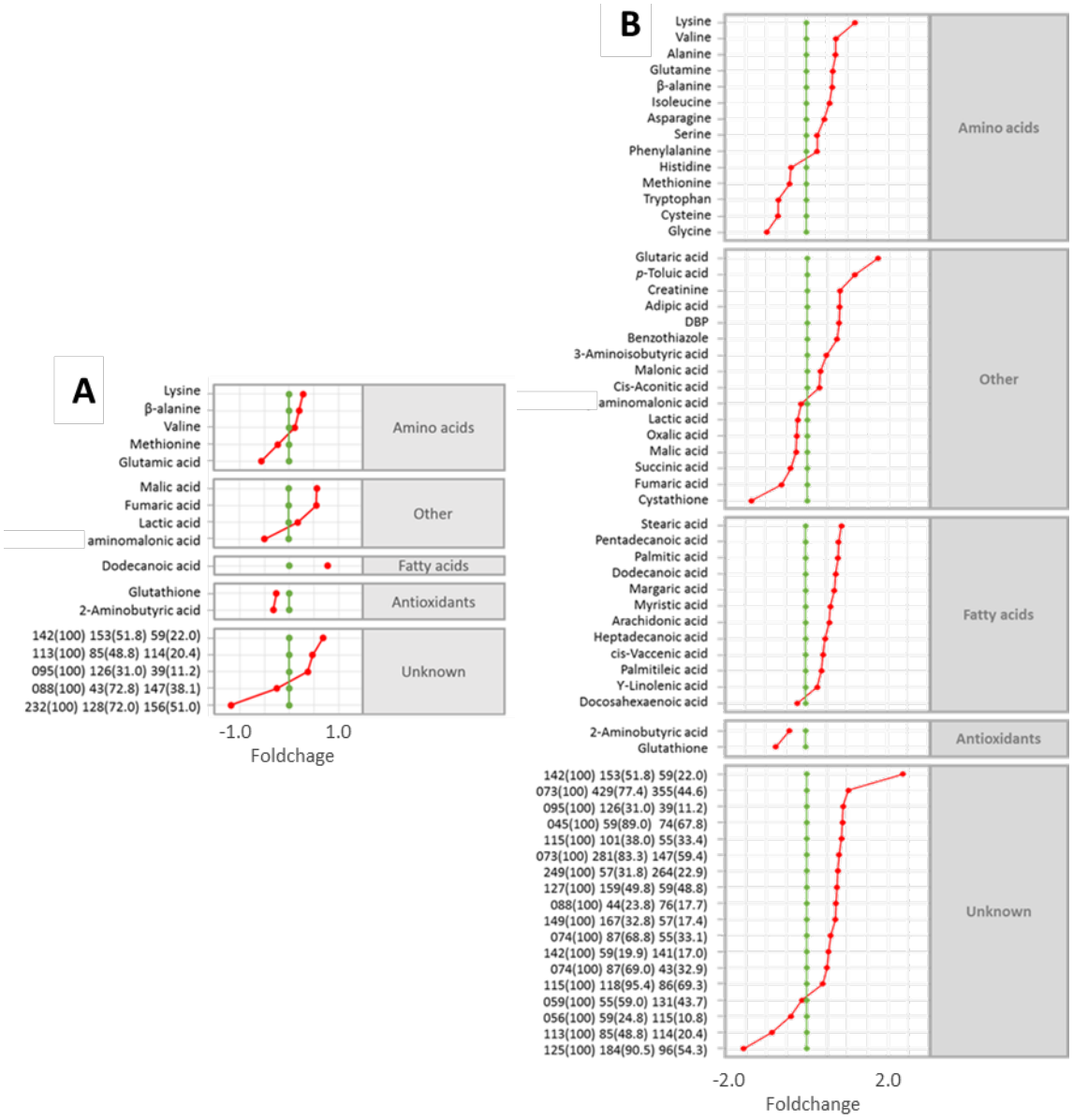

Figure 7.4. Lists of altered metabolites detected via SAM analysis in embryos exposed to: **A)** lethal-level Cu for 3 hrs (FDR = 0.11); **B)** lethal-level Cu for 18 hrs (FDR = 0.01). Red circles (●) indicate metabolite foldchanges in Cu-treated embryos relative to control embryos (green circles; ●) plotted using a $\log_2$ scale.

Comparing the 3 and 18 hr exposure data, the majority of metabolites that were differentially expressed after 3 hrs were also differentially expressed after 18 hrs (Figures 7.4A & B). In addition, many of these metabolites displayed similar foldchange directions. For example, lysine, β-alanine, valine, dodecanoic acid, and an unassigned feature (142[100] 153[51.8]…) were all up-regulated in the Cu-exposed groups; whereas methionine, aminomalonic acid, glutathione, and 2-aminobutyric acid were similarly

down-regulated. Conversely, a number of metabolites exhibited differential expressions which were dependant on the duration of Cu exposure. For example, after 3 hrs, the abundances of some energy-associated metabolites (malic, fumaric, and lactic acids) were all up-regulated compared to control embryos; but after a further 15 hrs of Cu-exposure, the expression patterns of these functionally-related metabolites switched into a coordinated mode of down-regulation. Quantitative enrichment analysis and network topology analysis of the 3 and 18 hr metabolite datasets (treatment vs. control) exposed some major changes in pathway regulation, which could be compared when overlaid (Figure 7.5). After 3 hrs Cu exposure, 8 pathways were identified as being differentially regulated compared to control embryos. These comprised of networks associated with energy production, glutathione metabolism, biosynthesis of aminoacyl-tRNA, and various subsets of amino acid metabolism.

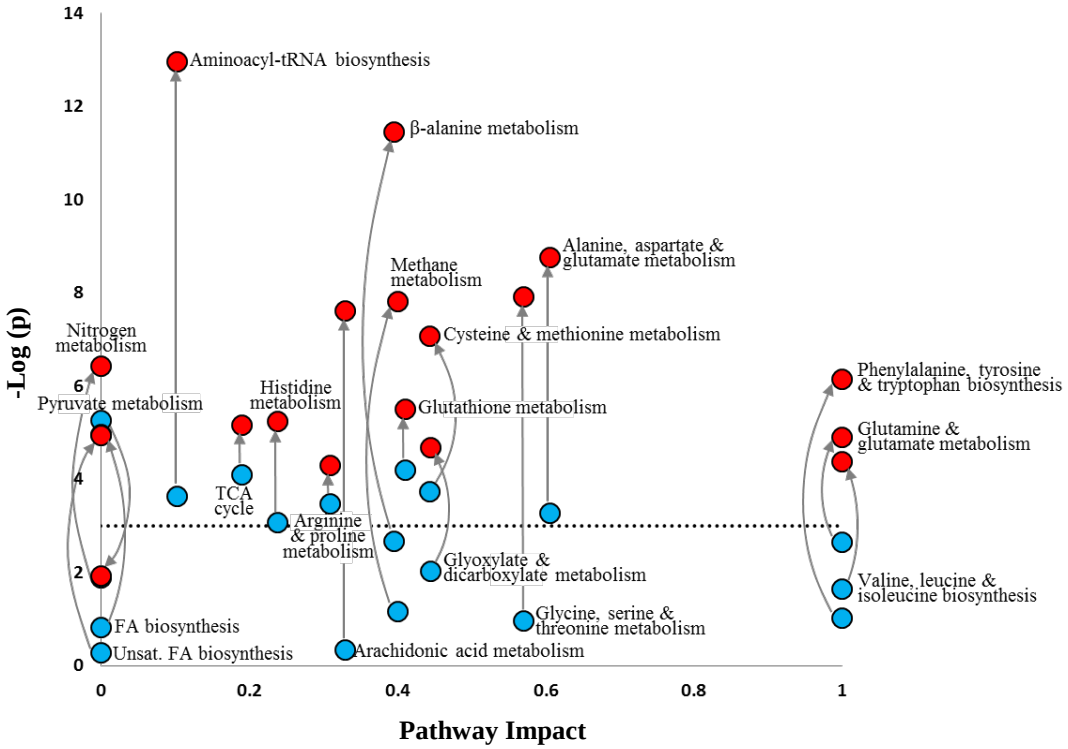

Figure 7.5. Pathway enrichment and topology analysis of the effects of lethal level bioavailable Cu (50.3 μgL^{-1}) on embryos after 3 hrs (blue circles) and 18 hrs (red circles) exposure, compared to their respective controls. The *y*-axis represents the −log of the raw *p*-value (i.e., *ln*[*x*]) associated with pathway enrichment analysis, and the *x*-axis represents the Pathway Impact (PI) score associated with topology analysis (a measure of metabolite centrality within the pathways). The black dotted line denotes the threshold *p*-value of significance, where pathways below the line ($p > 0.05$) were identified as not being significantly different from those functioning in control embryos. The effects of Cu exposure duration on differentially enriched pathways are seen as vertical shifts in significance.

After 18 hrs Cu exposure, all but one of these pathways (pyruvate metabolism) became more significantly disturbed compared to the metabolic baselines controls – as revealed by the pronounced vertical movement of the data. In addition, the extended exposure duration also resulted in a further 10 pathways displaying differential regulation compared to control embryos. These included networks associated with extra subsets of amino acid metabolism, methane metabolism, glyoxylate and dicarboxylate metabolism, arachidonic metabolism, and fatty acid biosynthesis. See Figure 7.6 for three examples of pathways identified as being differentially regulated after 18 hrs exposure of embryos to a lethal level of bioavailable Cu.

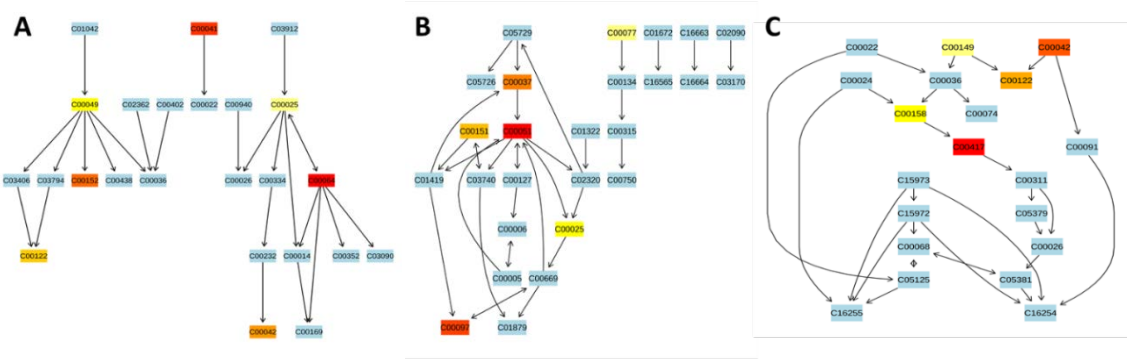

Figure 7.6. Examples of differentially enriched pathways, extracted from the KEGG database, in embryos exposed to lethal level bioavailable Cu (50.3 μgL^{-1}) for 18 hrs, compared to control organisms: (A) Alanine, aspartate and glutamate metabolism ($p < 0.001$; FDR < 0.001; PI = 0.61), (B) Glutathione metabolism ($p = 0.004$; FDR = 0.008; PI = 0.41), and (C) TCA cycle ($p = 0.006$; FDR = 0.010; PI = 0.19). Boxes which vary from yellow to red represent metabolites that were identified and quantified with our methods. Their colour indicates the level of significance (light yellow: $p > 0.05$, light orange to red: $p < 0.05$) from unpaired *t*-tests (control vs. treatment). Levels of light blue compounds in the pathways were not detected, but were used as background information for enrichment analysis to calculate the proportion of identified compounds within each pathway, and in topology analysis to determine the position (relative-betweenness centrality) and importance of each metabolite.

7.3.3.2 Sublethal exposure effects

Exposure of mussel embryos to sublethal levels of bioavailable Cu (0.04 and 1.10 μgL^{-1}) revealed that even low concentrations had substantial effects on metabolism (Figure 7.7). These metabolic responses were dependant both on the concentration and duration of Cu exposure. PLS-DA analysis of the sublethal metabolomics data across all samples revealed that there was a clear metabolic trajectory associated with developmental timing (Figure 7.7). For each Cu exposure duration, deviations from this baseline trajectory was apparent. The positioning of treated samples within the PLS-DA score plot, relative to their respective controls, indicated that different metabolites were responsible for variations in treatment-induced trajectories along the LV1 and LV2 axes.

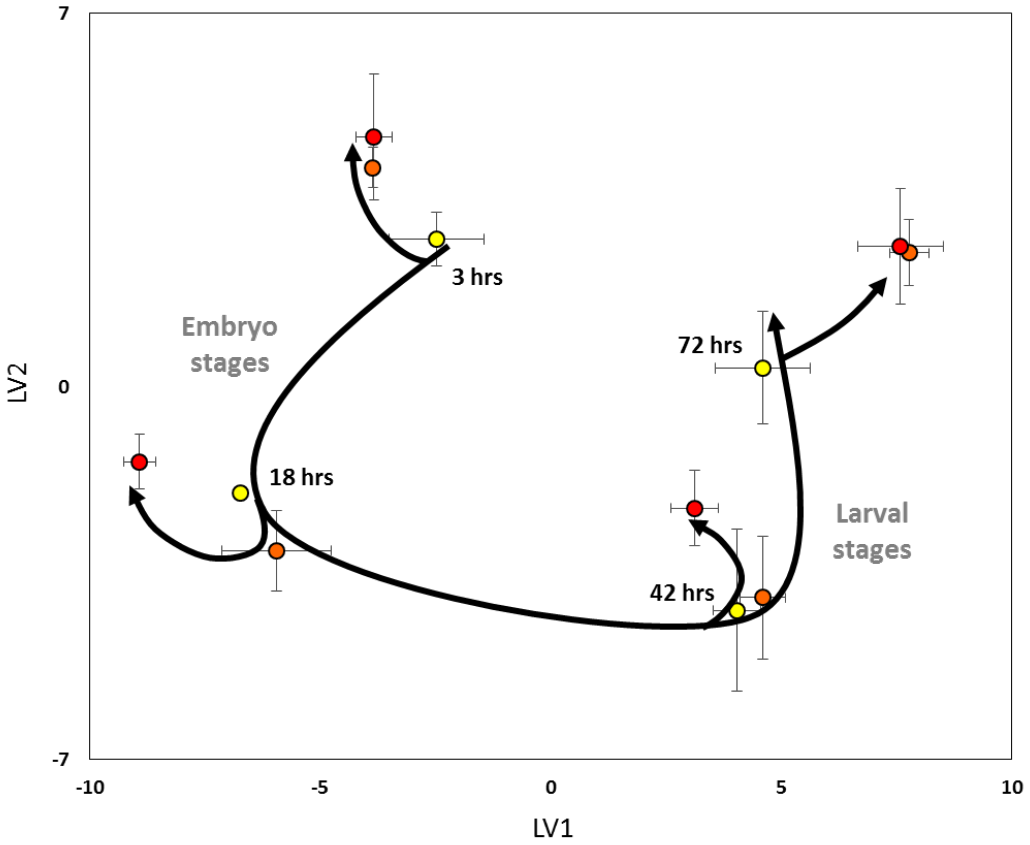

Figure 7.7. Metabolic trajectory analysis derived from PLS-DA of control embryos (yellow circles) and embryos continuously exposed to 0.04 gL^{-1}(orange circles) and 1.10 gL^{-1} (red circles) bioavailable Cu. Data are presented as means ± SD of triplicate samples. Black lines/arrows summarise the main trends/trajectories during baseline embryonic development and in response to sublethal Cu exposures for each treatment duration.

Due to the large effect of developmental timing on the embryonic metabolome, subtleties of some treatment effects were masked; especially when their trajectories aligned with the metabolic path during baseline development (i.e., exposure of embryos to 0.04 μgL^{-1} bioavailable Cu after 18 and 42 hr exposures). Thus, to remove unimportant components of the global variation contributing towards group partitioning in the model, and visually capture the effects of Cu treatments without developmental-induced biases, phenotypic normalisation was applied and the resulting data were replotted (Figure 7.8).

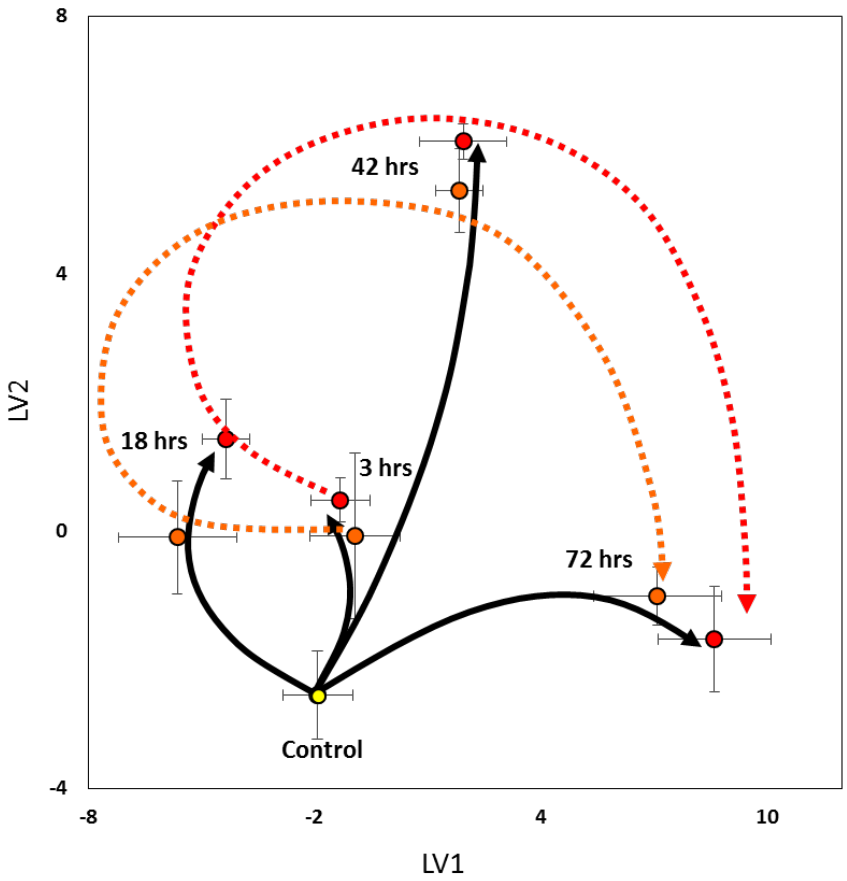

Figure 7.8. Phenotype normalised metabolic trajectory analysis derived from PLS-DA of control embryos (yellow circle) and embryos continuously exposed to 0.04 gL^{-1} (orange circles) and 1.10 gL^{-1} (red circles) bioavailable Cu. Control data are presented as the mean ± SD of 12 replicates (i.e., triplicate samples for each development stage/ exposure duration), whereas treatment data are presented as means ± SD of triplicate samples. Black lines/arrows summarise the main metabolic trends/trajectories in embryos/larvae for each exposure duration, and the coloured dashed lines summarise the metabolic trends/trajectories of embryos exposed to each Cu level during the experiment.

