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Modernising Conservation Through Technology: A metabolomic investigation of a critically endangered freshwater crayfish

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Modernising Conservation Through Technology: A metabolomic investigation of a critically endangered freshwater crayfish

This thesis is presented in fulfilment of the requirements for the degree of

Master of Science (Biological Sciences) by Research

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B.Sc. Biological Sciences (Conservation Biology)

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Abstract

BACKGROUND: The Anthropocene has seen extinction rates orders of magnitude higher than the background rate; a trend that has been seen in all ecosystems. As a result of human activities, freshwater resources, and many of the species dependent on them have become imperilled. Freshwater crayfish are a dominant aquatic invertebrate due to their significant biomass, and they are globally distributed, highly speciose, and ecologically important. They have been referred to, *inter alia*, as bioindicators, keystone species, ecosystem engineers, and umbrella species and are also a valuable human food source. However, currently one-third of freshwater crayfish species worldwide are classified as threatened under IUCN criteria, with many species facing possible extinction.

Cherax tenuimanus (hairy marron) is a critically endangered freshwater crayfish found only in a single river in the biodiversity hotspot of south-west Australia. Conservation efforts for this species have included a captive breeding program, which has been largely unsuccessful despite the successful breeding of sister taxon *Cherax cainii* (smooth marron) for aquaculture. Currently captive breeding, including aquaculture of crayfish, relies primarily upon traditional methods of investigating the impacts of environmental factors through gross trial and error, with little understanding of the physiological state of animals. This study tested the hypothesis that metabolomics could highlight potential biomarkers related to reproduction and stress in two congeneric freshwater crayfish, *Cherax tenuimanus* and *Cherax cainii*, for the purpose of providing information to assist with captive breeding.

HYPOTHESIS TESTING: In order to test this hypothesis, four sub-hypotheses were tested in this study. **Sub-hypothesis I**: *C. tenuimanus* can be induced to breed in aquaria. This hypothesis was supported, as mating occurred in both species of marron. Timing of reproductive behaviours was later in *C. tenuimanus* and fecundity was lower than *C. cainii.* Breeding behaviours were documented in detail. **Sub-hypothesis II:** The reproductive hormone methyl farnesoate (MF) can be measured in marron haemolymph as a non-lethal, low stress tool to monitor reproduction (i.e. as a targeted metabolomic approach). This hypothesis could not be confirmed or rejected, because MF was not detected using two extraction methods.

Sub-hypothesis III: Untargeted metabolomics using liquid chromatography–mass spectrometry (LC-MS) detects differences in the metabolome between species and sexes of marron. The profiles of *C. tenuimanus* and *C. cainii* were significantly different, as were the profiles between the sexes of each species.

Sub-hypothesis IV: Untargeted metabolomics using LC-MS detects differential responses in *C. tenuimanus* females and males in breeding pairs. The metabolite profiles supported this sub-hypothesis, where three patterns were identified by the behaviour of the metabolites. Metabolites either indicated a response to disturbance (change) where the response was transient or non-transient; differences between sexes where the differences remained unchanged whether the animals were housed on their own or with a potential mate; or a male response to female presence. Metabolites such as inosine, glutathione and arginine were recognised as potentially useful biomarkers.

CONCLUSIONS: This study demonstrates that metabolomics are useful in providing an informative profile and identifying biomarkers that have the potential to assist with the captive breeding of freshwater crayfish. Whilst a single metabolite (MF) could not be directly targeted in this study, an untargeted approach was successful, and by extension the overall hypothesis of this study was successful. Overall, 107 metabolites were detected in marron haemolymph: amino acids, lipids, nucleotides, and other compounds were successfully linked to biologically important processes in the marron life cycle. The metabolites identified by this approach showed differences between two congeneric species, between sexes and over time in response to an environmental stressor. The study highlighted potential biomarkers for targeted metabolomic studies that can be used to test a wide variety of hypotheses, especially when animals are kept in controlled conditions such as in this study. The investigations from this study also contribute to our understanding of the life history of *C. tenuimanus*, our knowledge of its reproductive biology and the differences with its sister species *C. cainii*, providing another piece to the conservation puzzle. These methods will be beneficial to species conservationists and aquaculturists alike.

Declaration

I certify that this thesis does not, to the best of my knowledge and belief:

i. incorporate without acknowledgment any material previously submitted for a degree or diploma in any institution of higher education;

ii. contain any material previously published or written by another person except where due reference is made in the text of this thesis; or

iii. contain any defamatory material.

iv. contain any data that has not been collected in a manner consistent with ethics approval.