The new PLS-DA score-plot (Figure 7.8) produced revealed that both the low and the medium bioavailable Cu treatment levels substantially perturbed embryo and larval metabolic signatures compared to controls. For each exposure duration, the divergent trajectory directions and lengths along the LV1 and LV2 axes further substantiate that the relative abundances of different metabolites are responsible for the observed pattern, and provides evidence that the toxic effect of Cu on the embryonic/larval metabolome is time-dependant. Furthermore, there appears to be similar temporal treatment effects between the two Cu treatment levels (dotted lines) which could not be discerned prior to phenotypic normalisation. This trend is consistently offset between the two Cu treatment levels (with the higher dose being further from away from controls than the lower dose) which indicates that higher Cu exposures cause more pronounced disturbances to baseline metabolism than the lower Cu dose regardless of exposure duration or development stage. However, since the positioning of the samples between Cu treatments within each exposure duration are located relatively close to one another, this suggests that the duration of exposure causes more pronounced effects than the concentration of toxin tested.

Comparing the 42 hr exposure and 72 hr exposure data in Figure 7.8, there are indications that some of the major metabolic changes which are induced by Cu after 42 hrs may return more closely to the metabolic baseline, or towards earlier exposure duration effects, along the vertical LV2 axis after continued toxin exposure; whereas other metabolites become more differentially perturbed due to the horizontal shift along the LV1 axis. These interpretations are supported by inspection of the metabolite foldchange values compared to their respective controls (Figure 7.9); combined heatmap and hierarchical cluster analysis of the mean metabolite foldchanges for each sublethal Cu treatment and exposure duration provides a visual summary of the relative metabolite abundance data.

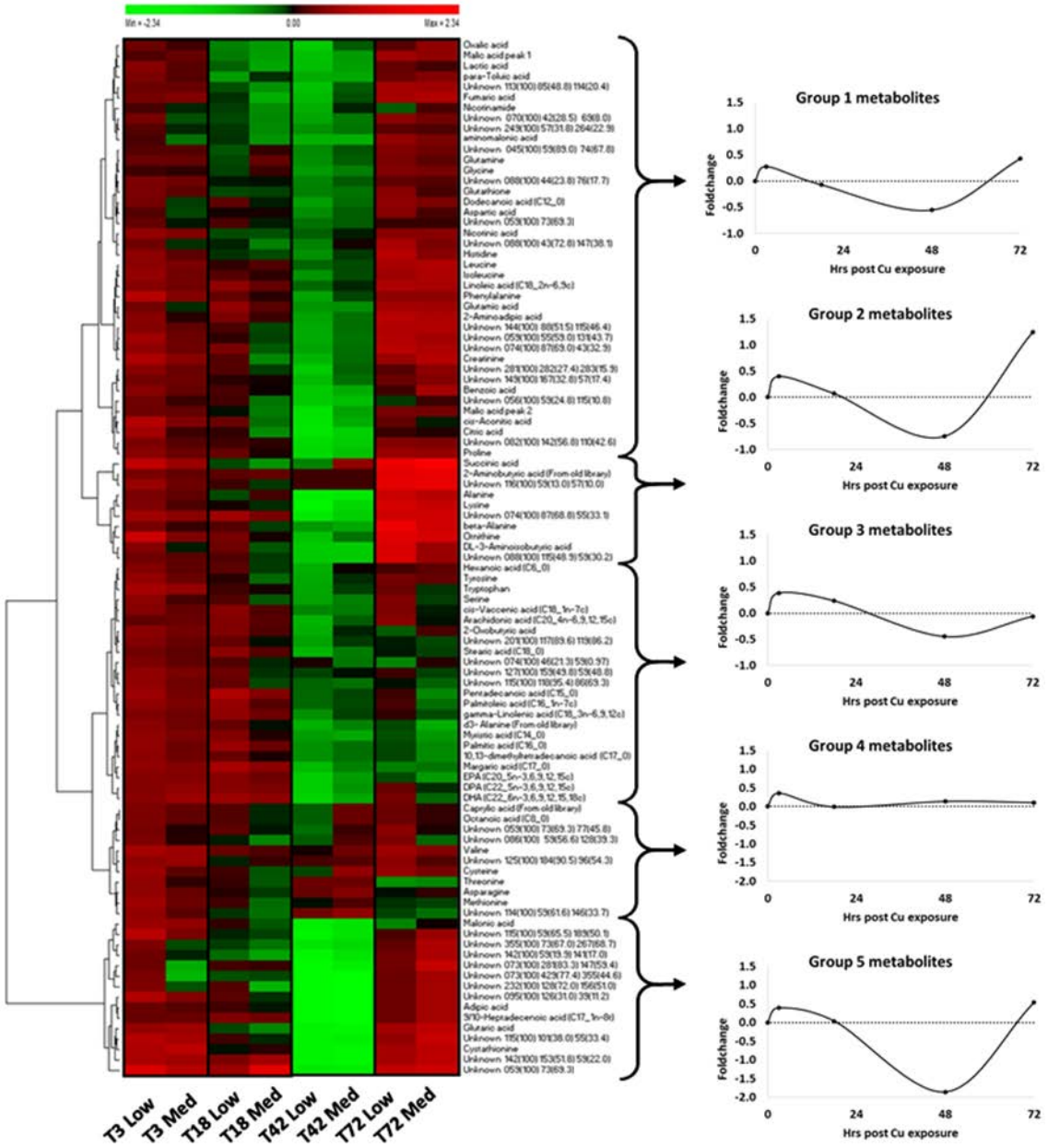

Figure 7.9. Heatmap and cluster analysis (left) of mean metabolite foldchanges in Cu-treated organisms relative to their respective controls, where: T*n* = exposure duration (hrs); Low = 0.04 µgL^{-1} bioavailable Cu; Med = 1.10 µgL^{-1} bioavailable Cu. Five general treatment-averaged foldchange trends are also displayed (line plots on right) based on the cluster analysis.

Although there are some treatment concentration effect variations for some metabolites (e.g., within the 18 hr exposure group), many of the metabolite responses are similar between the two different Cu doses (e.g., within the 3 hr exposure group). This correlates well with the separation of samples groups in the phenotype normalised PLS-

DA model (Figure 7.8), and provides contextual rationale for their spatial positions. Furthermore, some clear patterns can easily be discerned between the exposure durations.

After 3 hrs, most metabolites increased in their relative abundances compared to non-Cu treated controls. After 18 hrs, many metabolite levels within the low Cu dose exposure remained higher than in control embryos; whereas levels of various metabolites in the high dose exposure group had dropped below control values. After 42 hrs, the majority of metabolites consistently had negative foldchange values (ca. −2.0) compared to control organisms. These observations suggest that the higher of the two Cu doses began initiating the temporal change in metabolite profiles much earlier (after 18 hrs in trochophore-stage embryos) than the lower treatment dose (after 42 hrs in D-stage larvae). Continued exposure to sublethal Cu levels resulted in differential metabolite responses after 72 hrs. The relative abundances of a few metabolites (e.g., succinic acid, 2-aminobutyric acid) increased substantially above control values, many metabolites (e.g., glutamine, histidine, cystathionine) appeared to return to levels corresponding with those induced earlier after 3 hrs exposure, and a number of metabolites (e.g., asparagine, citric acid) had re-established back to the baseline controls. These general trends are summarised by plotting the mean Cu-induced foldchange values for each exposure duration based on the cluster analysis of metabolites which identified five broad response patterns (Figure 7.9 line plots).

7.4 Discussion

7.4.1 Bulk seawater composition

Most of the bulk seawater compositional features (Table 7.1) were within the range of expected values for nearshore waters in New Zealand. However, according to the Australian and New Zealand guidelines for marine water quality (ANZECC & ARMCANZ 2000), the total Cu level measured (2.9 μgL^{-1}) was more than two-fold higher than the 95% trigger value (1.3 μgL^{-1}). Guideline trigger values are concentrations that indicate a potential environmental problem if exceeded. The percentage value is the protection level that signifies the proportion of species expected to be protected if total Cu $\leq$ 1.3 μgL^{-1}. Taking into consideration the concentration of dissolved organic carbon (DOC), speciation modelling revealed that the majority (ca. 95%) of the total Cu was bound to humic substances. The resulting level of bioavailable Cu in the bulk seawater prior to EDTA additions was 0.133 μgL^{-1}.

Since the seawater had been through a treatment process and thermally equilibrated for 24 hrs prior to testing, it cannot be specified whether the source of the Cu was entirely from naturally-occurring background levels, elevated through a source of environmental contamination, or attributable to some aspect of the water treatment practices that was employed. Nevertheless, the measured level of total Cu was due to some inherent aspect of the seawater which would typically be used during commercial hatchery production of mussel larvae. This may be of concern for aquaculture operations at the facility; particularly for subsequent post-embryo incubation phases of larval rearing when EDTA additions are not normally applied (personal communication, N. Hawes; SPATnz, 2015).

7.4.2 Cu speciation, toxicity and macroscopic endpoints

Speciation and competition of trace metals in aquatic environments is complex and depends on a multitude of factors, including: pH, temperature, salinity, inorganic ion

composition, and DOC content (Deruytter et al. 2015). In seawater, the chemical speciation of Cu is largely dominated by the formation of organic complexes, with humic substances being the chief mediators (Kogut & Voelker 2001). When bound to these substances, Cu toxicity is greatly ameliorated since the free cupric ion is not available, or less available, for uptake by aquatic organisms (Lorenzo et al. 2005; Nadella et al. 2009). Remaining Cu comprises of free Cu^{2+} and an array of Cu-containing inorganic compounds. In these weakly-bound ionic forms, Cu is labile, bioavailable, and toxic (Sander et al. 2015).

To provide industry-relevant information in the current study, the addition of EDTA into the incubation tanks was included to simulate commercial hatchery conditions (Helm et al. 2004). Without EDTA, embryonic development and D-larval yield is routinely very low for this species (< 10%) with occurrences of cellular membrane degradation, detachment of microvilli, perivitelline space increases, changes in behaviour, and various enzymatic and macromolecular evidences of severe oxidative stress (Buchanan 1998; Gale et al. 2016). The precise reason for the consistently higher successes observed with EDTA supplementation during embryogenesis is not known. Although, it is possible that the chelator reduces toxic levels of bioavailable trace metals from contaminated source water (Liu et al. 2006). EDTA may alternatively be acting to reduce the activities of virulence factors associated with ubiquitous pathogenic bacteria such as *Vibrio* sp. (Hasegawa et al. 2008; Hasegawa & Häse 2009; Mersni-Achour et al. 2015), may be exerting its positive effects during embryogenesis through some currently unknown biological role, or could be functioning in a multifaceted manner.

The addition of EDTA to the embryo incubation water resulted in the relatively high levels of nominal total Cu^{2+} treatments (100 and 200 μgL^{-1}) exerting sublethal effects. Estimation of the bioavailable Cu fraction (free Cu^{2+} + $\sum$ inorganic labile species) within each sublethal treatment determined that 100 μgL^{-1} nominal total Cu = 0.04 μgL^{-1}

bioavailable Cu, and 200 μgL^{-1} nominal total Cu = 1.10 μgL^{-1} bioavailable Cu, with the vast majority of total Cu in these treatments being strongly bound as Cu-EDTA complexes. These findings may be of interest to the developing mussel industry in New Zealand because such levels of total Cu are beyond those which would normally be found within local coastal waters, even in highly polluted zones (e.g., within the Waitemata Harbour, Auckland [Gadd & Cameron 2012]), and supports the continued used of EDTA during the hatchery culture of this species as a precautionary safety measure against heavy metal contamination.

The chemical structures and relative toxicities of the various Cu species in estuarine and marine waters are difficult to accurately determine and continues to be an extremely active area of research (Sander et al. 2015; Benedict et al. in prep.). While the highly labile Cu species are considered most responsible for the toxic effects in this study, it is possible that other Cu-bound components also contributed to the behavioural, developmental, and molecular responses that were observed.

Although most Cu-organic complexes have low toxicities, those which are lipid-soluble can penetrate cell membranes, allowing both the Cu and the ligand to enter cells (Stauber et al. 1996). Indeed, stable isotope (^{65}Cu) labelling has been used to demonstrate that some Cu-DOC complexes can be taken up and accumulated within tissues of juvenile mussels (Zhong et al. 2012), and it is likely that they contribute towards Cu toxicity in mussel larvae (Deruytter et al. 2015). Furthermore, although EDTA substantially reduces Cu toxicity (via formation of the stable Cu-EDTA complex and resultant reduction in free Cu^{2+} concentration) toxic responses to the complex itself have been observed in fish, water fleas, ciliates, and bacteria (Schmidt & Brauch 2004). Cu-EDTA has also been shown to cause dysregulation of lipid metabolism in hepatic cell cultures (Kennedy et al. 2009), and induces differential expression of ROS-regulatory and stress-related enzymes in fish (Shen et al. 2004; Liu et al. 2006). Thus, it is important to highlight that the

concentrations of 'bioavailable Cu' reported herein may not fully represent the entire quantities of copper which were responsible for the developmental and biochemical effects observed.

The lower treatment levels were not lethal, but they did induce developmental retardation, behavioural abnormalities, and structural deformities in a dose-responsive manner. Under the experimental conditions that were employed, evidence from the larval development data reveal that the approximate 72hr EC10 of bioavailable Cu was around 1.10 μgL^{-1}. Comparative embryotoxcity information for the effects of Cu on *P. canaliculus* is thus far lacking. However, these results do fall within the ranges reported for embryos of other marine invertebrate species found in New Zealand. For example, the 72 hr EC10 values for Cu are 0.7 and 2.1 μgL^{-1} for the gastropod mollusc *Haliotis iris* and the sea urchin *Evechinus chloroticus*, respectively (Rouchon 2015), and the approximate 48 hr EC10 for the mussel *Mytilus galloprovincialis* typically ranges between 0.5–3.1 μgL^{-1} (Worboys et al. 2002; Beiras & Albentosa 2004; Fabbri et al. 2014; Deruytter et al. 2015). The longer term implications of these sublethal effects on subsequent growth, larval fitness, settlement and recruitment success, and potential post-metamorphic carryover effects are yet to be investigated.

7.4.3 Metabolomics

Metabolite profiling demonstrated that the embryonic and larval metabolomes of marine mussels are extremely sensitive to Cu-induced toxicological influences. These entire profiles are representative of unique 'metabolic fingerprints' that reflect health condition, and are thus in themselves biomarkers of physiological status. Based on the composition of metabolites within tissues, distinct metabolic trajectories associated with embryonic developmental timing, levels of Cu concentration, and the duration of Cu exposure were identified. Discrimination of treatment groups were obtained via unsupervised and

supervised multivariate analyses, with results showing the presence of intricate temporal metabolite variations.

One striking feature of the sublethal data was what appeared to be a general sine wave signal of metabolite foldchanges in Cu-exposed embryos compared to controls. This may suggest that an initial toxic shock, revealed by subtle accumulations of many metabolites in late-stage blastula embryos, was buffered via metabolic adaption, leading to transitional returns towards baseline levels through trochophore development. However, many of these metabolites fell below control levels in early D-stage larvae, indicating considerable metabolic dysregulation. This perturbation seemed to trigger an attempt to re-establish baseline metabolism during subsequent development to overcome possible deleterious effects of sublethal treatments.

Extended exposure of embryos to sublethal Cu beyond the experimental duration that was employed might result in further metabolic imbalances and transpire in newly significant departures from the metabolic baseline. Indeed, some metabolites at the end of the trial did appear to be on statistical trajectories which may support future divergences. In addition, although not explicitly illustrated in the line plots of the averaged metabolite foldchanges across the two sublethal treatment concentrations (Figure 7.9), closer inspection of the heatmap data revealed cases of treatment level-induced offsets in the sine wave responses. This indicates that the two sublethal Cu doses led to particular temporal differences; in some cases with the higher treatment seemingly inducing faster metabolic responses within the general trend (e.g., succinate, malonic acid, aconitic acid, and the unknown metabolite 088(100)43(72.8).

A lethal-level Cu dose triggered major alterations of many metabolites, and resulted in catastrophic metabolic failures and mortalities shortly after 18hrs of exposure. Pathway analysis revealed the occurrence of disturbances to a number of functionally-related metabolites within a range of known biochemical pathways. This approach

enabled unique temporal profiles dependant on exposure duration to be discerned which might not otherwise have been identified using more conventional procedures. As might be expected, dysregulation of these pathways became more severe with time. Eight out of 19 pathways showed signs of imbalance after 3hrs of high Cu exposure, whereas 18 out of the 19 pathways were likely affected after 18hrs exposure. Changes in metabolite compositions indicated dysregulation of energy production, occurrence of osmotic stress, potential neuro- and immuno-toxic associations, and numerous evidences for oxidative stress through disrupted regulation of reactive oxygen species (ROS).

7.4.3.1 Disturbance in energy metabolism

Under normal physiological circumstances, concentrations of TCA cycle intermediates remain almost constant (Sweetlove et al. 2010). When intermediates are removed to serve as biosynthetic precursors they are typically replenished in dynamic balance via anaplerotic reactions (e.g., glutamine → α-ketoglutarate) (Cheng et al. 2011). However, decreases in the abundance of TCA cycle intermediates can be considered indicative of increased pathway flux, ATP demand, utilisation for secondary biosynthesis, and/or reduced mitochondrial function; and vice versa (Chan et al. 2009; Aggio et al. 2010; Morita et al. 2013). After 42 hrs exposure, sublethal-level Cu treatments caused reductions in citrate, cis-aconitate, fumarate and malate. These data may suggest that low concentrations of Cu altered energy metabolism in early D-stage larvae through enhanced demands for ATP to fuel detoxification processes, such as synthesis of antioxidant enzymes. Indeed, sublethal trace metal exposures increase respiration rates in various organisms probably to account for enhanced ATP expenditure during toxin-induced requirements for defense and repair mechanisms whilst simultaneously maintaining basal metabolism (Romanowska et al. 2002; Vosloo et al. 2002; Handy 2003; Muyssen et al. 2006). The decreased pool of free amino acids at this time may also indicate enrichment

in protein synthesis and/or an enhanced requirement for energy through their oxidisation and utilisation as TCA cycle substrates.

Regardless of the precise mechanism responsible for the lowered abundances of TCA cycle intermediates after 42 hr sublethal exposures, these responses were transient, with levels of all detected TCA cycle intermediates returning, or adhering, to baseline levels by the end of the trial. This demonstrates a capacity for metabolic regulation of immunotoxicity in developing D-larvae. Since metabolite levels throughout the pathway were simultaneously reduced after sublethal level exposures, it appears that there was no particular break or obstruction stemming from inhibition of one or more TCA cycle enzymes; this is in contrast to lethal-level treatments.

Cis-aconitate (positioned between citrate and aconitate in the TCA cycle) is a key substrate for the enzyme aconitase. Exposure of embryos to lethal-level Cu induced an accumulation of this metabolite, which can be attributed to reduced aconitase activity (Cheng et al. 2013). Iron-sulphur-containing aconitase is a well-known target of Cu toxicity and, being the most sensitive TCA cycle enzyme to ROS-inhibition, its activity can be used as a biomarker of oxidative stress (Tretter & Adam-Vizi 2000; Bota & Davies 2002; Talbot & Brand 2005; Cherkasov et al. 2007; Lushchak et al. 2014). Enzyme inhibition in the case of Cu toxicity involves $O_2^{\cdot -}$ and $OH^{\cdot}$ produced via the Haber–Weiss reaction, a process analogous to the Fenton reaction (Winterbourn 1995; Muakami & Yoshino 1997; Kehrer 2000). Oxidative inactivation of aconitase promotes cluster instability, causing the enzyme to release redox-active Fe^{2+} and stimulating the associated formation of H_2O_2 which encourages Fenton reaction events and further production of $OH^{\cdot}$, thus creating a self-amplifying cycle if unresolved (Cantu et al. 2009; Rose et al. 2012). Aconitase therefore forms a direct link between ROS metabolism, iron homeostasis, the flow of metabolite intermediates in the TCA cycle, and energy production (Lushchak et al. 2014).