Signed: Emily D. Lette

Date: 31 March 2020

Co-authorship statement

This thesis contains one manuscript titled 'Metabolomic profiling of crayfish haemolymph: an investigation of differences between marron species and sexes' located in Chapter 4 and will be published in the journal *Freshwater Crayfish* 25(1), 2020. Emily D. Lette is the first author because of her role in contributing to the planning, preparation and execution of the research project and subsequent paper. Dr. Nathan Lawler is second author due to his contribution to the metabolomics analysis and interpretation of the research data. Dr. Quinton Burnham is a co-author as he supervised all aspects of the research and for contributions to editing the paper. For their expertise and provision of funding, Associate Professor Mary Boyce and Dr. Rodney Duffy are co-authors. The last authors are Associate Professor Annette Koenders who was co-supervisor for the research and Professor David Broadhurst who provided metabolomics analysis and statistical support; both had a role in identifying the research objective for this paper.

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Lette, E., Koenders, A., Burnham Q., Duffy R., Horwitz, P. (2018) Profiling the Metabolome of two *Cherax* species to investigate their Reproductive Physiology. [Abstract and oral presentation] International Association of Astacology 22nd International Symposium on Freshwater Crayfish, Pittsburgh, USA, 9-13th July 2018.

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Media:

Edith Cowan University. (2018, May 03). Getting Marron in the Mood: ECU Science Researchers and the critically endangered *Cherax tenuimanus* [video file]. Retrieved from https://youtu.be/5MSbAnLKoO0

Edith Cowan University News. (2018, May 03). Getting Marron in the Mood. Retrieved from https://www.ecu.edu.au/news/latest-news/2018/04/getting-marron-in-the-mood

Radio interview:

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Go confidently in the direction of your dreams, live the life you have imagined.

- Henry David Thoreau

Table of Contents

List of Figures

List of Tables

1 INTRODUCTION

Elevated extinction rates are occurring on a global scale (Comizzoli & Holt, 2019), creating a biodiversity crisis that is arguably the most critical conservation issue of our time (IPBES, 2018; WWF, 2018). Conservation biologists must tackle many challenges that are drivers for the decline in biodiversity, including habitat loss and degradation, human disturbance, overexploitation, pollution, disease, human-induced climate change (Butchart et al., 2010; Kearney et al., 2019), recurrent fires and predation (Lindenmayer, 2015). In Australia, the most dominant threats are ecosystem modification, invasive species and agriculture (Kearney et al., 2019), and where approximately 87% of terrestrial mammals (Woinarski et al., 2015), 45% birds, 93% reptiles, 94% frogs, 24% fish, 92% plants (Chapman, 2009; DOEE, 2016), and 90% of invertebrates (Williams et al., 2001) are endemic, the threat of species loss is great. Recent events, such as record breaking temperatures and severe drought in Australia followed by catastrophic levels of bushfires have affected the habitats of many threatened species (Morton, 2020). In order for threatened species to recover when their natural environment is also under threat and no longer a viable location for their survival, conservationists (including any relevant government departments, regulations, wildlife managers, etc.) need to look outside the box to find the best tools for conservation (Corlett, 2017; Greenwood et al., 2016). Conservation of biodiversity means that the conservation of all species should be a priority, and most biologists recognise that a single approach is not adequate to save a species (Roth & Swanson, 2018); this is reflected in species recovery plans worldwide. For species recovery it is not 'one size fits all' as each have unique requirements so in order to save a species it is likely that more than one approach is necessary (Roth & Swanson, 2018). Reserve systems and recovery plans created for threatened species need to carefully consider the threats to ecosystems and the natural habitat, populations of species, and whether they focus on a single umbrella species or just those most at risk and then determine which conservation approaches are most suitable (Braby, 2018; Trayler et al., 1996).