Cheng et al. (2013) recently demonstrated via aconitase gene knockdown in a *Drosophila* model that reduced mitochondrial aconitase activity impairs glycolysis and the TCA cycle, and decreases generation of ATP. Specific markers of impaired energy metabolism caused by reduced aconitase activity included an increase in levels of cis-aconitate, and substantial concomitant decreases in downstream levels of succinate, fumarate and malate. Results of the current study match this metabolite expression pattern for the group of embryos exposed to lethal-level Cu for 18 hrs, with the potential Cu/ROS-induced inhibition of aconitase limiting the synthesis and turnover of downstream TCA cycle intermediates under severe oxidative stress conditions.

7.4.3.2 Amino acid metabolism

Compositional alterations in the free amino acid (FAA) pool in Cu-exposed embryos were dependant on exposure duration and level of treatment. FAAs play a number of diverse roles in marine organisms; they represent the pool from which new proteins can be synthesised during early development, and they serve as important fuels for energy acquisition in embryos and larvae (Rønnestad & Fyhn 1993, Rønnestad et al. 2003). The FAA pool is also the receiving depot for the catabolism of endogenous protein during embryogenesis in some bivalves (da Costa et al. 2011), as well as the proteinaceous components of food once feeding-competency has been attained. Total free essential amino acid contents can be used as a rough proxy for protein turnover (Waterlow 2006), and these patterns likely reflect in part differential capacities and/or requirements for protein production through embryogenesis, and between treatments and controls. Indeed, with major perturbations to most of the FAAs detected, aminoacyl-tRNA biosynthesis being identified as a significantly affected pathway was a likely outcome due to its pivotal role in delivering amino acids to ribosomes during protein synthesis.

FAAs also have diagnostic capabilities for early detection of various pathologies, and can serve as biomarkers for a number of physiological states (Combs et al. 2013). Particular amino acids may contribute toward antioxidant protection as small peptides (e.g., glutathione) (Wu et al. 2003; Elias et al. 2008), some act as signalling molecules or precursors for their synthesis (e.g., glutamate and tyrosine) (Hyde et al. 2003; Meijer & Dubbelhuis 2004), whereas others have important biochemical roles specific for the adaption of aquatic organisms to flourish in marine environments. For example, marine osmoconformers such as bivalve molluscs utilise certain FAAs as organic osmolytes to balance intracellular osmolality with the external medium (Kube et al. 2006).

7.4.3.3 Osmotic disturbance

Free glycine, alanine and β-alanine serve as organic osmolytes in bivalves and their levels are associated to changes in salinity (Powell et al. 1982; Sansone et al. 1987; Hosoi et al. 2003), with sodium-dependant cotransport being responsible for their accumulation or release from tissues to maintain cell volume (Preston 1993; Kube et al. 2006). These osmolytes were differentially regulated in Cu-exposed embryos, compared to controls. Heavy metal toxicity appears to be strongly dependant on the abilities of some organisms to regulate intracellular osmolality. However, there are still many questions surrounding the osmoregulatory mechanistic basis for salinity-dependant Cu toxicity, and especially so in regards to marine osmoconformers (reviewed by Grosell et al. 2007). Nevertheless, exposure of marine bivalves to Cu and other metals are known to induce compositional variations in FAAs with osmolytic function (Wu & Wang 2010; Zhang et al. 2011; Kwon et al. 2012; Ji et al. 2015a,b), and some new mechanistic advances have progressed our understanding of Cu-induced toxicity in these organisms. Although it has been suggested that Cu-toxicity in marine molluscs is more related to effects on acid-base equilibrium

and ammonia excretion rather than on imbalances in ion regulation, intracellular ion regulation plays a key mechanistic role (Grosell et al. 2007).

Exposure of marine clam mantle and gill cells to Cu results in reductions of intracellular Na^+, K^+, and Cl^- concentrations, which is presumed to be associated with inhibition of Na^+/K^+-ATPase activity and competition by Cu for cell membrane ion transport systems, such as Na^+ channels and the $Na^+/K^+/2Cl^-$ cotransporter (Lopes et al. 2011; Nogueira et al. 2013). On the other hand, although hemolymph osmolality and concentrations of Na^+ and K^+ are not altered by Cu exposure in clams, Na^+/K^+-ATPase expression and total activity increases in gills and digestive glands (Boyle et al. 2013; Jorge et al. 2016). This suggests that increased Na^+/K^+-ATPase expression may be a compensatory physiological response to ameliorate the metal-induced inhibition of enzyme activity. Additional Cu-induced changes on intracellular levels of divalent cations (Mg^{2+} and Ca^{2+}) have also been observed in clams (Jorge et al. 2016), and Ca^{2+} flux has been linked with organic osmolyte transport systems in mussels and clams (Bishop et al. 1994; Pierce 1994). Thus, it is becoming clearer that Cu is an ionoregulatory toxicant in marine bivalves, affecting ionic and volume control at the cellular level; although further work is needed to tease out the intricacies of Cu-induced intracellular ion modulation and co-dependant associations with organic osmolytes such as those detected in the present study. In order to fill some of these knowledge gaps, a comprehensive approach incorporating multiplatform metabolomics to specifically assess a broader range of organic osmolytes and associated metabolic features, combined with targeted analyses of enzyme activities/expressions and intracellular ion concentrations, could substantially enrich our understanding of the osmoregulatory mechanisms involved in Cu toxicity.

7.4.3.4 Neuorotoxicity

Neurotoxicity, defined as 'the capacity of chemical, biological, or physical agents to cause adverse functional or structural changes in the nervous system' (NRC 1992), may have been an important mechanistic feature contributing towards deteriorating health in Cu-exposed embryos. It has recently been established that neurogenesis in *P. canaliculus* commences early on during development, with expressions of solitary FMRF-amidergic cells being identifiable via immunochemical labelling at the trochophore stage (18 hrs post-fertilisation) (Rusk et al. 2017). By the early D-larval stage (42 hrs post-fertilisation), immunoreactivity patterns within neural compartments are consistent with initial formation of the cerebral ganglion, which quickly progresses to include the emergence of the peripheral system and mantle nerve within a couple of days. In the current study, a number of amino acids associated with neurotransmission were found to be differentially regulated. Pathway enrichment analysis also substantiated this with detection of significant network alterations to glutamine and glutamate metabolism, as well as phenylalanine, tyrosine and tryptophan metabolism.

Phenylalanine and tyrosine are precursors for the biosynthesis of L-DOPA and the catecholamine signalling messengers, dopamine and epinephrine. These amino acids and/or their neuroactive derivatives can modulate larval behaviours in different mussel species, including *P. canaliculus* (Young et al. 2009; Alfaro et al. 2011; Sanchez-Lopez et al. 2012; Young et al. 2015). Such metabolites also have abilities to regulate larval swimming activities and developmental timing of other molluscan taxa through their actions on ciliary beat frequencies of innervated velar tissues and morphogenetic programmes, respectively (Alfaro et al. 2014; Young et al. 2016). Although a targeted analysis of neurotransmitters was not performed in the present study, levels of phenylalanine and tyrosine are tightly coupled with production of endogenous catecholamines in molluscs, and are thought to regulate neuronal processes in developing

embryos and larvae. For example, enhancement of tyrosine or L-DOPA concentrations in molluscan tissues leads to increased catecholamine synthesis (Boadle-Biber & Roth 1972; Pires et al. 2000; Zhu et al. 2005), and inhibition of the catecholamine re-uptake system in oyster embryos leads to developmental retardation, reduction in growth rate, and formation of shell structure abnormalities in early D-stage larvae (Di Poi et al. 2014).

Neurotoxic effects of heavy metals on metabolite intermediates associated with catecholamine biosynthesis have been described in studies using various model invertebrates. Exposure of the nematode *Caenorhabditis elegans* to sublethal Pb levels reduces whole animal tissue concentrations of free phenylalanine and tyrosine, and is suggested to contribute towards toxicity through deleterious neuronal consequences of atypically enhanced catecholamine production (Sudama et al. 2013). Short term exposure of water fleas (*Daphnia magna*) to sublethal levels of metals (Cu, Li and/or Cd) for 24–48 hrs also causes a reduction in phenylalanine content, increases tyrosine hydroxylase gene expression, and enhances production of L-DOPA and dopamine (Poynton et al. 2011; Nagato et al. 2013). These coordinated precursor-product alterations are highly supportive of a mechanistic link, and the upregulation of this pathway may be a general response to metal stress (Nagato et al. 2013).

Reductions in phenylalanine content after 48 hrs exposure of mussel embryos to sublethal Cu treatments were closely mirrored by levels of tyrosine. This may be suggestive of enriched catecholamine synthesis. Interestingly, the developmental abnormalities and altered swimming behaviours that were observed in approximately 10% of the D-larval population are consistent with the effects observed by Di Poi et al. (2014) in oyster larvae due in part to elevated catecholamine concentrations in synaptic clefts, and the cilioinhibitory function of catecholamines (Carol & Catapane 2007), respectively. Towards the end of the trial, free phenylalanine and tyrosine contents in mussel larvae appeared to have gone through a period of recovery, potentially indicating

metabolic adaption to prolonged low-level Cu exposure. In contrast, phenylalanine content in embryos exposed to the highest Cu treatment for 18 hrs was greater than in control organisms, which may indicate disruption of baseline catecholamine biosynthesis in moribund embryos as one of the catastrophic metabolic failures contributing towards mortality.

Tryptophan is the precursor for the biosynthesis of the neurotransmitter serotonin. The role of serotonin in mollusc development is also very important early on during embryogenesis. Its presence, along with enzymes responsible for its production and the receptors to which it binds, are found in various embryonic stages of diverse molluscan taxa (Dickinson et al. 2000; Buznikov et al. 2003; Franchini 2005; Filla et al. 2009). Serotonin is involved in oocyte maturation, fertilisation, and cleavage divisions, and also regulates embryonic rotations, is associated with embryo/larval sensory organs, and has been implicated in controlling development rates and larval behaviours (Geurrier et al. 1993; Kempf et al. 1997; Filla et al. 2009; Young et al. 2009). Cu-induced neurotoxicity via modulation of tryptophan metabolism and disruption of the serotonergic nervous system has not yet been investigated in molluscan embryonic forms. However, such mechanisms have been shown in other aquatic models. For example, Cu exposure reduces levels of serotonin in fish (De Boeck et al. 1995), whereas supplementation of fish feeds with tryptophan increases serotonin synthesis (Lepage et al. 2002; Morandini et al. 2015), and also substantially enhances resistance to the metal (Hoseini et al. 2012; Fatahi & Hoseini 2013). The benefits of tryptophan towards ameliorating Cu toxicity are thus thought due in part to its role in upholding serotonin production for normal physiological functioning. The lowered abundance of free tryptophan that was observed after exposure of embryos to lethal-level Cu after 18 hrs, and sublethal levels after 42 hrs, may have been due to a concerted metabolic effort to retain serotonin concentrations within neuronal cells and synapses, thereby reducing neurotoxic symptoms.

Glutamine is the common precursor for the biosynthesis of glutamate and γ-amino butyric acid (GABA). Glutamate is considered a major excitatory neurotransmitter, whereas GABA is a major inhibitory neurotransmitter across vertebrate and invertebrate taxa. However, they can act in both excitatory and inhibitory modes specifically in the case of particular molluscan neurons (Mathieu et al. 2014). When applied exogenously to seawater, these metabolites modulate larval swimming and attachment behaviours of molluscs (Stewart et al. 2008; Young et al. 2009; Alfaro et al. 2014), and induce fast synchronous metamorphosis of sea urchin larvae (Naidenko 1996; Sato & Yazaki 1999). Glutamatergic neurons are also identifiable in the peripheral and central nervous systems of ascidian and molluscan larvae, with presumed functions in mechanical and sensory processes (Hatakeyama et al. 2007). Together, these observations signify various neuroactive roles during early marine invertebrate development.

Glutamine and glutamate have previously been suggested as useful biomarkers of neurotoxicity in adult mussels exposed to heavy metal contamination (Wu & Wang 2010, 2011). Differential regulation of glutamine and glutamate metabolism as a neurotoxic response to Hg, Zn and Cd has also been demonstrated in fish and clams (Liu et al. 2011a,b; Kim et al. 2016), and Cd and/or Cu exposure influences glutamine synthetase mRNA expression and/or glutamate dehydrogenase activity in mussels and fish (Casanova et al. 2013; Ventkata & Radhakrishnaiah 2013). Excessive levels of Mn, Hg and Pb trigger neurotoxicity though similar disruptions of the glutamine/glutamate-GABA cycle, which underlines a common mechanism of metal toxicity (Fitsanakis & Aschner 2005; Sidoryk-Wegrzynowicz & Aschner 2013). Although information regarding molluscan embryonic forms is thus far limited, Wirbisky et al. (2014) recently demonstrated that Pb exerts neurotoxicity in fish embryos by influencing the GABAergic system in a dose responsive and temporally variable manner through embryogenesis. Our results indicate that glutamine and glutamate biosynthesis in mussel embryos are

similarly perturbed by Cu exposure which is consistent with the findings of these studies, and further suggests that this system may also be a useful biomarker of metal-induced neurotoxicity in molluscan embryos. This disturbance from baseline metabolism may cause direct neurotoxic effects through altered glutamine/glutamate signalling, and/or indirect deleterious effects through downstream influences on GABA biosynthesis.

7.4.3.5 Pyrimidine catabolism

The pyrimidine nucleotides, thymine and cytosine, are catabolised in mussels to 3-aminoisobutyric acid (BAIBA) and β-alanine through its uracil derivative, respectively (Awapara & Allen 1959). Both of these degradation products were found to be higher in mussel embryos exposed to lethal-level Cu. Since β-alanine serves osmoregulatory functions in marine bivalves associated with Cu-induced toxicity and sodium-dependant cotransport as previously discussed, tissue concentrations are likely to reflect the net outcome of interplay between these systems. On the other hand, BAIBA can be used as a rough indicator for rate of DNA and tRNA turnover (Nielsen et al. 1974). Increases in BAIBA have been found in humans, mice and marmosets exposed to lead poisoning (Farkas 1987; Tomokuni et al. 1991, 1992), and in fruit flies exposed to insecticides (Brinzer et al. 2015); with toxin-induced DNA/RNA damage thought responsible for the elevated levels observed. The higher levels of pyrimidine degradation products measured in our study are indicative of shifts in nucleotide metabolism, with potential adverse implications for DNA/RNA production, protein synthesis and organismal growth. Links between pyrimidine metabolism and oxidative stress have been implicated in a number of disease pathologies and metal toxicity responses (Van Kuilenburg et al. 2004, 2006; Glahn et al. 2008; Gemelli et al. 2013; Bo et al. 2014; Liu et al. 2015; Miccheli et al. 2015), although establishing potential mechanistic associations between oxidative DNA damage and pyrimidine degradation will require more focused investigation.

7.4.3.6 Oxidative stress, redox homeostasis and metal chelation

Generation of reactive oxygen species (ROS) and oxidative stress is one of the most well-known mechanisms of metal toxicity in aquatic organisms (reviewed by Valavanidis et al. 2006; Sevikova et al. 2011). A number of amino acid metabolite signatures associated with oxidative stress responses and/or with roles in maintenance of redox homeostasis or metal chelation were identified. These included changes in aminomalonic acid levels, variation in histidine metabolism, alterations to the transsulferation pathway, and differential regulation of glutathione (GSH) metabolism.

Aminomalonic acid (AMA) showed variable flux in embryos exposed to Cu. When protein/peptide-bound, AMA has possible origins stemming from errors in protein synthesis and oxidative damage to amino acids (Copley 1992). The moiety is generated by free radical oxidation of proteins, and can arise due to hydrogen atom abstraction from the α-position of glycine residues (Dean et al. 1997). The dicarboxylic acid can also be derived from metal-induced cysteine oxidation via β-elimination of the sulphur residue (Kang et al. 2003). Reductions in free AMA within rabbit hepatic tumors are suspected due to depletion of cysteine stemming from enhanced requirements for GSH to alleviate oxidative stress (Ibarra et al. 2014), and toxin-induced changes in the free metabolite have previously been recorded in water fleas exposed to polyromantic hydrocarbons (Vandenbrouck et al. 2010).

Free histidine can serve as a high affinity chelator of metals, and it has been suggested that elevation of histidine levels as a response to Cu toxicity may provide an energetically low-cost detoxification mechanism for invertebrates (Gibb et al. 1997); as is the case for plants (Krämer et al. 1996; Wycisk et al. 2004). However, Bundy et al. (2008) comprehensively tested the hypothesis that Cu exposure upregulates histidine metabolism in earthworms and found a weak negative correlation between levels of free histidine and Cu dose; which also appears to be the case for freshwater microalgae

exposed to Cd (Chia et al. 2015). Furthermore, Digilio et al. (2016) recently conducted a metabolomics-based investigation of Cu toxicity in adult mussels and found a parallel trend of subtle histidine reductions in haemolymph samples with increasing doses of Cu. Exposure of mussel embryos to lethal-level Cu in the present study similarly caused histidine levels to decrease in mussel embryos.

Although it is unascertained why Cu appears to reduce levels of free histidine, it is possible that the metabolite is being incorporated into histidine-rich glycoprotein (HRG) as a detoxification strategy against excess metal concentrations. HRGs are important immunoresponsive macromolecules which are widely distributed across bivalve taxa and have very high capacities to bind and detoxify divalent metal ions such as Cu (Abebe et al. 2007; Devoid et al. 2007). HRGs also serve other immune functions due to their antibacterial properties, and their ability to clear necrotic cells by inducing phagocytosis (Rydengård et al. 2007; Poon et al. 2010).

The transsulferation pathway involves the conversion of methionine to cysteine, via cystathionine, and is affected by metal exposure to a range of organisms, including rats (Sugiura et al. 2005), nematodes (Hughs et al. 2009), and midges (Jeppe et al. 2014; Long et al. 2015). In the current study, cysteine and methionine metabolism was differentially regulated in mussel embryos exposed to Cu, with relative metabolite levels involved indicating depletion of methionine through enrichment in transsulferation, and subsequent downstream metabolism of cysteine.

Cysteine is an important structural component of glutathione (GSH), and also the rate-limiting precursor in its biosynthesis (Aquilano et al. 2014). When the demand for GSH is high due to generation of reactive oxygen species (ROS), the transsulferation pathway can act as a reserve pathway that channels methionine towards cysteine (through initial conversion to homocysteine, subsequent conjugation with serine to form cystathionine, and then final cleavage and transsulferation of the carbon-sulphur bond)

(McBean 2012). In its free form, cysteine's thiol group gives it an effective capacity to chelate metal ions. However, when bound to redox-active metals, such as copper, free cysteine is quickly oxidised and the reduced metal may undergo a Fenton-like reaction to form highly toxic hydroxyl radicals (˙HO); thus, keeping free cysteine levels low is necessary to protect against these harmful oxidants (Jozefczak et al. 2012). Conversely, as a residue within GSH, cysteine's amino group is blocked via conjugation with glutamate, and thiol oxidation by transition metals is greatly diminished so as to reduce deleterious Fenton-like reactions from occurring, while simultaneously providing a tripeptide with tremendous antioxidant properties (Jozefczak et al. 2012).