Standard practice for conservation of a threatened species is to protect habitat first, creating a safe haven *in situ* for the species that are most at risk, remove the threatening process(es), then improve the habitat as required (DOEE, 2015; Greenwood et al., 2016). On a large scale these are government managed and funded biosphere reserves and national parks and range to smaller scale government, corporate and privately managed and funded wildlife sanctuaries, refuges, and community reserves. Habitat management and protection are the most important factors for conservation but where

protected areas are lacking, threatened species will need to be managed by removing other stressors where possible (Hannah, 2011). Other forms of *in situ* management options include assisted migration or translocation of species to a habitat that can support the population and assist with recovery (e.g. use of Ark sites) (Bowkett, 2009; Braby, 2018; Hannah, 2011). When *in situ* management is not enough, managers need to take a more hands-on approach to species conservation and intervene to avert extinctions, which may include *ex situ* options (Braverman, 2014; DOEE, 2015; IUCN, 2014). It is important that *ex situ* conservation programs are integrated with and support *in situ* management so the two approaches complement each other (Bowkett, 2009; Braby, 2018; IUCN, 2014).

Ex situ conservation practices are used to protect an endangered plant or animal species outside its natural habitat, often with human involvement or control of some aspect(s) of the organism's environment (Braby, 2018; IUCN, 2014; Kasso & Balakrishnan, 2013). These practices have the potential to address the causes of primary threats, offset the effect of threats and buy some time for a species, with the ultimate aim to restore wild populations (IUCN, 2014). The role of *ex situ* conservation may be a temporary rescue or long-term change for a population. For animals, *ex situ* management may include, but is not limited to, the use of zoos, captive breeding programs, artificial insemination, embryo transfer and *in vitro* fertilisation, reintroduction, cryopreservation, and gene banks: all of which could play a critical role in preventing the extinction of a species (Braby, 2018; Kasso & Balakrishnan, 2013; Pukazhenthi & Wildt, 2004). Although species rescue through *ex situ* conservation practices, such as captive breeding programs, require intensive management and can be costly, they can also be the difference between survival and extinction (Hannah, 2011; Mawson, 2004; Snyder et al., 1996)

Conservation activities including zoo-based breeding programs are often considered the last chance for a species on the brink of extinction (Hogg, 2013). Success stories such as giant panda *Ailuropoda melanoleuca* (Swaisgood et al., 2010), California condor *Gymnogyps californianus* (Snyder et al., 1996) and locally, numbat *Myrmecobius fasciatus*, chuditch *Dasyurus geoffroi* and the Western swamp tortoise *Pseudemydura umbrina* (Hogg, 2013; Mawson, 2004), to name a few, have highlighted the importance of captive breeding and its contribution to conservation of threatened species. However, some species have not shared the same successes and it is often the cute and cuddly species that attract funding and attention (Braby, 2018; Colléony et al., 2017), as indicated by the numbers of managed programs with 57% mammal, 32% bird, 5% reptile, 2% amphibian, 2% fish, and 2% invertebrate, contributing to these programs (Hogg, 2013). Challenges for these programs besides

funding include maintaining genetic diversity and avoiding inbreeding, behavioural changes that can affect breeding and/or cause reintroduction issues and the controversy as to whether animals should be left to breed in the wild; however with high extinction rates, loss of biodiversity mixed with disturbance and loss of habitat, many species face near impossible odds of recovery (Bowkett, 2009; Snyder et al., 1996). Therefore, these programs are an insurance to prevent extinction in the wild and provide some hope (Hannah, 2011), especially where the program can be used with reintroduction of an organism within its previous native range (Corlett, 2016). For these reasons, captive breeding programs as a part of a species recovery plan are used globally to increase numbers of wild stock and assist with the long-term preservation of fauna (Coughran & Furse, 2012; DOEE, 2015; Hogg, 2013). An example of a recovery plan utilising *ex situ* approaches to conservation is the freshwater crayfish, *Cherax tenuimanus* (hairy marron) (Duffy & Day, 2015).

1.1 Marron: a case study of success and failure of captive breeding

Freshwater crayfish are globally distributed and highly speciose, with over 640 species worldwide including 148 species in Australasia (Coughran & Furse, 2012; Crandall & Buhay, 2008), and are a dominant aquatic invertebrate due to their significant biomass (Westhoff & Rosenberger, 2016). They are ecologically significant and have been referred to, *inter alia*, as bioindicators, keystone species, ecosystem engineers, and umbrella species (Brown & Lawson, 2010; Coughran & Furse, 2012; Horwitz, 2010; Richman et al., 2015) and are also a valuable human food source (FAO, 2018; Piper, 2000). Despite their significance, freshwater crayfish face a range of threats to their existence. Conservation concerns for freshwater crayfish worldwide include: climate change, habitat loss or modification, overfishing, pollution and environmental toxins, biological invasion, displacement by introduced species, hybridisation, and the spread of pathogens (Horwitz, 2010; Richman et al., 2015; Westhoff & Rosenberger, 2016). Latest figures estimate that one-third of the crayfish species worldwide are classified as threatened under IUCN criteria with some species potentially facing extinction (Richman et al., 2015).

In south-western Australia (SWA) there are six species of freshwater crayfish in the genus *Cherax*; two of which are commonly referred to as ‘marron’. Marron are the third and fourth largest freshwater crayfish in the world and arguably the largest endemic organism in their environment (Austin & Ryan, 2002; Beach & Talbot, 1987; Beatty et al., 2003). *Cherax tenuimanus* Smith, 1912 is endemic to the Margaret River and the species sometimes referred to as “Margaret River hairy marron” to

differentiate them from *Cherax cainii* Austin, 2002 that has a wide range throughout SWA, and is known as “smooth marron” (Bunn et al., 2008).

Breeding trials conducted on *C. cainii* showed that they can breed successfully in captivity, and as such an industry has been created for farming them over the past 30-40 years (Lawrence, 2007; Luckens, 2015; Morrissy, 1970, 1992; Morrissy & Cassells, 1990). Currently Western Australia produces the most marron in the world at 60 tonnes per annum (Stanley, 2016), and the industry hopes to increase production and expand further into world markets. *C. cainii* have been introduced to many bodies of water throughout SWA and also into other areas in Australia for the purpose of farming commercially and recreational fishing (Beatty et al., 2016; Morrissy, 1992). One of the water bodies where *C. cainii* has been introduced is the Margaret River, where they are displacing *C. tenuimanus*, likely through competition and hybridisation (Bunn, 2004). It has been suggested that there is a difference in fecundity between these two species and that there is also a difference in the timing of reproduction between them in the wild; these two factors have been proposed as potential mechanisms for the displacement of one by the other (Austin & Ryan, 2002; Bunn et al., 2008; Duffy, Ledger, Dias, & Snow, 2014). This has led to the listing of *C. tenuimanus* as Critically Endangered under the Western Australian Wildlife Conservation Act 1950 (Wildlife Conservation (Specially Protected Fauna) Notice 2015), nationally under the Environment Protection and Biodiversity Conservation Act 1999, List of Threatened Fauna of Australia, as well as Critically Endangered under the IUCN red list of threatened species criteria since 2010 (Austin & Bunn, 2010). With an estimated population of only 400 individuals remaining in the wild (R. Duffy, pers. comm.) *C. tenuimanus* are now found in just three sites in the upper reaches of the Margaret River (Austin & Bunn, 2010; Duffy et al., 2014) in an area estimated to be less than 10km^2 (*Figure 1.1)* (Duffy et al., 2014).