Other studies documenting the impact of Cu toxicity on the transsulferation pathway in molluscs is limited. However, Jeppe et al (2014) recently reported that exposure of aquatic midge larvae to Cu resulted in differential expression patterns of transsulferation genes, advocating that Cu induces an increased flux of cysteine into GSH synthesis, whilst at the same time diminishing pools of cystathionine. Our results appear to be consistent with these findings.

Lethal-level Cu treatment caused free methionine, cystathionine, and cysteine to decrease markedly in mussel embryos, which may have been a metabolic attempt to alleviate oxidative stress caused by the metal. Such responses could reduce Fenton reactions from occurring, and also be indicative of an enhanced requirement for GSH production and antioxidant activity. Interestingly, it appears that Cu, Cd and Zn may have quite diverse mechanisms of toxicity regarding their particular impacts on transsulferation pathway components (Jeppe et al. 2014; Long et al. 2015). For example, Cu and Cd differentially modulate transsulferation pathway metabolites, suggesting that different pathway components are being targeted by the toxins (Jeppe et al. 2014). Although many transition metals share some mechanistic similarities in regards to their toxic actions, it has been suggested that the transsulferation pathway could be used as a specific diagnostic

biomarker suite for detecting the identity of distinct metal stressors (Long et al. 2015). Detailed characterisation of this pathway in molluscan embryos/larvae at various molecular levels, and in response to different environmental contaminants, could be an exciting avenue for uncovering new mechanisms of toxicity, and identifying novel ecotoxicological biomarkers with forensic capacities.

Pathway enrichment and topology analysis identified GSH metabolism as being differentially regulated after lethal-level Cu exposure. The network includes the metabolites cysteine, glycine, glutamic acid, GSH, ornithine, and generic collectively termed L-amino acids; all of which were detected by our methods. Cu exposure caused levels of GSH to fall which was an expected response to Cu toxicity since metals increase GSH oxidation; resulting in a depletion of cellular GSH under high oxidative stress conditions (Jozefczak et al. 2012). A number of other metabolomics-based studies which have incorporated pathway analyses to investigate toxicity mechanisms in aquatic organisms have similarly identified perturbed GSH metabolism as a key response to various toxins. For example, Tufi et al. (2015) combined targeted neurotransmitter analysis with an untargeted metabolomics-based approach to explore sublethal effects of a pesticide, imidacloprid, in the central nervous system of pond snails (*Lymnea stagnalis*). Similar secondary bioinformatics procedures were used to identify a number of differentially regulated pathways, including GSH metabolism and biogenic amine synthesis, to generate a novel mechanistic neurotoxicity hypothesis.

Lower level Cu exposures to mussel embryos caused transient responses in GSH pathway metabolites which were indicative of variations between oxidative stress-induced GSH demand, biosynthesis, and oxidation. However, many of these changes were not statistically different and additional analyses at other levels of biological organisation are recommended to confirm the presence of oxidative stress at sublethal exposure concentrations. In Chapter 6, metabolic profiles of oyster larvae infected with

Ostreid herpesvirus microvariant displayed subtle indications of mild oxidative stress at the metabolite level in response to the virus. However, contrary to initial expectations based on *a priori* knowledge of host-virus interactions, secondary pathway analysis did not detect significant alterations to GSH metabolism. Thus, it was suggested that using only metabolomics-based approaches to characterise pathways which tightly regulate metabolite levels within confined ranges in well-functioning systems may be a methodological limitation. As such, changes in the expressions or activities of enzymes which regulate these processes may offer additional information. Thus, targeted analyses of a suite of oxidative stress biomarkers (enzymes and macromolecular oxidation products) in mussel embryos exposed to Cu have further been conducted in Part B of Chapter 7 to test the current interpretations of the metabolomics data as a validatory exercise.

7.4.3.7 Lipid metabolism

Prior to attaining feeding competency, lipids are the most important sources of energy during embryonic and larval development for many bivalve species (Gireesh et al. 2009; Matias et al. 2010; Sánchez-Lazo & Martínez-Pita 2012). When energy requirements are high, lipases hydrolyse triacylglycerols and release fatty acids (FAs). These free fatty acids (FFAs) are rapidly metabolised to acetate by β-oxidation in the mitochondria, and ultimately enter the TCA cycle under optimal conditions to supply ATP for various essential cellular process (anabolic reactions, active transport, cell division, motility, muscular contraction, etc.). Increased lipid catabolism, and/or enriched FA biosynthesis, and/or reduced FFA consumption can all lead to apparent rises in the total FFA pool, and vice versa.

Bivalve oocytes and embryos contain high levels of maternally-derived FA desaturase mRNA for altering the degree of FA saturation during early development, and

can therefore perform certain fatty acid conversion tasks (e.g., C20:4[n-3] to C20:5[n-3]) (Liu et al. 2014; da Costa et al. 2015). However, they typically do not have appreciable levels of elongase activity prior to attaining feeding competency, and are therefore unlikely able to generate fatty acids from acetyl-CoA subunits, or from smaller fatty acid precursors (Pettersen et al. 2010). Thus, variations in levels of FFAs in the current study are more likely indicative of altered lipid metabolism, rather than being a reflection of changes in *de novo* FFA biosynthesis.

With increases in FFA pools and greater demands for energy under enhanced oxidative stress conditions, it seems reasonable to suggest that lipid catabolism was being upregulated in certain Cu-treated groups. At the same time, reduced TCA cycle flux due to extreme Cu toxicity on enzyme activities may have simultaneously blockaded FFA consumption, leading to extreme FFA accumulations; as was indicated under lethal-level Cu exposures by the relative metabolite levels of the TCA cycle intermediates previously discussed. However, additional lipid-based analyses and/or targeted enzymatic assessments would be required to qualify these premises.

7.4.3.8 Immunotoxic associations

FFA accumulations are associated with mechanisms of immunotoxicity. It has been reported that significant increases in the FFA pool are toxic to organisms; inducing oxidative stress by generating ROS, and stimulating inflammation by increasing levels of the redox-sensitive transcription factor nuclear factor-[kappa]B (NF-κB) (Paolisso et al. 1996; Tripathy et al. 2003a,b;). These processes are not exclusive of one another. FFA-induced oxidative stress and harmful proinflammatory responses are inextricably linked since cross-talk can occur between ROS and the Nf-κB pathway. For example, while ROS have various inhibitory or stimulatory roles in NF-κB signalling, certain NF-κB-regulated genes serve major functions in mediating cellular ROS levels (Morgan & Liu 2011). The

NF-κB pathway is crucially involved in many biological processes, including innate immune responses in molluscs (Zhou et al. 2013; Li et al. 2015), inflammation (Baker et al. 2011), cell proliferation and differentiation (Janssen-Heininger et al. 2000; Biswas et al. 2004), apoptosis (Hayden & Ghosh 2011), and embryonic morphogenesis (Gilmore & Wolenski 2012). In addition, it has been established that Cu-induced immunotoxicity involves activation of the NF-κB pathway via direct or indirect ROS-mediated effects on the transcription factor (Persichini et al. 2006; Korashy & El-Kadi 2008). Thus, the large increases in the total FFA pool that was observed in moribund embryos exposed to lethal-level Cu both indicates and promotes a high level of oxidative stress and inflammation. This consequence was not apparent from the metabolomics data after short-term (3 hr) lethal-level exposure, or significantly at any time during sublethal treatments. This may suggest that the response occurs due to severe failures of the system only under chronically high Cu dose conditions. Under sublethal conditions, FFA consumption and conversion through a less negatively impacted TCA cycle to deliver the required energy demands may have been responsible for circumnavigating such potentially damaging immunotoxic effects of excessive FFA accumulations.

7.4.3.9 Lipid peroxidation

Particular FFAs or their derivatives can also be used as biomarkers for lipid oxidation. For example, dicarboxylic acids are classically derived from medium and long-chain monocarboxylic fatty acids by ω-oxidation, followed by β-oxidation, and reflect the end-products of oxidative attack on both free and esterified unsaturated fatty acids (Ferdinandusse et al. 2004). Thus, their presence partly is reflective of the usual lipid catabolic pathway. However, some of these metabolites are substantially increased in particular situations involving ROS-induced lipid peroxidation, and their higher-than-normal abundances can be used as biomarkers of oxidative stress/damage (Inouye et al.

2000; Hammond et al. 2008). For example, adipic acid (or hexanedoic acid) is a final product of lipid peroxidation which is nonenzymatically mediated by ROS (Jana et al. 2013), and is found at high levels under certain pathophysiological conditions associated with high oxidative stress environments (He at el. 2016). This metabolite was accumulated in moribund embryos which supports the involvement of severe oxidative stress.

7.4.3.10 Other metabolite variations

Malonic acid levels were enhanced by lethal-level Cu exposure. Elevated malonic acid levels have recently been reported in shrimps and oysters from sites contaminated by metals from industrial discharges (Ji et al. 2016a,c; Xu et al. 2016). Malonic acid is a C3 dicarboxylic acid and is well-known for its role as a regulator of cellular respiration by competing for the active site of succinate dehydrogenase/ respiratory complex II (SDH/CII) which catalyses the oxidation of succinate to fumarate in the tricarboxylic acid cycle (Kim 2002; Wojtovich & Brookes 2008). Inhibition of this enzyme would reduce the amount of fumarate being synthesised, which is in accordance with the diminished levels of fumarate that were detected in the Cu-exposed embryos. The induced mitochondrial dysfunction caused by SDH/CII inhibition can in turn trigger ROS production which depletes GSH and NADPH; thus overwhelming mitochondrial antioxidant capacity and resulting in lipid peroxidation and mitochondrial swelling (Fernandez-Gomez et al. 2005). Malonic acid is also associated with osmotic adjustment and secondary neuronal excitotoxicity, and can potentiate release of cytochrome c which induces apoptosis (Döbrössy et al. 2011; Fernandez-Gomez et al. 2005).

The C5 dicarboxylic acid glutaric acid is an endogenous toxin also known to induce oxidative stress (de Oliveira Marques et al. 2003). Lethal-level Cu caused levels of glutaric acid to increase in mussel embryos, whereas sublethal exposures did not cause

statistically significant effects. Although the mechanisms involved are not yet fully characterised, increased metabolite levels have been associated with decreased GSH concentrations, reduced GSH peroxidase activity, depressed total antioxidant reactivity, and increased lipid peroxidation and protein carbonylation (Fighera et al. 2006; Latini et al. 2007). Glutaric acid also plays roles in apoptotic pathways (Tian et al. 2014), and can impair energy metabolism through inhibition of Na^+/K^+-ATPase and enzymes involved in oxidative phosphorylation (Fighera et al. 2006).

2-aminobutyric acid (AABA) can also be considered a biomarker of oxidative stress (Soga et al. 2006). Under cysteine deficiency caused by considerable oxidative stress and high GSH demand, the first enzyme (Glutamate cysteine ligase) of the GSH biosynthetic pathway utilises AABA instead of cysteine (due to its similar structure) and causes the production of ophthalmic acid instead of GSH (Fujii et al. 2011; Dello et al. 2013). In such circumstances, decreases in both GSH and AABA would simultaneously be observed. Precisely, lethal-level Cu quickly induced considerably lower co-displayed levels of GSH and AABA, which decreased further upon continued exposure. Thus, reductions in AABA provide further evidence within the metabolomics data for severe dysregulation of the redox system under conditions of high Cu contamination. This effect was not observed after sub-lethal Cu exposures, with ROS homeostasis and GSH demand seemingly being maintained within certain acceptable boundaries.

7.4.3.11 Xenobiotics

During pre-processing and integrity checking of the metabolomics data, the presence of two xenobiotic compounds, benzothiazole (BTH) and dibutylpthalate (DBP), were identified in particular data subsets. BTH and its derivatives are widely used chemicals in various industries for the manufacture of rubber, textiles, plumbing components, pesticides, and a range of household products. They are highly water

soluble, they have been classified as emerging organic pollutants in marine environments, and some are considered at ubiquitous water contaminants (Bester 1997; Herrero et al. 2014). BTH is a common vulcaniser and major component that can leach from rubber, asphalt and polyethylene pipes; it is environmentally stable and can be found at relatively high concentrations within surface runoff and domestic wastewaters, which has led to its use as a tracer for aquatic pollutant inputs into urban coastal ecosystems (Spies et al. 1987; Zeng et al. 2004; Kloepfer et al. 2005). BTH is known to cause respiratory dysfunction in fish larvae (Evans et al. 2000), and induces ROS production in fish gill cell cultures (Zeng et al. 2016). DPB is used extensively as a plasticiser in resins and polymers, and especially in polyvinylchloride to render it flexible. Other industrial applications include manufacturing of adhesives, surface coatings, cosmetics, and as a solvent in insecticides. DPB is a widely distributed marine pollutant, readily bioaccumulates in organisms due to its lipophilic properties, and is highly toxic to marine mollusc embryos and larvae (Mackintosh et al. 2004; Xie et al. 2007; Liu et al. 2009; Zhou et al. 2011).

The presence of BTH and DPB within the metabolomics dataset was originally discounted and ear-marked as potential artefacts or plastic/rubber-derived leachates from the incubation tanks, sample vials, pipework or water treatment system at the research facility, or as background contaminates within the bulk seawater that was used. However, closer inspection of the data revealed that the prevalence of these xenobiotics were associated only with moribund embryos exposed to lethal-level Cu. Thus, in combination with the other assessments of embryonic health and pathophysiology, further consideration led to the decision to retain these compounds for further analysis. Elevated tissue concentrations of BTH and DPB were identified as being important (but not crucial) during PLS-DA modelling for sample class discrimination of embryos exposed to high bioavailable Cu for 18 hrs. As previously outlined, moribund embryos showed strong

signs of commencing apoptosis with probable failures in lipid membrane stability and permeability (i.e., high incidences of cellular budding, shrinkage and cellular dissociation, and metabolite markers for oxidative lipid damage). The presence of such xenobiotics only within these specific embryo samples are thus telling of a decreased capacity to maintain selective exclusion of exogenous compounds across the cellular membrane through deteriorating membrane transport systems and/or disruption of the lipid bilayer prior to sampling and cell death. This is in line with the detrimental effects of severe Cu-induced oxidative damage to membrane integrity and permeability (Wong-Ekkabut et al. 2007; Repetto et al. 2012), resulting in a gateway for BTH and DPB in the seawater to penetrate cells and form xenobiotic-contaminated embryos. Assessment of cellular viability through the neutral red uptake assay showed that although these cells were in poor condition, they were indeed live. Unfortunately, due to logistical constraints, this analysis was not extended to characterise membrane stability by measuring the lysosomal retention of the stain over time which would have provided additional information on lipid bilayer integrity.

It was surprising to have detected BTH and DPB in some of the samples since the aquaculture research facility where the experiment was conducted is not adjacent to sources of urban run-off. Although the origins of these compounds are unascertained, it appears they were derived from some aspect of the experimental set-up or as environmental pollutants in the source seawater rather than from downstream contamination during sample processing. Being found in moribund-only samples suggests that low background levels of BTH and DPB unlikely poses any immediate threat to healthy embryos or larvae during development in culture or in the wild. However, it is possible that deteriorating health condition and/or cell membrane instability induced by other factors which cause oxidative stress, such as heavy metal

contamination or attack by ubiquitous marine pathogens, may be exasperated due to enhanced cellular penetration and bioaccumulation of organic marine pollutants.

7.4.4 Metabolomics as a health assessment tool

The capacity of metabolomics to discriminate larval groups in this study was far more powerful than using routine visual assessment methods. The lowest sublethal-level copper exposure caused some (~10%) changes in D-larval swimming behaviour (i.e., slowed swimming and altered motion trajectories) towards the end of the trial (66 hrs post exposure), but did not impact the rate of embryonic development. On the other hand, metabolomic analysis of these samples identified significant compositional changes in embryonic metabolite profiles after only 3 hrs exposure, and similarly could discriminate treated embryos/larvae at every subsequent sampling point from non-treated controls. This fact highlights the exceptional sensitivity that metabolomics can offer to detect changes in metabolic processes days before organisms display any visually observable phenotypic traits of altered health state.

It could be argued that the metabolic shifts detected after very short term toxin exposure were not representative of the subtle signs of 'poor health' (i.e., altered swimming behaviours) that were observed almost three days later. However, these initial deviations that were detected in embryonic metabolism may well have been reflective and/or responsible for the cascade of subsequent metabolic shifts that ultimately concluded in altered behaviour, and the delayed developmental timing in other treatments. Regardless of the precise mechanism, these findings also suggest that metabolomic approaches can be used for the early detection of poor larval health, as well having potential capacities for prognosis and diagnosis.

Within the context of mollusc aquaculture, there is certainly positive scope for the application of metabolomics to monitor larval health during hatchery culture, and also to

identify precise temporal points during production runs when external factors first start influencing larval physiology; thus helping to determine causation for poor health and occurrences of mass mortality (reviewed in Chapter 1). Metabolomics is clearly useful for characterising mechanisms of toxicity (e.g., this study; Bando et al. 2011; Go et al. 2014; Brandão et al. 2015; Boyles et al. 2016), but there is also scope for the approach to be incorporated into ecotox test methods to provide information earlier than traditional biological endpoints, such as '% D-larval yield' and mortality. With further development and biomarker validation, this could provide additional measures of toxicity, enhance test sensitivity, and reduce test duration.

7.5 References

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Part B

Targeted analysis of oxidative stress biomarkers

Abstract

In Chapter 7 Part A, an untargeted metabolomics approach was successfully applied to characterise the metabolic basis of copper toxicity during marine mussel embryonic and larval development. Among a range of toxin-induced effects (e.g., developmental retardation, initiation of apoptosis, cell membrane degradation, changes in glutathione metabolism, osmotic disturbance) a substantial number of metabolite variations pointed strongly towards the occurrence of oxidative stress. Herein Chapter 7 Part B, a comprehensive and targeted survey of nine traditional oxidative stress biomarkers have subsequently been performed to test the previous interpretations and validate the metabolomics data. To this end, a novel assay was developed to monitor levels of reactive oxygen species (ROS) *in vivo*, the activities of five enzymes involved in ROS regulation and glutathione turnover were measured, and ROS-induced oxidative damage to lipids, protein, and DNA were assessed. Effects of copper on the oxidative stress biomarkers were dose-dependent, with results confirming oxidative stress as a key mechanism of copper toxicity in developing embryos, thus validating the metabolomics data.