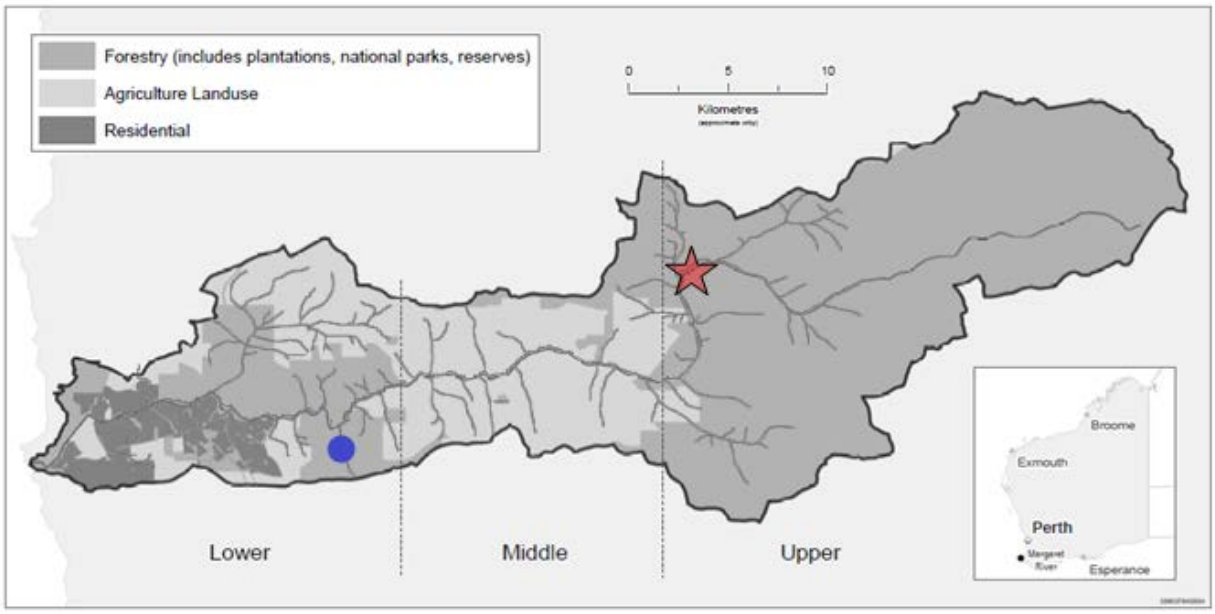


Figure 1.1. The former range of *Cherax tenuimanus* in the Margaret River, Western Australia, is outlined above; the current range is marked with a red star. The Margaret River system is separated by land use into lower, middle and upper reaches (Duffy & Day, 2015). A reservoir of *Cherax cainii* located in the lower reaches at Ten Mile Brook dam is marked with a blue circle.

Due to the threats facing *C. tenuimanus*, recovery actions began in 2009, and a draft recovery plan for the species came into effect in 2015 (Duffy & Day, 2015). The plan to protect *C. tenuimanus* initially involved regular removal of *C. cainii* from the critical habitats of *C. tenuimanus* in the upper reaches of the Margaret River by Department of Primary Industries and Regional Development (DPIRD) staff and volunteers, as well as community awareness activities to assist with the conservation and survival of the wild *C. tenuimanus* populations (Duffy & Day, 2015; South West Catchments Council, 2015). These practices are standard for species conservation as described earlier but despite these efforts, numbers continued to decline (DPIRD, unpub. data). Captive breeding of *C. tenuimanus* began in 2007; this *ex situ* conservation activity was incorporated into the recovery program with the intention to increase numbers for reintroduction and to reduce the risk of extinction (Duffy et al., 2014; Duffy & Day, 2015).

A captive population of approximately 500 *C. tenuimanus* is currently held by the DPIRD Pemberton Freshwater Research Centre (PFRC) for the purpose of retaining a genetically verified pure brood stock for population enhancement (Duffy & Day, 2015). One of the criteria for success of the Hairy Marron Recovery Plan is to establish a self-sustaining captive breeding population with at least 1000 breeding animals of which 50% are female (Duffy & Day, 2015); however, this has proven difficult to achieve as the number of offspring being produced in this population is generally low and declining annually

(Duffy, unpub. data). This is despite the fact that PFRC facility has a long history of successfully breeding *C. cainii* and the breeding methods for *C. cainii* are widely used in commercial-scale aquaculture (Duffy & Day, 2015; Fotedar et al., 2015; Lawrence, 2007; Morrissy, 1970).

Triggers for spawning in many crayfish are environmental cues such as changes in seasonal photoperiod and water temperature (Aiken, 1969; Beatty et al., 2003; Daniels et al., 1994; Huner, 1994; Karplus et al., 2003; Morrissy, 1970; Morrissy & Cassells, 1990; Westin & Gydemo, 1986) and it may be that a difference in water temperature and/or the rate at which the water temperature changes between the Margaret River and the PFRC ponds are factors in their reproductive performance. However, the current knowledge of the reproductive biology of *C. tenuimanus*, such as spawning times and ideal breeding conditions, is largely based on *C. cainii*, and often assumptions are made based on other *Cherax* species such as *C. destructor* (yabby) and *C. quadricarinatus* (redclaw) (for example Morrissy & Cassells, 1990). It is known that *C. cainii* are spring spawners and that mating can be induced earlier in warmer waters (Beatty et al., 2003) and, if housed in climate controlled tanks, they can be induced when water temperatures and photoperiods are increased from winter to spring conditions (Morrissy, 1992). The current conservation paradigm for *C. tenuimanus* therefore relies on *ex situ* breeding, which in turn is reliant upon a version of captive breeding that is entirely dependent on what is known about *C. cainii* and other *Cherax* species.

1.2 New approaches to improve captive breeding

Currently aquaculture and captive breeding of crayfish (and captive breeding generally) rely largely upon traditional methods of investigating the impacts of gross manipulation of environmental variables (e.g. temperature, light, food, stocking densities, water quality etc.) on overall biomass (Huner, 1994; Lawrence, 2007; Lawrence & Morrissy, 2000; Luckens, 2015; Morrissy, 1970, 1992). With this approach there is little understanding of the actual physiological state of animals and the relationship between stress and reproductive success. What is needed for improved captive breeding are methods that can provide the missing data and allow for alternative hypotheses to be tested (i.e. to develop the field as a rigorous scientific endeavour). New methods of investigating physiology that can provide valuable insights for *ex situ* conservation include non-lethal monitoring of reproductive hormones (Pukazhenthi & Wildt, 2004) and biotechnologies targeting proteomic, transcriptomic, genomic, and most recently metabolomic data (Corlett, 2017).

Whilst still new to the aquaculture industry and captive breeding of aquatic organisms (Alfaro & Young, 2016), the use of metabolomics has provided valuable insight to some areas of developmental and reproductive biology for mammals by identifying biomarkers to evaluate the viability and quality of sperm (Menezes et al., 2019), oocytes (Bertoldo et al., 2013), embryos (Cortezzi et al., 2013) and reproductive disorders (Courant et al., 2013). Studies have assessed the health of stock with metabolomics by identifying biomarkers for stressors to environmental conditions for aquatic organisms, including decapod crustaceans, such as oxidative stress in *Callinectes sapidus* (Schock et al., 2010), *Procambarus clarkii* (Izral et al., 2018); oxidative and thermal stress in *Crassula aequilatera* (Alfaro et al., 2019); nutritional stress in *Procambarus clarkii* (Izral et al., 2018), *Astacus leptodactylus* (Costantini et al., 2018); anthropogenic stressors such as metals and contaminants in *Orconectes virilis* (Izral, 2016); and overall health in high density stocking of *Litopenaeus vannamei* (Schock et al., 2013).

Metabolomics is the field of science that identifies and interprets metabolites; the small, low molecular weight compounds (<1500 Da) such as amino acids, nucleic acids, fatty acids, sugars, vitamins, co-factors, pigments, etc. in biological samples such as cells, tissues, biofluids, or the entire organism (Lankadurai et al., 2013; Miller, 2007). Metabolites have functions as substrates, intermediates and products in metabolic pathways (Lin et al., 2006; Miller, 2007; Viant, 2007). As cells function they leave behind metabolites as a biological signature; therefore, the metabolome of an organism is in essence a metabolic snapshot or profile of the condition of an organism (Bundy et al., 2009; Kosmides et al., 2013; Lankadurai et al., 2013; Peng et al., 2015). The metabolome can be affected or altered by changes in an organism's environment as the metabolites have impacts associated with cellular functions and biological pathways (Bundy et al., 2009; Lin et al., 2006; Peng et al., 2015; Roessner & Bowne, 2009).

Crustacean haemolymph constituents have been shown to change during the life cycle (moulting and reproduction) and can be affected by environmental conditions, nutritional changes and health status (Mai & Fotedar, 2018). Haemolymph is affected by the molecules that it transports throughout the body of a crayfish in an open circuit system shifting metabolites from cells to elimination sites, hormones to target cells, as well as distributing oxygen and nutrients (Martin & Hose, 1995). Haemolymph levels of metabolites may not be as concentrated as in surrounding tissues (e.g. hepatopancreas, muscle) but they are detectable (Izral et al., 2018) and therefore suitable for monitoring the biochemical changes in response to a stressor. These responses can provide a clearer picture and provide valuable information about the physiology and the reproductive biology of an

organism (Bundy et al., 2009; Dunn, Broadhurst, Atherton, et al., 2011). It is for these reasons that using a metabolomic approach for captive breeding, particularly with conservation programs for threatened species can assist in assessing the overall health of a captive bred animals to ensure the delicate balance of nutrition, water quality (for aquatics) and other environmental conditions necessary to survive and thrive.

Metabolomic approaches can be targeted; for example, identifying a specific suite of metabolites, or even a single metabolite. If the metabolite is chosen for a specific purpose (i.e. a hormone directly linked to reproduction) it may be useful to help identify issues with captive breeding programs. Metabolomic approaches can also be exploratory (otherwise referred to as profiling); where vast numbers of metabolites are identified to create baseline profiles initially and then to identify changes to these profiles as various factors are manipulated. Furthermore, an exploratory approach can be used to inform a subsequent targeted approach where a single metabolite or subset of metabolites are focused upon. Whilst the circumstances regarding *C. tenuimanus* are unfortunate and their future is under threat, an opportunity for research presents itself as we are able to compare the isolated and critically endangered *C. tenuimanus* to the relatively wide-spread and successfully aquaculture-farmed *C. cainii*, which is a closely related congeneric species.