7.6 Introduction

Oxidative stress is caused by an imbalance between oxidants and antioxidants in biological systems. When the system favours production of oxidants, such as reactive oxygen species (ROS), the cellular redox balance is disrupted and oxidative damage to cellular components can occur (Sies & Jones 2007). Reactive oxygen species (ROS) is a collective term used to describe various chemical species with high oxidative capacities. These include the hydroxyl radical ($OH^{\cdot}$), singlet oxygen ($^1O_2$), superoxides (compounds containing the superoxide anion $O_2^{\cdot -}$), and peroxides (H_2O_2 and compounds containing an oxygen–oxygen single bond or the peroxide anion O_2^{2-}) (Valavanidis et al. 2006; Lushchak 2011). Cellular ROS are generated during the process of mitochondrial oxidative phosphorylation, and play important roles in enzymatic reactions, signal transduction, activation of nuclear transcription factors, gene expression, and the antimicrobial action of macrophages (Frädrich et al. 2016). Thus, maintaining a certain level of basal ROS is crucial to normal physiological functioning.

Regulation of ROS is controlled by nonenzymatic mechanisms, such as the major cellular redox buffer glutathione (GSH), which has a high antioxidant capacity to scavenge excess ROS levels (Schafer & Buettner 2001). A number of enzymatic ROS scavenging mechanisms also facilitate ROS detoxification, and include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Apel & Hirt 2004). SOD is the first line of defense against excess ROS by dismutating $O_2^{\cdot -}$ into either ordinary molecular oxygen or H_2O_2. The H_2O_2 can subsequently be detoxified directly by CAT, or indirectly by GPx, through conversion to H_2O and O_2. GPx catalyses the oxidation of GSH to glutathione disulphide (GSSG) and simultaneous reduction of H_2O_2 (Forman et al. 2009). When this system is functioning optimally, GSSG can then be recycled via glutathione reductase (GR) to replenish GSH levels. However, when ROS is generated beyond the capacity of cells to maintain homeostatic control, the excess ROS

can oxidise and critically damage lipids, proteins, and DNA (Temple et al. 2005). Under moderate oxidative stress conditions, repair mechanisms are activated to alleviate the resulting cellular damage. For example, GPx also serves to reduce toxic lipid hydroperoxides (formed through ROS-induced lipid oxidation) to their corresponding non-toxic alcohols (Knapen et al. 1999). Under high oxidative stress conditions, both the ROS regulatory system and the repair mechanisms involved in ameliorating ROS-induced lipid, protein, and DNA damage are overwhelmed. This can lead to cell death, and expiry of the organism under severe conditions.

Oxidative stress is one of the most well-known mechanisms of metal toxicity. Normal and elevated levels of $O_2^{\cdot -}$ and H_2O_2 can interact with transition metals, such as copper and iron, to form more-toxic $OH^{\cdot}$ radicals via metal catalysed Haber–Weiss/Fenton reactions (Birben et al. 2012). In addition, high levels of metals can also indirectly cause intracellular ROS to accumulate through its inhibitive effect on antioxidant enzymes involved in ROS regulation (e.g., SOD, CAT, GPx, GR) (Pigeolet et al. 1990; Nzengue et al. 2011). Targeted analysis of these enzymes and/or the degradation products of macromolecule oxidation (e.g., lipids → lipid hydroperxides, proteins → protein carbonyls, DNA → 8-hydroxy-2'-deoxyguanosine) can provide a means to specifically measure and characterise the level of oxidative stress in an organism exposed to metal contamination (Valavanidis et al. 2006). Indeed, copper toxicity and oxidative stress in marine molluscs has been fairly well studied for post-metamorphic life stages of shellfish, such as mussels (Regoli & Principato 1995; Company et al. 2008; Maria & Bebianno 2011; Trevisan et al. 2011), oysters (Jing et al. 2006), clams (Geret et al. 2002; Peng et al. 2015), and scallops (Regoli et al. 1997). However, much less is known about the regulatory mechanisms of toxicity in early life-stage embryonic and larval forms.

In Chapter 7 Part A, a broad discovery-based untargeted analysis of metabolites was conducted in newly-fertilised mussel embryos exposed to various level of bioavailable copper. Among many other metabolic and physiological toxin-induced consequences, the metabolomics-based analysis identified a number of metabolite alterations, which indicated the occurrence of an oxidative stress response to the metal. These included signatures of nucleotide degradation, lipid peroxidation, xenobiotic uptake via cell membrane destabilisation, changes in fatty acid composition, enrichment of the transulphuration pathway, perturbation to glutathione metabolism, and effects on tricarboxylic acid cycle intermediates likely due in part to ROS-induced inhibition of the enzyme aconitase. Together, these findings provided multiple and complimentary evidences at the metabolite level for Cu-induced oxidative stress.

Although the specific signatures of metabolite changes in Part B were not hypothesised in detail, the general results were not entirely surprising considering that copper tends to promote ROS production in various animal models (Ercal et al. 2001; Valko et al. 2005; Rhee et al. 2013; Regoli & Giuliani 2014; Atli & Grosell 2016; Periera et al. 2016). However, it has been advocated that oxidative stress should not be universally quantified by any single biomarker nor level of biological organisation since various degrees of oxidative stress are represented by different molecular and biochemical perturbations, which can have diverse physiological outcomes (Dotan et al. 2004). Thus, particular types or severities of oxidative stress should ideally be evaluated using different indices. Indeed, in Chapter 6, metabolite signatures which were potentially indicative of low-level oxidative stress in oyster larvae as a response to Ostreid herpesvirus infections were identified; but these biochemical changes were so subtle (hypothetically due to a well-functioning redox balance system) that additional analyses of ROS-regulatory enzymes was needed to obtain more concrete evidences. Taking this into consideration in the current chapter, we have further applied a targeted analysis of nine different known

measures of oxidative stress in an attempt to compliment the metabolomics data and validate our interpretations.

7.7 Methods

7.7.1 Experimental design summary and sampling

Newly fertilised mussel (*Perna canaliculus*) embryos were exposed to four levels (0.0, 0.04, 1.10, 50.3 μgL^{-1}) of bioavailable Cu in the presence of 4 µM EDTA and sampled repeatedly during a 72 hr exposure trial, as detailed in Chapter 7 Part A. Samples for Part B of this chapter were obtained simultaneously with the embryo and larval samples taken for metabolomics analysis in Part A using exactly the same methods to obtain similar amounts of biomass (one sample for DNA extraction and analysis, and one sample for sequential lipid and protein extraction and analysis). Samples were immediately snap frozen in liquid nitrogen and stored at −80°C until analysis.

7.7.2 Protein, lipid and DNA analysis

Prior to extraction, embryo and larval samples (each containing approximately 200,000 individuals) were thawed in a 20°C water bath for 1 min. Total protein was extracted on ice by adding 500 µL of ice-cold enzyme extraction buffer (50 mM potassium phosphate [pH 7.2], containing 0.1 mM Na_2EDTA, 1 mM EGTA, 125 mM KCl and 1 mM phenylmethylsulfonyl fluoride) to the samples, followed by mixing with a vortex-mixer and homogenising for 1 min using a Mini-Beadbeater-1 and Zirconia/Silica beads (Biospec Products: Bartlesville, OK, USA). The homogenate was centrifuged (Ependorf 5417R: Eppendorf South Pacific Pty. Ltd.: North Ryde, NSW, Australia) for 15 min at 20,800*g* and 4°C. The supernatant, now referred to as the protein extract, was then transferred to a 1.5 mL microcentrifuge tube. The pellet of cell debris was retained for lipid extraction and the protein extract was subjected to ultrafiltration using Vivaspin® 500 Centrifugal Concentrators with 10,000 MWCO membranes (Sartorius Stedim

Biotech Gmbh: Goettingen, Germany) according to the manufacturer's instructions. The ultrafiltered protein extract was reconstituted with ice-cold 100 mM potassium phosphate (pH 7.2) and protein contents were determined using the Lowry protein assay (Fryer et al. 1987). Samples were then diluted as required with 100 mM potassium phosphate (pH 7.2) prior to targeted enzyme analyses. Protein carbonyls levels in the reconstituted protein extracts were determined via reaction with 2,4-dinitrophenylhydrazine (DNPH) according to Reznick and Packer (1994) and expressed as nmols of carbonyls per mg protein.

Lipids were extracted from the pellet of cell debris (remaining after protein extraction) by adding 500 µL of methanol-chloroform solution (2:1 v/v) to the tube. Each sample was left to stand for 1 min and then 200 µL of chloroform was added and vortex-mixed for 30 sec. Deionised water (200 µL) was added and the extract mixed again for 30 sec. To separate the phases, the resulting homogenate was centrifuged (Eppendorf 5417R) twice for 1 min at 20,800*g* at ambient temperature. Lipid hydroperoxide levels were determined using the ferric thiocyanate method described by Mihaljević et al. (1996), adapted for measurement in a microtitre plate reader. Lipid hudroperoxide levels were determined by measuring the absorbance at 500 nm. A calibration curve with t-butyl hydroperoxide was used and the lipid hydroperoxide content was calculated as nmol of lipid hydroperoxide per sample.

The above assays were carried out using a multilabel counter (Wallac Victor 1420, Perkin Elmer: San Jose, California, USA) controlled by a PC and fitted with a temperature control cell (set to 25°C) and an auto-dispenser. Data were acquired and processed using the WorkOut 2.0 software package (Perkin Elmer).

DNA was extracted using an ISOLATE II Genomic DNA Kit (Bioline: N.S.W, Australia) following the manufacturers' instructions for standard samples, but with minor modifications. After addition of the pre-lysis buffer, samples were homogenised for 1 min

using a Mini-Beadbeater-96 and Zirconia/Silica beads (Biospec Products). In addition, the pre-lysis was supplemented with 5 mM deferoxamine and 20 mM EDTA, and the lysis buffer was supplemented with 5 mM deferoxamine. All solutions were deoxygenated by gently bubbling with nitrogen gas for 5 min.

Digested DNA samples were analysed using high-performance liquid chromatography (HPLC) followed by UV detection of guanine and coulometric detection of 8-hydroxy-2'-deoxyguanosine (8-OHdG). The procedure was performed using a C18 reverse-phase column (5 mm, 4.6 mm × 250 mm) (JASCO: Tokyo, Japan), an HPLC system (PerkinElmer: Boston, USA) and an electrochemical detector (5100 Coulochem, ESA: MA, USA). Separation of DNA was achieved using an isocratic mobile phase (50 mM potassium phosphate [pH 5.5] and 10% methanol) at a flow rate of 1 mL min^{-1} with the column being maintained at 30°C. The analytical cell oxidation potentials of the electrochemical detector were set to 150 mV and 350 mV for electrodes 1 and 2, respectively, with the guard cell potential set at 400 mV. Unmodified nucleosides were detected by their absorbance at 260 nm. Peak data were collected and analysed using a DataCenter 4000 (DataworkX: Brisbane, Australia) general-purpose laboratory data interface, and Delta 5.0 chromatography data acquisition and analysis software (DataworkX). The retention times for guanine and 8-OHdG were 12 and 17 min, respectively. Pure solutions of 8-OHdG and guanine (Sigma-Aldrich: St Louis, MO, USA) were prepared in HPLC-grade water (Merck: Darmstadt, Germany) and sterilised by passage through 0.22 mm filters (Millipore: Bedford, MA, USA) to be used as standards. The amount of DNA injected onto the column for each sample was estimated using the signal for guanine, and 8-OHdG was quantified by comparison to external standards.

7.7.3 Antioxidant enzyme analysis

Total protein was extracted as previously described for protein carbonyl measurements, and followed by enzymatic antioxidant assays for superoxide dismutase (SOD: EC 1.15.1.1), catalase (CAT: EC 1.11.1.6), glutathione peroxidase (GPx: EC 1.11.1.9), glutathione reductase (GR: EC 1.8.1.7), and glutathione-S-tranferase (GST: EC 2.5.1.18) activity using a Perkin Elmer Wallac Victor 1420 multilabel counter.

SOD activity was determined using the microplate assay (Banowetz et al. 2004), with minor modifications. A 50 µL of protein extract or standard (prepared from bovine liver SOD [Sigma-Aldrich] where one unit of SOD corresponded to the amount of enzyme that inhibited the reduction of cytochrome c by 50% in a coupled system with xanthine oxidase at pH 7.8 and 25°C) was mixed with 125 µL of freshly prepared reaction solution containing PIPES buffer, pH 7.8, 0.4 mM of *o*-dianisidine, 0.5 mM of diethylenetriaminepentaacetic acid, and 26 µM of riboflavin. Absorbance at 450 nm was measured immediately (t = 0 min), then samples illuminated with an 18 W fluorescent lamp placed 12 cm above the plate for 30 min and measured again (t = 30 min). A regression analysis was used to prepare a standard line relating SOD activity to the change absorbance. Superoxide dismutase activities in the extracts, calculated with reference to the standard line, are expressed as units SOD per mg of total protein.

CAT activity was determined using the chemiluminescent method, adapted for 96-well microplates (Janssens et al. 2000). A 50 µL of extract or standard (purified bovine liver CAT [Sigma-Aldrich] in homogenisation buffer) was mixed with 100 µL of 100 mM phosphate buffer (pH 7.0) containing 100 mM of Na_2EDTA and 50 µL of 1 µM H_2O_2. Samples were then incubated at 25°C for 30 minutes, after which 50 µL of a solution containing 20 mM luminol and 11.6 U/ml of horseradish peroxidase (Sigma-Aldrich) was injected into each well. Light emission, the intensity of which was proportional to the amount of H_2O_2 remaining in the mixture, was measured. A regression analysis was used

to prepare a standard line relating standard CAT activities to the intensity of light emission. CAT activities in the extracts were calculated with reference to the standard line and expressed as µmol of H_2O_2 consumed per min per mg of total protein.

GPx activity was determined using the spectrophotometric method (Paglia & Valentine 1967), adapted for a microplate reader. A 20 µL of extract or standard was mixed with 170 µL of assay buffer containing 50 mM of Tris-HCl buffer (pH 7.6), 5 mM of Na_2EDTA, 0.14 mM of NADPH, 1 mM of GSH, and 3 U/mL of wheat germ glutathione reductase (EC 1.6.4.2) (Sigma-Aldrich). The reaction was initiated by the addition of 20 µL of *t*-butyl hydroperoxide to give a final concentration of 0.2 mM. The consumption of nicotinamide adenine dinucleotide phosphate was monitored at 340 nm every 30 sec for 3 min, with the plate shaken automatically before each reading. The GPx activities in the extracts were calculated with reference to a standard line constructed with GPx purified from bovine erythrocytes (Sigma-Aldrich) in extraction buffer. Data are expressed as nmol per min per mg of total protein.

GR activity was determined using the method of Cribb et al. (1989), with minor modifications. Briefly, 50 µL of extract (diluted extract or standard GR from wheat germ [Sigma-Aldrich] in homogenisation buffer) was mixed with 150 µL of 100 mM sodium phosphate buffer (pH 7.6) containing 0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid) and 10 µL of 12 mM NADPH. The reaction was initiated by the injection of 10 µL of 3.25 mM oxidised glutathione and the absorbance was measured at 415 nm every 30 sec for 3 min, with the plate shaken automatically before each reading. The rate of absorbance increase per min was calculated and a calibration curve relating standard GR activities to the change in absorbance was constructed. GR activities in the extracts were calculated and expressed as nmol of oxidised glutathione reduced per min per mg of total protein at pH 7.6 and 25°C.

GST activity was determined using the photometric 1-chloro-2,4-dinitrobenzene (CDNB) method according to Habig et al. (1974), with minor modifications. The absorbance at 340 nm was measured every 30 sec for 3 min, with the plate shaken automatically before each reading. The change in absorbance per minute was calculated and converted into nmol of CDNB conjugated to GSH per min per mg of total protein using the extinction coefficient (E_{340} = 9.6 mM^{-1} cm^{-1}) of the resulting S-2,4-dinitrophenylglutathione.

7.7.4 Reduced glutathione analysis

Glutathione (GSH) was extracted and identified during untargeted metabolomics-based analysis in Chapter 7 Part A. Due to it high relevance in mechanisms of oxidative stress, all glutathione data are presented herein Part B, along with the other biomarkers that were targeted. GC/MS-derived peak intensity values were normalised against the L-Alanine-d4 internal standard (i.e., [glutathione peak height / int. std. peak height] x int. std. concentration) to obtain semi-quantitative GSH data.

7.7.5 ROS analysis

ROS production was measured *in vivo* using the cell-permeant ROS-detecting fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate (H_2-DCFDA) (Invitrogen – Molecular Probes, D399: Eugene, OR, USA). Upon cleavage of the acetate groups by intracellular esterases and oxidation, the non-fluorescent H_2-DCFDA is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF), which can be measured via fluorescence spectroscopy. At 18, 36 and 66 hrs post-fertilisation, approximately 60,000 embryos or larvae from each of the 15 incubation tanks were concentrated on a 15 µm mesh screen, transferred via pipette to a 50 mL Falcon™ tube, and made up to 25 mL with FSW. Six 210 µL subsamples (technical replicates) of each larval solution, containing approximately 500 larvae, were then transferred to wells of a 96-well microplate. Blank

seawater controls contained 210 µL of FSW. Immediately prior to performing each ROS assay, a pre-frozen stock of the H_2-DCFDA probe (1 mM in DSMO) was thawed and a 20 × dilution was prepared in FSW. 40 µL of this diluted H_2-DCFDA working solution was pipetted into all wells and the plates were incubated in the dark at 19 ± 0.5°C for 120 min. ROS production was assessed by measuring the DCF fluorescence intensity on a Perkin Elmer microplate reader (Ex/Em: 490/520 nm). Variations in intensity values are reflective of relative differences in ROS production among treatments.

7.7.6 Statistics and data presentation

To identify differential expressions/activities of targeted oxidative stress biomarkers in Cu-exposed larvae compared to control organisms, each biomarker was assessed separately via *t*-test analysis for each development stage and treatment level using SPSS v22.0 (IBM Corporation: Armonk, NY, USA). To facilitate visualisation of the trends on a common scale, data have been autoscaled and each biomarker presented separately in line plots. In addition, a summary of results is also displayed in the form of a table with colour coding; where up/down arrows represent statistically different ($p < 0.05$) biomarker expressions/activities in the Cu-exposed embryos and larvae compared to non-exposed control organisms.

7.8 Results

Exposure of mussel embryos to various levels of copper induced a number of significant changes in the oxidative biomarkers assayed. These changes were dependant on both the dose and duration of exposure. To contextualise the following results, an overview of the relationships between the oxidative stress biomarkers analysed in this study and the ROS-regulatory system is provided in Figure 7.10 as a visual summary.

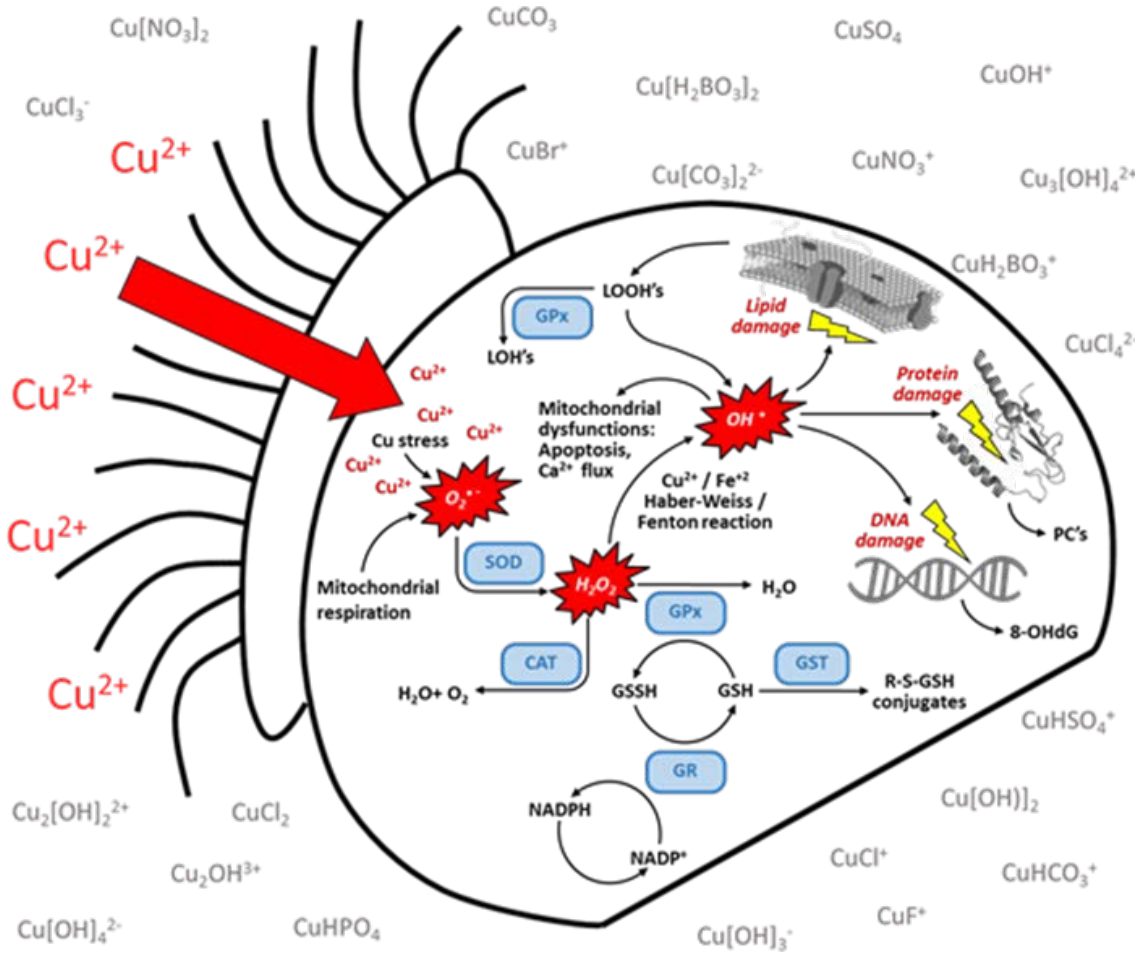

Figure 7.10. Stylised schematic representation of Cu-induced oxidative stress and ROS-regulatory mechanisms in an early D-stage mussel larva.

ROS production in recently fertilised mussel embryos after 18, 42 and 66 hrs post-exposure to Cu treatments is shown in Figure 7.11. There was a positive linear trend in the generation of ROS during progression of baseline ontogeny from trochophore through to D-stage development (control values in Figure 7.11A–C). Significant increases (*t*-test; $p < 0.01$) in Cu-induced ROS production were detected after 36 hrs exposure to sub-lethal Cu concentrations, compared to controls (Figure 7.11B).

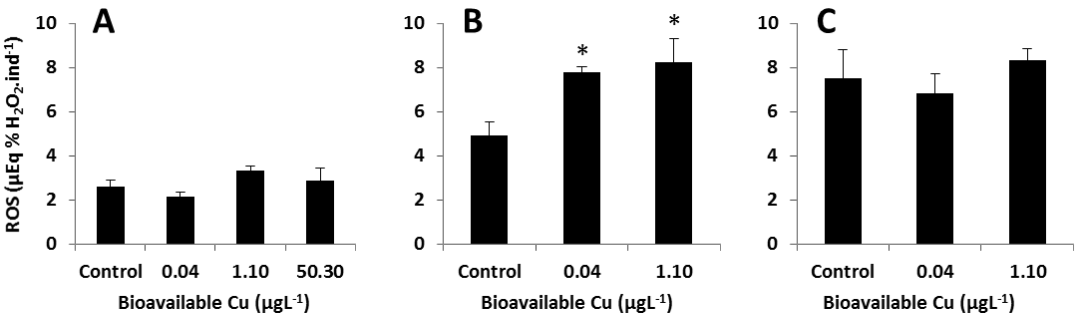

Figure 7.11. Production of reactive oxygen species (ROS) in embryos and larvae reared under different levels of bioavailable Cu. **A)** 18 hrs post-exposure, **B)** 36 hrs post-exposure, and **C)** 66 hrs post-exposure. ROS levels are expressed as % H_2O_2 micro equivalents per individual. Asterisks denote significant differences compared to controls (*t*-test, $p < 0.05$).

The effects of Cu exposure on the suite of targeted oxidative stress biomarkers (and glutathione from Chapter 7 Part A) are displayed in Figure 7.12 and Table 7.3. Exposure of embryos to low bioavailable Cu (0.04 μgL^{-1}) for 42 hrs resulted in very subtle signs of lipid damage (ca. 13% higher LOOHs than control values). However, after 72 hrs exposure, levels of lipid peroxidation products were comparable to those in control organisms. This demonstrates that the low dose treatment did not result in lasting negative effects associated with the characteristic mechanism of heavy metal toxicity via ROS-induced oxidative damage to DNA, proteins and lipids. Evidenced by the slight response of the ROS-regulatory system via GSH, it appears that the embryos/larvae were able to alleviate any substantial adverse effects, either solely through this system, or in combination with other unknown metabolic regulatory mechanisms.

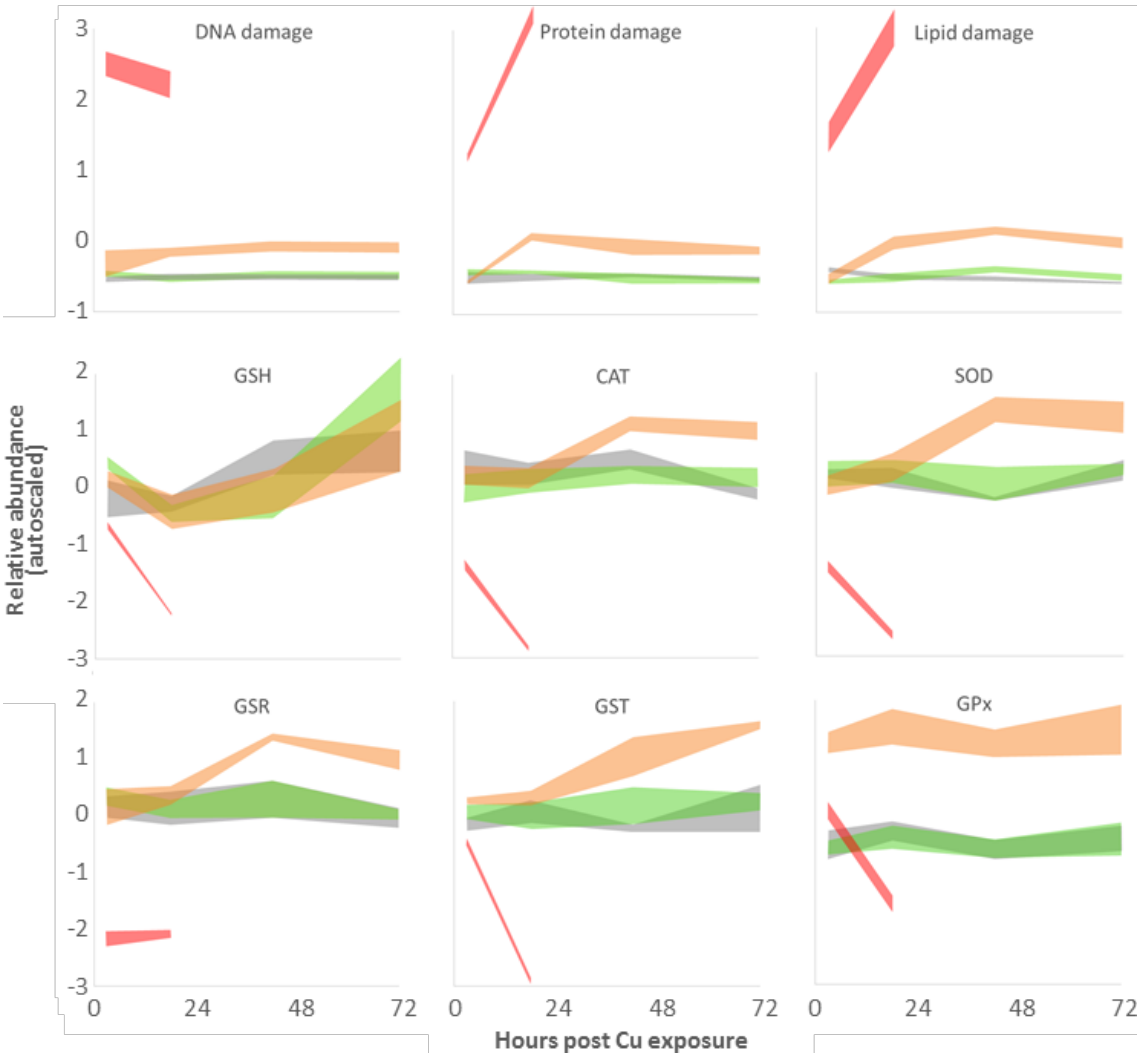

Figure 7.12. Targeted analysis of oxidative stress biomarkers in response to Cu exposure over time. Data are autoscaled and the width of the coloured lines (grey = controls; green = 0.04 μgL^{-1} bioavailable Cu; orange = 1.10 μgL^{-1} bioavailable Cu; red = 50.3 μgL^{-1} bioavailable Cu) are bounded by the standard error of the means of triplicate samples.

Table 7.3. Relative temporal changes in oxidative stress biomarkers across Cu treatments, compared to controls. Up/down-arrows represent statistical differences (*t*-test; $p < 0.05$) between treatments and controls displayed in Figure 7.12 at different time points; hyphens represent no statistical difference.

Biomarker	0.04 μgL^{-1}				1.10 μgL^{-1}				50.3 μgL^{-1}	
	3h	18h	42h	72h	3h	18h	42h	72h	3h	18h
DNA damage	-	-	-	-	-	↑	↑	↑	↑	↑
Protein damage	-	-	-	-	-	↑	↑	↑	↑	↑
Lipid damage	↓	-	↑	-	-	↑	↑	↑	↑	↑
GSH	-	-	-	-	-	-	-	-	↓	↓
CAT	-	-	-	-	-	-	↑	↑	↓	↓
SOD	-	-	-	-	-	-	↑	↑	↓	↓
GR	-	-	-	-	-	-	↑	↑	↓	↓
GST	-	-	-	-	↑	-	↑	↑	-	↓
GPx	-	-	-	-	↑	↑	↑	↑	-	↓

In contrast, exposure of larvae to the medium level Cu dose (1.10 μgL^{-1}) resulted in the fast upregulation of GST and GPx after 3 hrs exposure. By 18 hrs post Cu exposure, evidence of oxidative damage to all three macromolecule biomarkers had occurred. After 42 hrs exposure, DNA, protein and lipid damage persisted and further modulation of the ROS regulatory system was apparent, supported by additional inputs from the increased co-expressions of GR, SOD, and CAT. These strong indications of Cu-induced oxidative stress continued to 72 hrs post-exposure. GSH levels were more variable than other biomarkers, but were not statistically different compared to control values at any time.

The high dose Cu treatment (50.3 μgL^{-1}) quickly induced extensive damage to DNA, protein and lipids in embryos after only 3 hrs exposure. Unlike the sublethal data, down-regulation of other enzymatic oxidative stress biomarkers (i.e., GR, SOD, and CAT) at the first sampling point was detected, indicating severe toxicological effects. By 18 hrs post-exposure, these negative impacts had worsened and included simultaneous reductions in levels of GST and GPx, providing clear evidence of a disrupted/failing ROS regulatory system.

7.9 Discussion

Production of pro-oxidant reactive oxygen species (ROS) and antioxidant glutathione (GST) were assessed, and the activities of key enzymes involved in the regulation of ROS homeostasis were measured (i.e., glutathione S-transferase [GST], glutathione peroxidase [GPx], glutathione reductase [GR], superoxide dismutase [SOD], and catalase [CAT]). Oxidative damage to lipids, proteins and DNA were also evaluated to investigate consequential cytoxic and genotoxic effects via analysis of macromolecular oxidation products (i.e., lipid hydroperoxides [LOOHs], protein carbonyls [PCs], and 8-hydroxy-2'-deoxyguanosine [8-OHdG]). In general, it appears that oxidative stresses caused by sublethal Cu treatments were able to be mostly alleviated by homeostatic control mechanisms of the redox balance system, whereas lethal-level Cu resulted in severe cellular damage beyond the adaptive metabolic abilities of developing embryos. These toxin-induced consequences are summarised in Figure 7.13.

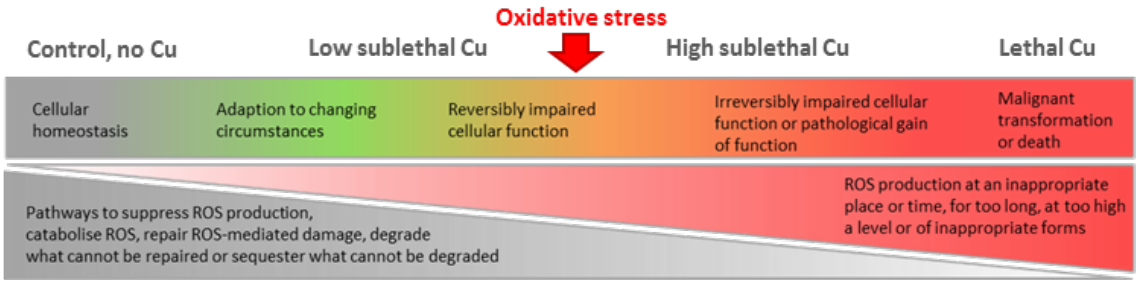

Figure 7.13. General effects of Cu-induced oxidative stress on mussel larval physiology.

7.9.1 Reactive oxygen species

Baseline 'steady-state' ROS production during mussel embryogenesis increases with development to the D-stage larval phenotype; at least in terms of the relative ROS quantities produced per individual. Normal embryonic development is highly dependent on redox status due to its wide-ranging influence on multiple cellular processes, including

cell proliferation, differentiation, and signalling (Dennery 2007). A certain level of ROS production is crucial for controlling apoptosis and cell elimination, and for regulating key transcription factors that alter embryonic gene expression. The inability to increase oxidative stress at specific phases can lead to abnormal developmental sequences and metabolic dysregulation during embryogenesis (Dennery 2007).

The particular redox status at a given embryonic stage alters the fate of cells towards proliferation, differentiation, apoptosis, or necrosis; where a reduced state leads to proliferation, mild oxidation leads to differentiation, and further oxidation to cell death (Schafer & Buettner 2001). Thus, the pattern of ROS production that was detected in control embryos is in accordance with early embryonic cell proliferation, followed by differentiation of shell field and retractor muscle cells in emerging D-stage larvae, and commencement of organogenesis during subsequent larval development (Rusk et al. 2016).

In the case of the sublethal ROS data, there was evidence of acute oxidative stress occurring at 36 hrs post-exposure with increased spikes in ROS being detected, compared to controls. By 66 hrs of exposure, ROS levels were again similar to control values, revealing high cellular antioxidant potential during this period and a certain ability to limit oxidative damage. This is supported by the increased activities of ROS-regulatory enzymes that were detected shortly thereafter (72 hrs post-exposure), and the enhanced but static levels of lipid peroxidation and protein carbonylation between 42 and 72 hrs post-exposure.

ROS levels after 18hrs of lethal-level Cu exposures were surprisingly similar to control levels. This could be indicative of oxidative stress having previously occurred and then being re-established towards the baseline by ROS-regulatory enzymes and antioxidant metabolites. Alternatively, in extreme circumstances and after initial oxidative stress-induced spikes, decreased levels of ROS may occur due to initiation of

cell death. The mitochondrial apoptotic pathway primarily responds to internal insults such as oxidative stress and DNA damage (Aronis et al. 2003). During Fas-mediated apoptosis (a common mechanism of programmed cell death during embryonic development), reduced ROS production occurs early on in the death cascade before significant cell death has occurred (Zou et al. 2000; Aronis et al. 2003). Considering the collective results across the many biological endpoints that were assessed in Chapter 7 Part A (i.e., embryo/larval mortality, cellular viability, microscopical observations showing signs of cellular budding, and the various metabolite changes) and Chapter 7 Part B (i.e., decreased activities of ROS regulatory enzymes indicative of severe oxidative stress, and increased oxidative DNA damage), initiation of apoptosis and necrosis is a plausible explanation for the lower-than-expected ROS levels observed at this time.

7.9.2 Glutathione

GSH is an efficient antioxidant and provides protection against oxidative stress by conjugating with electrophiles and reducing ROS (Wang & Ballatori 1998). During this process, GSH is converted into its oxidised form (GSSG) via catalysis by GPx (Forman et al. 2009). GSH is also involved in the formation of GSH-S conjugates with ionic forms of Cu (forming linear II covalent complexes) (Rabenstein et al. 1988), and acts as an intracellular chelator to prevent the nuceophilic interaction of Cu with the main cellular structures (Maracine & Segner 1998). In the current study, levels of GSH were minimally disturbed by sublethal-level Cu exposures (as previously discussed in Chapter 7 Part A).

Under low-to-moderate oxidative stress conditions, GSH concentrations may be replenished through biosynthesis from precursor metabolites and/or through the NADPH-dependant reduction of GSSG (Lu et al. 2009; Marí et al. 2009). Indeed, maintenance of basal-level GSH flux during sublethal Cu treatments is indicative of a well-functioning GSH homeostatic control mechanism. Unfortunately GSSG was not detected via GC-MS

in Chapter 7 Part A, which could have provided empirical evidence for GSH utilisation under sublethal Cu treatments had accumulations of the oxidised form been observed.

During lethal-level exposure, GSH levels decreased substantially and very quickly. Such a response was not surprising since excessive metal-induced oxidative stress is well-known to cause rapid GSH depletion in molluscs (Regoli & Principato 1995; Regoli et al. 1998; Canesi et al. 1999; El-Gendy et al. 2009; Zhu et al. 2011; Nugroho & Frank 2012). The decrease in GSH indicates increased utilisation to stabilise Cu in its oxidative state (thus preventing redox cycling and free radical generation), and an inefficient GSH regeneration capacity resulting in a reduced ability to scavenge free radicals, and raising the general cellular oxidative potential (Elia at al. 2003). For further discussion of GSH and its metabolism, refer to Chapter 7 Part A since differential network alterations were also detected via pathway enrichment analysis of functionally-related metabolites.

7.9.3 Enzymatic biomarkers of oxidative stress

ROS homeostasis and GSH turnover is regulated by an elaborate system of enzymes, antioxidants, and pro-oxidants. Cu-induced toxicity responses of different biochemical components accountable for controlling redox balance in aquatic organisms can vary widely depending on the duration of Cu exposure, the Cu concentration, the type of tissue assessed, the cellular localisation of the biomarkers themselves, and also the particular enzyme isoform/s being considered (Geret et al. 2002; Bigot et al. 2010; Maria & Bebianno et al. 2011; Zhang et al. 2012; Atli & Grosell 2016).

Although different studies have employed various experimental designs that make comparing results difficult, effects of low-dose or short-term Cu exposures in adult bivalves frequently include increased antioxidant enzyme activities as a detoxification strategy, whereas inhibition of antioxidant enzymes are typically characteristic of

prolonged exposure durations or higher Cu doses (Regoli et al. 1998; Matozzo et al. 2001; Jing et al. 2006; Sabatini et al. 2011; Gomes et al. 2012; Lewis et al. 2016). Similar activity patterns of GSH regulatory enzymes in response to Cu have also been observed (Doyotte et al. 1997; Canesi et al. 1999; Sabatini et al. 2011; Jorge et al. 2013). Our findings in mussel embryos are consistent with these general trends, revealing that such mechanisms of Cu toxicity are highly conserved through ontogeny from fertilisation.

GPx serves to detoxify H_2O_2 (oxidising GSH to GSSH in the process) and also plays an important role in ameliorating ROS-induced lipid damage (Pisoschi & Pop 2015). GPx was the only ROS-regulatory enzyme that was affected very quickly to any appreciable degree by sublethal-level Cu exposures. The increased GPx activity after 3 hrs Cu exposure indicates an initial over production of H_2O_2 during early embryogenesis. It is uncertain as to the exact effectiveness of the GPx-GSH couple to remove ROS at this time, but other biomarkers were not affected suggesting a reasonable level of efficiency, and ROS levels themselves were similar to control levels 15 hrs after this point. However, it is possible that this particular system was under pressure and latent effects were being realised between sampling times since at 18 hrs post Cu-exposure, some damage to lipids, proteins and DNA were detected. Activities of other ROS-regulatory enzymes seemed to subsequently react to these damages as a potential counter measure, albeit with a significant lag phase.

Embryonic GR, GST, CAT and SOD generally responded similarly to one another during the Cu exposure period, and in a dose-responsive manner. The higher sublethal Cu treatment increased enzyme activities, but the response was delayed and a plateau was reached around 42 hrs post-exposure which indicates stabilisation or re-equilibration of the ROS-regulatory system. On the other hand, a lethal-level Cu dose inhibited enzyme activity very quickly during the first few hours of cleavage (4- to 16-cell stage embryos) indicating severe oxidative stress which intensified with time.

Superoxide ($O_2^{\cdot -}$) is among the most abundant and toxic ROS produced by the mitochondria, but is involved in important cellular signalling pathways (Landis & Tower 2005). Under moderate-to-high oxidative stress scenarios, SODs are usually the first defence against oxygen radicals and are essential for eliminating excess $O_2^{\cdot -}$ via catalytic breakdown into less toxic H_2O_2 and water to protect cells from damage (Fridovich 1995). SODs also play a critical role in inhibiting oxidative inactivation of nitric oxide, thereby reducing formation of the strong oxidant peryoxynitrite, and preventing mitochondrial dysfunction (Fukai & Ushio-Fukai 2011). Paradoxically, some SODs (Cu/ZnSOD) require Cu to function effectively, and Cu deficiency can lead to oxidative stress and DNA damage (Gaetke & Chow 2003). However, low level oxidative stress caused by excess Cu increases *Sod* gene expression through redox-sensitive transcription factors such as NF-κB (Miao & Clair 2009), whereas higher dose exposures are inhibitive potentially due to a direct effect on the *Sod* gene or indirect effect mediated via an increase in superoxide radicals themselves (Khatun et al. 2008). The H_2O_2 produced during $O_2^{\cdot -}$ detoxification by SOD is further eliminated by CAT through conversion into water and O_2. Relative SOD and CAT activities thus tend to accompany one another (Warner 1994), which is consistent with our findings.

GSTs function to detoxify electrophiles, such as by-products of ROS activity and certain xenobiotics, by conjugating them to GSH (Hayes & Strange 2000). However, in the case of Cu, conjugation to the thiol group on the cysteine residue within GSH occurs spontaneously, and participation of GST is apparently not required (Dierickx 1986). Nevertheless, Cu exposure does modulate GST activities in marine molluscs to varying degrees (Regoli & Principato 1995; Canesi et al. 1999; Moreira & Guilhermino 2005; Cunha et al. 2007). Many GST isoenzymes also exhibit GPx activity and catalyse the reduction of hydroperoxides (e.g., fatty acid, phospholipid, and DNA hydroperoxides) to their corresponding alcohols via nucleophilic attack by GSH on electrophilic oxygen

(reviewed by Hayes & Pulford 1995). Since these are generated by lipid peroxidation and oxidative damage to DNA, GSTs therefore additionally have roles in combating downstream effects of oxidative stress. GSTs are relatively well-studied, and some mechanisms have been put forward which may explain the differences in embryonic enzyme activites that were detected (Tang et al. 1996; Salazar-Medina et al. 2010; Letelier et al. 2006, 2010; Ahmed et al. 2016).

The enhanced embryonic GST activity in response to low-level Cu exposure likely reflects ROS-induced upregulation of *Gst* gene transcription, as demonstrated for marine polychaetes (Rhee et al. 2007a,b). These authors also demonstrated that *Gst* gene (and presumably GST) expression increases in a dose responsive manner. However, at high Cu concentrations, metal-induced enzyme inhibition can reduce total GST activity regardless of the higher transcription. The mechanism of Cu-induced GST inhibition in molluscs has been proposed to occur in two steps; Cu first binds to the enzyme, and then locally generated free radicals modify amino acid residues within the active centre, thus reducing its activity (Tang et al. 1996). More recently, Ahmed et al. (2016) reported that non-specific and irreversible binding of Cu to thiol groups on the enzyme also can accelerate inter- and intra-disulphide bond formation, leading to major conformational changes and inactivation; but potentially also acting to detoxify free Cu^{2+} (Tang et al. 1996). These primary ROS-induced oxidation and secondary non-specific Cu^{2+} binding mechanisms cause more severe effects on GST activity and are different from that of other metals (e.g., cadmium, which occupies the GSH-binding site and prevents the enzyme-substrate complex from forming through stearic hindrance with the γ-carbon of the glutamic side chain of GSH) (Letelier et al. 2006; Salazar-Medina et al. 2010; Ahmed et al. 2016).

7.9.4 Macromolecular biomarkers of oxidative damage

Oxidative stress caused by Cu induces structure modifications and functional modulation of nucleic acids, proteins, and lipids. Lipid peroxidation changes the physiological functions of cell membranes. If the process cannot be terminated by antioxidants such as GSH, GPx and CAT, major cellular damage occurs. LOOHs are formed as first stable primary products from ROS-initiated lipid oxidation of polyunsaturated fatty acids (Ayala et al. 2014). Lipid peroxidation decreases bilayer thickness, increases permeability, and alters fluidity of most cellular membranes, including mitochondria, microsomes, peroxisomes and plasma membrane (Wong-Ekkabut et al. 2007; Rapetto et al. 2012). Furthermore, various products of severe lipid oxidation (e.g. hydroperoxides or their aldehyde derivatives) can also inhibit protein synthesis, and alter enzyme activity and chemotactic signalling (Ayala et al. 2014).

At the lowest sublethal Cu treatment, a very subtle but statistical increase in lipid peroxidation was transiently detected after 42 hrs exposure, which was completely alleviated upon continued exposure. Higher sublethal Cu treatment intensified lipid peroxidation after 18 hrs. However, LOOH levels thereafter remained relatively constant upon continued exposure. In accordance with the patterns of ROS production, these findings indicate that the endogenous antioxidant potential was high enough to re-equilibrate the system and terminate the peroxidation process with minimal membrane damage. Indeed, levels of antioxidant GPx and CAT were simultaneously enhanced, providing a protective capacity. Conversely, lethal Cu treatment more than doubled generation of LOOHs very quickly, and resulted in levels reaching >450% of control values in moribund embryos, with a high likelihood of substantial membrane damage occurring. These findings draw a parallel with the behavioural, cellular morphology, and other biochemical (e.g., increased lipid peroxidation metabolites) endpoint assessments reported in Chapter 7 Part A. Lipid damage was mirrored very closely by evidences of

protein and DNA damage, which also correlates well with the microscopical and metabolomics data in Part A, and the enzyme data in Part B.

ROS promotes primary protein carbonylation via direct oxidation of side chain amino acids, such as lysine, arginine, proline, and threonine residues, to form reactive ketone or aldehyde groups (Suzuki et al. 2010). Oxidative damage can lead to protein fragmentation, unfolding, and misfolding; all of which results in activity loss (Pisoschi & Pop 2015). Indeed, the activity loss of the ROS-regulatory enzymes measured herein as a response to the high Cu dose exposure is due in part to this process. Such major influences on protein function will clearly have detrimental impacts on most enzyme mediated reactions, resulting in catastrophic failures of all cellular processes. Similarly, at a lethel-level Cu dose, substantial DNA oxidation was quickly induced, which undoubtedly contributed to the failing embryonic system. Interestingly however, the level of DNA damage did not worsen with time, which may suggest that considerable repair efforts were being made.

7.10 Conclusion

Simultaneous profiling of a suite of targeted enzymatic biomarkers associated with maintenance of ROS homeostasis, and non-enzymatic biomarkers associated with the effects of oxidative damage to key cellular components, clearly demonstrated the occurrence of oxidative stress in mussel embryos as a response to Cu. These data also verify some of the interpretations gained from the metabolomic analysis in Chapter 7 Part A, and demonstrate that untargeted and targeted analytical strategies can be used very effectively to gain highly complementary information. Metabolomic analysis was more sensitive at discriminating copper-exposed embryos/larvae from non-exposed organisms compared to analysis of oxidative stress biomarkers, since the lowest Cu dose altered the embryonic metabolome, but not the ROS-regulatory system. This indicates that metabolic

processes can be affected by Cu as a first consequence in the absence of an oxidative stress mechanism. This also suggests that metabolic regulation of low-level Cu toxicity can be achieved effectively without significant inputs from the primary redox balance system. Although Cu-induced oxidative stress has previously been studied at the protein level in adult marine molluscs, such comparative comprehensive analyses have not yet been conducted during embryonic or larval development. Thus, this work additionally provides the first such investigation to deliver novel information regarding oxidative stress mechanisms of the innate immune system during early ontogenic development of a molluscan model. In conclusion and corroborating the metabolomics data, oxidative stress does indeed play a critical role in the mechanism of copper toxicity for greenshell® mussel embryos and larvae. ROS imbalance is a consequence of various internal and external influences, and can be used as a non-specific stress indicator. Metabolomics is a useful approach to characterise oxidative stress mechanisms and associated metabolic dysfunction reflective of poor health status.

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Chapter 8 Thesis synthesis

Discussion and conclusion

8.1 Thesis background

"With earth's burgeoning human population to feed we must turn to the sea with understanding and new technology. We need to farm it as we farm the land" – Jacques Yves Cousteau

Global aquaculture production, a long-recognised source of economic sustenance and a more recently sought solution to future food shortages, has expanded greatly in the past two decades, and recently overtook capture fisheries in terms of annual harvestable biomass. However, if we are to continue this growth to meet projected demands, we must do it right, and we must do it sustainably. Bivalve mariculture is key to this success. Context-dependant, Jacques was wrong about one thing – we distinctly need not to farm the seas as we have the land; raped with unwanton destruction of the terrestrial environment through naivety and greed! But he was right about turning towards the sea, and doing it with understanding and new technology – precisely so that we do not make the same mistakes again. We need to comprehensively understand how we as a species influence marine ecosystems, and how marine organisms interact with their physical environments and with one another. It is important that we gain this knowledge so that we can further develop and promote sustainable and environmentally beneficial aquaculture practices. We need to embrace not only new technologies, but also some new conceptual changes in the way we think about the functioning of biological systems and the way we approach and conduct scientific research.

Compared to other biological research fields, such as agriculture and even general marine biology, aquaculture research has in the past tended to lag behind in terms of applied technologies towards understanding animal physiology. But this is changing. With shared needs to solve global issues, more acceptance towards collaborative relationships, and less of a focus on the potential for losing commercial advantages, multinational and multidisciplinary research teams from industry and academia are coming together around the world to progress the future of aquaculture. This includes incorporation of advanced molecular technologies, such as omics-based approaches. However, there is much room for improvement.

An essential step forward is to work on bridging the gap between farmers, industry managers, aquaculture researchers, molecular biologists, biochemists, and funding decision makers. For progress to be made efficiently, it is crucial that this bridge is broad-spanning and with information flowing in all directions. Academic-based researchers need to know about the specific problems industry are suffering from in order to investigate and provide best possible advice and outcomes, and industry-based researchers need to be aware of what tools and technologies are available and they need to be informed as to how they can be used to solve particular issues. This Thesis was developed to assist in bridging the technological knowledge gap which exists among researchers and those with industry-affiliated interests (Chapters 2 & 3), and also to expand current applications and usages of omics-based approaches in aquaculture research and general larval biology (Chapters 4–7).

8.2 Core chapter philosophies

Chapter 2

> **"It is the framework which changes with each new technology and not just the picture within the frame**" – Marshall McLuhan

This is certainly the case for recent advances in mass spectrometry and bioinformatics. The ability to quickly analyse very complex biological matrices has evolved into the new metabolomics approach to study biochemistry, and the advent of such omics-based approaches also brings along a new conceptual framework to interrogate and understand biological systems. This framework has been extensively incorporated into aquaculture research through various transcriptomic and proteomic efforts, thus receiving considerable attention. However, although metabolomics is a burgeoning field in aquaculture, very few seem to be aware of its existence, let alone value. To facilitate awareness, a survey of the published literature was conducted to identify metabolomics-based investigations which have applications to the global aquaculture industry in various sectors (e.g., fish, molluscs, crustaceans) and categories (e.g., hatchery culture, nutrition, health). The purpose of this was to construct a timely first formal review of metabolomics in aquaculture research, identify the knowledge gaps, and provide some suggested lines of enquiry for the future. This review also aimed to broadly disseminate and educate aquaculture scientists and other interested parties about the advantages of incorporating metabolomics into an existing or upcoming research programme in the hope that more investigators will start taking on the approach.

Chapter 3

"Information about the package is as important as the package itself"
– Frederick W. Smith

We've all been there, putting together flatpacked furniture or attempting to program a new washing machine without instruction or in a foreign language with uninterpretable diagrams… all too easy to give up and walk away. We live in a sea of information. Information is a source of learning. But unless it is well-organised, processed, simple to understand, and available to the right people in a format for decision making, it is not useful. To supplement the first 'applications' review, a second companion literature review was conducted in Chapter 3 to assess various metabolomics strategies (e.g., analytical platforms, statistical analysis techniques, interpretation tools) which are typically used for such studies. The purpose of this second review was two-fold. Firstly, to identify which strategies would be most-suitable for the case studies that were conducted in this Thesis, and secondly to provide an easy-to-read primer for aquaculture scientists to educate and facilitate them in conducting a metabolomics-based investigation for the first time. Only time will tell, but I sincerely hope that these two reviews will have a significant impact on the future of aquaculture research and lead to an increase in the number of studies incorporating metabolomic-based components.

Experimental case studies

The primary research of this PhD Thesis was specifically developed into four case studies with differing applications and incorporating different strategies, with some overlaps (Figure 8.1). The purpose of designing these case studies in this manner was to demonstrate that metabolomics could successfully be used for wide-ranging research questions/problems in larval biology and aquaculture. To this end, different taxa (i.e., mussels and oysters), lifestages (i.e. embryos and larvae), sampling regimes (i.e., single point and temporal), research themes (e.g., baseline development, growth variation, culture conditions, health, immunology, toxicology), data formats (i.e., metabolite ratios, single metabolites), and research goals (e.g., sample classification, candidate biomarker identification, predictive modelling, biochemical mechanism characterisation) were investigated or employed. In addition, different data analysis/visualisation/interpretation techniques were used when appropriate (e.g., univariate and multivariate analysis, heatmap analysis, correlation analysis, pathway analysis), and a validation-of-interpretation exemplar was incorporated (i.e., targeted enzyme/biomarker analysis). Each of these case studies delivered unique biological insights in their own rights, and together provide broad scope examples for how metabolomics can be used for a variety of different resolutions.

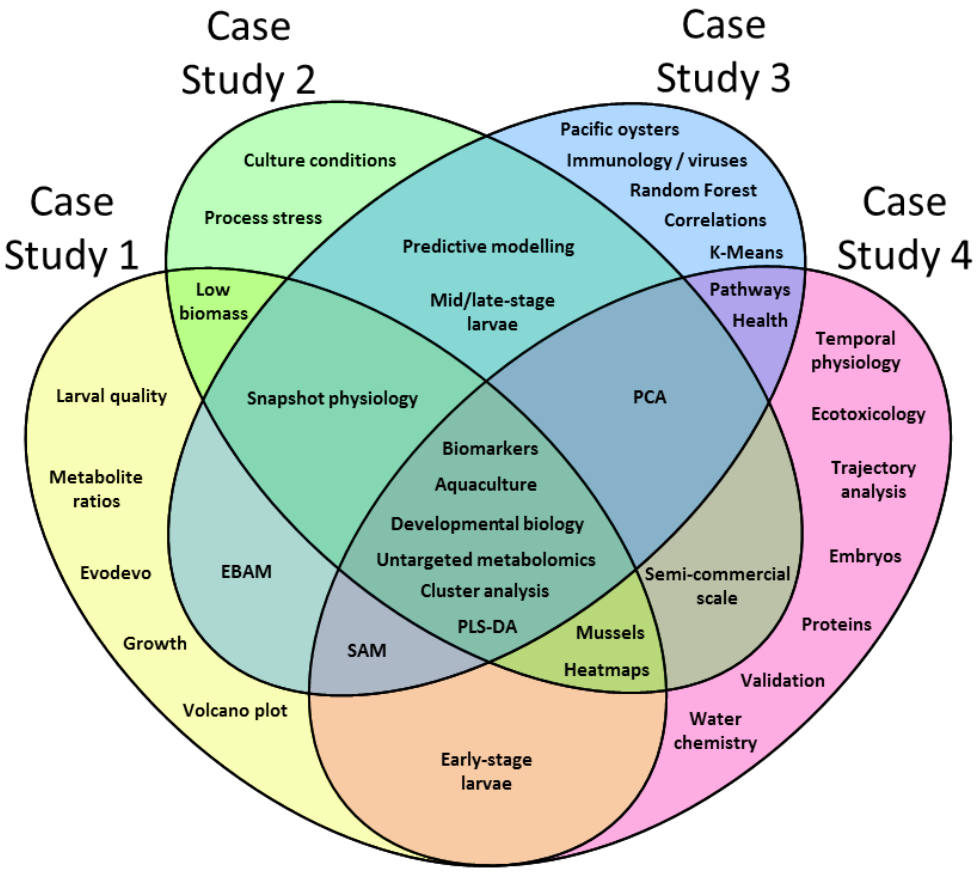

Figure 8.1. Venn diagram of the different metabolomic approaches and their applications for each case study conducted within this Thesis.

Chapter 4

“What?! I'm not small! It's the world that's too big!!”
– Hiromu Arakawa: Fullmetal Alchemist

Sometimes your perspective of the world is not what matters; unfortunately it is the perspective of others who make the rules. Slow-growing larvae are considered inferior in quality and are typically screened from the population every two days during commercial larval culture since they increase the bell curve of development which can cause subsequent production problems and inefficiencies (e.g., through delayed settlement and metamorphosis). This provided an excellent opportunity to examine if currently used quality indices could be discriminated at the metabolite level, and was the first test of metabolomics as an approach to study early lifestages of marine molluscs. Matrices of metabolite ratios were thus compared based on data from slow- vs. fast-growing larvae. Four separate feature reduction techniques were employed and only those features which were identified by all methods were selected for further analysis. Four metabolite ratios were ultimately identified which were best able to discriminate larval classes. Based on the composition of metabolites, this case study demonstrated that larvae from the same cohort and age but with different size phenotypes are metabolically different, and provided provisional insights into energetic roots. Extending the scope, the results of this particular case study also has significant relevance within an evolutionary development framework. The precise mechanisms responsible for intraspecific variations in larval growth and development of marine invertebrates have not yet been fully elucidated, but is a widely-researched and controversial topic (Pechenik et al. 1996; Pace et al. 2006; Hedgecock et al. 2007; le Cam et al. 2009; Meyer & Manahan 2010; Pan et al. 2016). With further metabolomics-based focus and careful control of parental genetics, teasing apart the physiological basis for larval growth variation could be achieved.

Chapter 5

"Future shock is the shattering stress and disorientation that we induce in individuals by subjecting them to too much change in too short a time"
– Alvin Toffler: Future Shock

In this case study, alterations in the mussel larval metabolome were assessed in response to potential handling stress and after being reared under different culture conditions. What constitutes stress and disorientation, what 'too much change' is, and how to define 'too short a time' is challenging, but are key questions/considerations for shellfish hatchery managers. Initial expectations were that a harsh water exchange process with aerial exposure would induce undue stress which would be reflected by changes in key anaerobic metabolites. However, this was not the case and it seems that the highly turbulent process did little to disorientate larval metabolism given a little time to re-equilibrate. On the other hand, different culture conditions resulted in quite different metabolic profiles indicative of altered lipid and protein metabolism, but did not produce visually different phenotypes. This suggests that larvae can metabolically buffer 'scope for growth' reasonably well. Further efforts to evaluate influencing factors, determine the precise tipping point where this adaptive strategy becomes compromised, and characterise the mechanism/s which buffer growth could help to improve and model larval performance under different scenarios and culture conditions. This study also demonstrated that metabolomics can evaluate visually non-observable traits which reveals the high sensitivity of the larval metabolome to particular environmental influences.

Chapter 6

"Indeed, I cannot think why the whole bed of the ocean is not one solid mass of oysters, so prolific the creatures seem"
– Arthur Conan Doyle: Sherlock Holmes

And prolific they indeed once were, detective! But now we are facing a global epidemic of marine virus infections, and the successful future of the oyster aquaculture sector is in jeopardy. This case study sought to investigate early lifestage responses of the oyster metabolome during an ostreid herpesvirus (OsHV-1) infection to inform upon mechanisms of the host-virus interaction. Larvae are excellent models for this purpose since they are the most susceptible lifestage to OsHV-1. Results revealed unique insights into how the virus hijacks host metabolism for its benefit, and how the larval host attempts to counter the viral attack. Distinct links were able to be drawn between larval physiology and that of mammalian macrophages and cancer cells. Personally, the most exciting finding for me was a strong implication for the involvement of immunoresponsive gene 1 (*Irg1*) through the observed metabolic consequences which result from an upregulation in its transcription. Being able to draw previously unknown parallels between invertebrate and vertebrate immunity, and especially in early lifestages, offers exciting new avenues of research. It is hypothesised that *Irg1* is crucially involved during infections, and is central to immunometabolism in oyster larvae. The various hypotheses which were constructed during data interpretation can now be put to the test, and there is considerable scope for this initial work to pave the way for improving the outcomes of selective breeding programs. We, as a global scientific community, will find a solution to secure the future of the oyster aquaculture sector, and we will strive to ensure that the losses suffered by farmers and their families over the past decade do not occur again.

Chapter 7

"The best way to detoxify is to stop putting toxic things into the body and depend upon its own mechanisms" – Andrew Weil

Organisms have evolved over hundreds of millions of years, during which time they have had many opportunities to trial various adaptive strategies which enable them to exist in hostile and changing environments. Oxygenation of our early atmosphere was a catastrophic event for most living organisms and led to a loss of iron bioavailability and the oxidation of insoluble Cu(I) to soluble/bioavailable Cu(II) (Chrichton & Pierre 2001). The presence of dioxygen, the increase in attacks by oxygen radicals, and the loss of iron presented a need for a new redox active metal – copper was ideally suited! This event coincided with the arrival of multicellular organisms and a new iron-copper biochemistry/ redox balance system. Copper is now an essential trace element for all living organisms, but is also highly toxic. Special detoxification mechanisms have thus evolved; some are taxon-specific and some are poorly understood. Metabolomics was applied in this case study to investigate applications in early lifestage mollusc toxicology. Results revealed that mussel embryos and larvae successfully employed various strategies to detoxify the metal. First, as revealed by the metabolomics data, through a form of metabolic regulation at very low dose concentrations or short exposure durations; then, at higher exposure levels, through coordinated assistance of the ROS-regulatory system which involved typical antioxidants and enzymes associated with characteristic oxidative stress mechanisms of metal toxicity in post-metamorphic stages. This study provided novel insights into the metabolic consequences of copper toxicity in developing embryos, and validated the metabolomics data interpretations through targeted analysis of oxidative stress biomarkers. Metabolomics is a very powerful tool for toxicological studies.

8.3 Study limitations

The replication of n = 3–6 may appear to be low in this research, but sample availability was restricted and the Metabolomics Standards Initiative (reviewed in Chapter 3) have proposed a minimum of triplicate biological sampling with n = 5 preferred (Sumner et al. 2007). Broodstock and embryos were kindly provided in-kind by SPATnz and the Cawthron Institute, depending on the specific study. Early on during the research, the hatchery was operating at low production capacity with very high relative value placed on biomaterial, and with other commercial objectives. Thus, for Case Studies 1 (Chapter 4) and 2 (Chapter 5), relatively low total biomass could be obtained. However, each sample analysed was composed of 80,000+ pooled individual organisms (from phenotypically homogenous cohorts) which was considered a good representation of the larval population, and variability between biological tank replicates was low compared to between-class variation in most cases. In Case Studies 3 (Chapter 6) and 4 (Chapter 7), sample biomass availability was higher due to larger animals being used or up-scaling of culture facilities, respectively. Thus, higher replication could be achieved and was implemented in Case Study 3. For Case Study 4, although higher initial biomass was available, the incorporation of a temporal sampling design across different development stages and multiple treatments restricted biological replication. However, between-replicate variability was again relatively low and we are confident that the data obtained reflects real group differences. In future studies, it is strongly recommended that biological replication is maximised when possible, or experimentally evaluated via power analysis if budget and sample availability allows.

Another potential limitation of the research conducted in this Thesis regards the general strategy of employing metabolomics in isolation. However, this is not restricted to the specific case studies which were investigated herein, and it is important to note that this is more of a paradigmatic consideration for omics-type research in general (and also

for many targeted analytical investigations). Due to the inherent nature of biological organisation, 'things' happen on different time scales, at different relative levels, with different inducing factors, and with different final consequences. Inter-relationships with known and unknown regulatory feedback mechanism further complex how well the expression of particular cellular components (e.g., genes, proteins, metabolites) correlate with one another or influence each other, and how well they might correlate with, or more importantly be responsible for, an observable phenotypic trait at a particular point in time. Thus, any single omics approach (e.g., transcriptomics, proteomics, metabolomics, lipidomics [an extension of metabolomics], fluxomics [the study of metabolic fluxes]) in isolation will not, and cannot, provide a complete picture. Albeit a slight exaggeration, this is perhaps best metaphorically summarised as depicted in Figure 8.2 by our modern adaption of the ancient Indian parable of 'The Blind Men and the Elephant'.

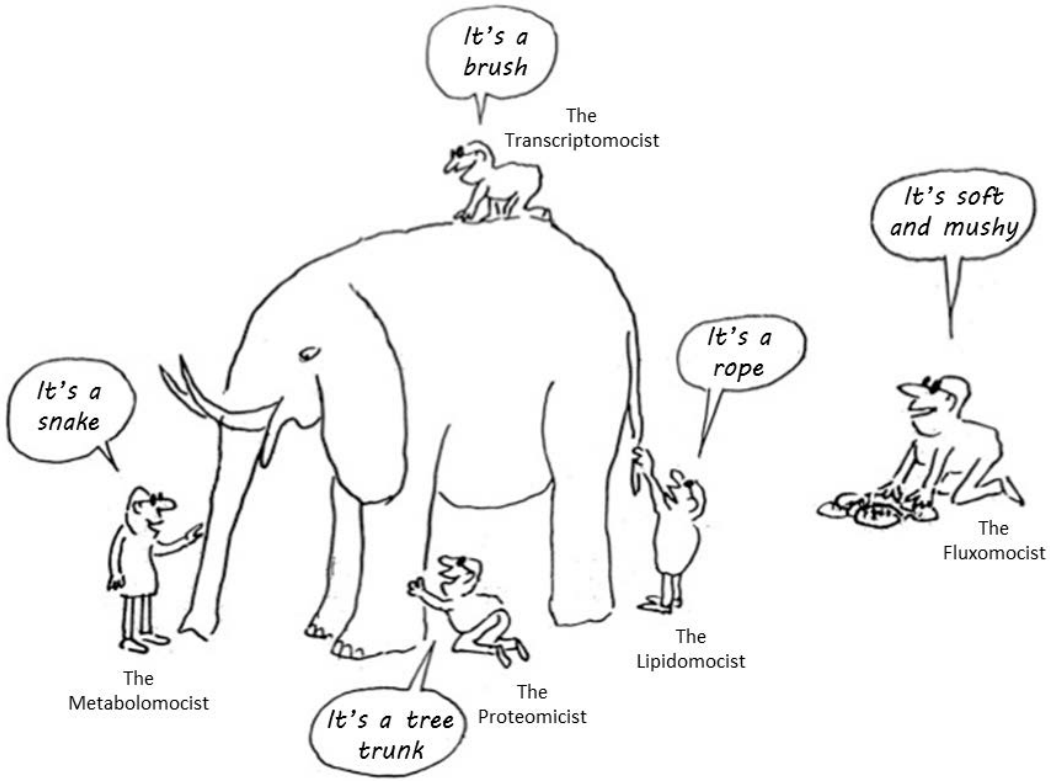

Figure 8.2. 'The Blind Men and the Elephant'. Adaption of an ancient Indian parable to illustrate how biases can blind us, preventing us from seeking a more complete understanding of the nature of things.

However, it is important to point out that there have been many thousands of cases in the published literature where metabolomics (and other omics-based approaches) have been used in isolation to gain very important biological/mechanistic insights, to construct hypotheses which have been rigorously tested and confirmed, to identify biomarkers which have gone through scrutinising validation procedures, and for predictive modelling purposes regardless of biological understanding. It is also important to reiterate here that each of the omics approaches also have their own unique applications and advantages. Without the intention to detract from the power and application of metabolomics, the integration and synergy of multiple omics-based techniques undoubtedly offers the most powerful progression forward.

8.4 Future metabolomics research

There are many potential avenues for future metabolomics-based research in early lifestage investigations of marine molluscs (and other invertebrates). One of the principal rationales for investigating mussel embryos and larvae in this Thesis was due to the urgent need for a better understanding of how these organisms function, what their fundamental requirements are, and what factors may be responsible for variations in larval growth, health, and settlement/metamorphic success during hatchery culture. These broad questions are 'big topics' and although the specific case studies investigated in this Thesis did not set out specifically to provide answers to these questions, the results of the current research undoubtedly provides positive scope for metabolomics-based approaches to help find solutions to various industry-related problems.

In terms of the New Zealand mussel sector, causation for larval batch crashes during hatchery culture needs to be identified and remediated. We have demonstrated that the mussel larval metabolome is dynamic and sensitive to both endogenous and exogenous influences, and we believe this sensitivity can be harnessed to identify reasons

for production variability. We suggest that a dedicated and long term biobanking programme be initiated at the SPATnz hatchery to consistently collect temporal embryo/larval samples from every batch. Indeed, dissemination of the findings in this Thesis has sparked significant interest from concerned parties within the New Zealand aquaculture industry, which recently led to a substantial commercial contract for the AUT Aquaculture Biotechnology Group. Routine biobanking of embryo and larval samples is now being conducted at the SPATnz hatchery every two days over numerous production runs, during different seasons, and with different selectively bred families.

Metabolomics-based analysis of carefully chosen larval samples from multiple good vs bad batches will commence soon, with the ultimate aim to identify causation for poor health and performance. Samples are also being taken for gene, protein, and bacterial community analysis with the intention to conduct an integrated omics-based analysis. It is our expectation that these data will be able to inform precisely when a larval population begins to deviate from the 'optimal' metabolic baseline trajectory well before phenotypic traits are visually observable, thus providing temporal point accuracy for managers to investigate possible process-derived causes (e.g., variations in physical parameters, water quality, handling procedures) and/or biological influences (e.g., variations in genetics, nutrition, maternal investment, pathogen prevalence).

In addition, with the success of Case Study 3 (Chapter 6: mollusc host-pathogen interaction) and a suspected role of bacterial pathogens (i.e., *Vibrio* spp.) during mussel larval mortality events (personal communication, R. Roberts, SPATnz, 2016), experimental infection trials have recently been completed with the aim to identify specific host metabolite biomarkers to use as a reference for Vibriosis disease (Young et al. unpublished data). One of the next key questions we aim to investigate is to determine whether incidences of high bacterial loading is a cause of poor larval health or an opportunistic consequence of metabolic deficiencies (i.e., a pre-existing condition) in

larvae which favours pathogen infection and subsequent proliferation. Such knowledge could be used to focus remedial attention towards external factors or more inherent aspects of larval physiology.

In terms of a successful future for global oyster aquaculture, incidents of herpesvirus infections in larvae, spat and juveniles needs to mitigated. We view Case Study 3 (Chapter 6) as a first pilot investigation for metabolomic-based applications in this area, and foresee wider usages of metabolomics by other research groups to advance the state of knowledge in the near future. Our study provided a brief snapshot of the host-virus interaction, and while this led to some unique insights and intriguing findings, a more detailed analysis is advised to determine precisely which mechanisms are activated, at which time, and in which sequence. We recommend that fine-scale temporal assessments be conducted (e.g., at 30–60 min intervals) during the infection process with multiple samples being taken for integrated omics-based analyses (i.e., transcriptomics, proteomics, multi-platform metabolomics, lipidomics). A comprehensive and temporal survey would promote the use of highly advanced bioinformatics and statistical procedures to identify relationships within and between various levels of biological organisation. A wide range of targeted analytical assays should then be developed and conducted to validate particular implications of the data (e.g., focussing on *Irg1* activation, the Warburg effect, fatty acid metabolism, ROS regulation, and/or other mechanisms).

Another avenue for further OsHV-1 work should involve identifying mechanisms of viral resistance by assessing molecular and biochemical responses of selectively bred families which display varying susceptibilities to infection. A few omics-based studies have already progressed this area, but we believe metabolomics should also be incorporated as part of the general strategy. As a first step to advance metabolomics-based utilisation, we have recently performed two experiments in collaboration with Cawthron

Institute, University of Auckland, and University of Otago in New Zealand. From 40+ selectively bred families (3rd generation), 10 new families were created based on virus susceptibility and growth performance of their parents. A large number of spat were reared under different feeding regimes and subjected to virus and hypoxia challenges to investigate differences in the host-virus interaction and determine the effect of growth phenotype and anaerobic capacity on virus susceptibility. A range of physiological assessments were made to calculate a basic energy budget to determine Scope for Growth, and numerous samples taken for omics-based analysis and targeted enzyme work. Samples are currently being processed. As a final comment on promoting and advancing OsHV-1 research, we also recommend more synergy be developed between primary research groups in this area (e.g., teams from New Zealand, France, Italy, UK, Australia), and start looking towards integrating omics data.

How metabolomics might be further applied and developed to assist the aquaculture industry in general is an exciting unknown. However, the future of biotechnology in aquaculture will undoubtedly benefit from building upon the knowledge which is starting to come from comprehensive exploratory metabolomics-based studies. Due to its infancy, we are still well within the discovery phase of research and the full extent in which metabolomics will be applied is yet to be realised. We suggest three avenues in which metabolomics should be applied in the future of aquaculture to enhance production, increase efficiency and help supply the global demand for food.

- ***Knowledge discovery:*** Use of highly sensitive and precise analytical platforms/technologies within specialised laboratories should be enhanced in order to continue the expansion of knowledge in aquatic biology and the discovery of new applications. This high-level research requires broad expertise and access to state-of-the art equipment, and it is likely to be

performed in collaboration with academic institutions. We are currently facilitating this goal by collaborating with other aquaculture and capture fishery sectors (e.g., salmon, abalone, clams) and industry partners (King Salmon Ltd, OceanNZ Blue Ltd, Cawthron Institute) to provide a metabolomics-based service to industry, and we are expanding the range of available analytical tools by developing an NMR-based capability.

- ***Development of biochemical assays:*** By building upon the knowledge gained from *'knowledge discovery'*, the identity of single or multiple biomarkers which reflect the physiological condition (nutritional status, stress, health, disease) of cultured organisms can be used to develop simple assays for monitoring organism status and for forensic purposes. These assays will ideally be accurate, cheap, easy to use, require low user skills, be applicable in field and hatchery environments, and deliver immediate results. For aquaculture, this goal is a long way off due to requirements for comprehensive validation studies. However, the medical field is already reaping the rewards from simple biomarker assays which have been validated and commercially developed due to exploratory-based metabolomic studies conducted in the last decade. The road to commercialisation for aquaculture may very well be sooner.

- ***Incorporation of alternative metabolomics-based platforms:*** Presently, metabolite fingerprinting/profiling is performed in well-equipped laboratories. However, new technologies are continually emerging. Use of lower resolution platforms (e.g., IR spectroscopy) with proven application, sensitivity and robustness are being made accessible for field measurements at a fraction of the costs of current systems. We believe that with development

and validation of multivariate predictive classification models and advanced forecasting algorithms, IR instruments could be the technology which brings metabolomics-based platforms from the hands of scientists and into the hands of farm workers. This will also pave the way to provide and support a unique opportunity for method development and knowledge discovery to be performed in-house with minimal training and expenditure. In order to progress this concept, we have recently put together a multidisciplinary team of researchers from various institutes and are currently assessing IR capabilities and developing novel data analysis procedures and pipelines. This work is providing data to support a large funding proposal to the New Zealand Ministry of Business, Innovation and Enterprise.

8.5 Conclusion

Metabolomics was successfully applied to investigate early lifestages of marine molluscs, with demonstrated applications in aquaculture, immunology and toxicology. We are still at an early stage in the application of metabolomics in aquaculture, and it is envisioned that more streamlined procedures and strategies will be generated in the coming years to facilitate implementation of this powerful approach. Some of those advances will involve the development of extensive metabolite biomarker libraries, easy-to-use bioinformatics packages, small robust analytical platforms for use in the field, improvements in analytical sensitivities and metabolite coverage, and integration with other omics-based approaches. But more importantly, our future challenge will no doubt be to translate the clear potential of this approach into practical solutions to significantly improve the commercial aquaculture sector.

8.6 References

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