1.3 Hypotheses and significance

The overall hypothesis of this work was that metabolomics would identify potential biomarkers related to reproduction and stress in two congeneric freshwater crayfish species. Changes detected in the metabolome of the crayfish during this study represent real-time biochemical changes within the animals and this study will facilitate further development of metabolomic approaches to conservation and captive breeding.

This thesis has four sub-hypotheses:

Sub-hypothesis 1 – Marron housed as male/female pairs in glass aquaria with controlled lighting and temperature will undertake mating (Chapter 2).

Conditions that marron were held in during this laboratory study are described, including housing and maintenance requirements as well as observations relating to reproduction and mating. Marron were housed in glass aquaria in a climate-controlled laboratory where the physical conditions were

manipulated to imitate the day length and water temperatures which occur during the natural breeding season. Two different temperature treatments were used, and the marron were kept at two stocking densities, as individuals and with a potential mate.

Sub-hypothesis 2 – The reproductive hormone (methyl farnesoate) can be detected in marron haemolymph and used as a non-lethal, low stress tool to predict reproductive success (i.e. as a targeted metabolomic approach) (Chapter 3).

An assay to detect the reproductive hormone methyl farnesoate was adapted and employed as a targeted approach to better understand their reproductive physiology using gas chromatography-mass spectrometry (GC-MS).

Sub-hypothesis 3 – Untargeted metabolomics can detect differences in the metabolome between sexes and species of marron (Chapter 4).

An untargeted approach to metabolomics was trialled using liquid chromatography-mass spectrometry (LC-MS) to see if the sexes and species *C. cainii* and *C. tenuimanus* will be differentiated from their haemolymph metabolome.

Sub-hypothesis 4 – Untargeted metabolomics can detect changes between sexes of marron when they are placed in breeding pairs (chapter 5).

Using the same method as chapter 4, the metabolome of pairs of *C. tenuimanus* were analysed over a 5-week period during breeding season to identify and compare differences in metabolites between the sexes, over time, and due to sex and time interactions with the purpose of identifying useful biomarkers.

Once these four hypotheses have been tested the overall hypothesis (i.e. that the metabolome of *C. cainii* and *C. tenuimanus* provides potential bioindicators related to reproduction and stress) will have been tested.

2 Breeding a large, critically endangered freshwater crayfish in aquaria: methods, observations, and challenges.

2.1 INTRODUCTION

For some endangered species *in situ* conservation is not sufficient and/or feasible, which usually prompts attempts at *ex situ* methods such as captive breeding, often as a last chance for species facing extinction (Hannah, 2011); for this reason the Margaret River hairy marron Recovery Plan (2015-2020) stipulates the need for successful captive breeding of the species (Duffy et al., 2014). However, captive breeding faces challenges due to factors such as an organism's body size, required habitat size, generation time, habitat specificity, and environmental and/or behavioural mating cues, to name but a few (Snyder et al., 1996). Conservation is costly and often the aforementioned issues are exacerbated by a lack of financial support to keep endangered animals in captivity. Any issues that lead to delays in successfully breeding increase a species' likelihood of extinction. Therefore, it is important for the success of any captive breeding program to efficiently identify the needs of the organism at risk and to ensure they are met to encourage mating and reproduction. Imitating the natural environment can be difficult to achieve as it has many variables and replicating it in a different (artificial) habitat type, such as ponds, tanks, or aquaria in a laboratory may not be possible; as such, considerable thought must go in to the design of housing and the environment the animals will experience.

This chapter describes the aquarium conditions in which marron were held during the experimental period of this study, and tests sub-hypothesis 1: Marron housed as male/female pairs in glass aquaria with controlled lighting and temperature will undertake mating. To test this statement observations of behaviours relating to reproduction and mating were recorded for *C. cainii* and *C. tenuimanus.*

2.2 METHOD

2.2.1 Transfer and processing of marron

On two occasions, sexually mature crayfish (i.e. 2+ years of age) were randomly selected from captive bred stock at the DPIRD Freshwater Research Centre in Pemberton, Western Australia (PFRC), 335km south of Perth (34.443° S 116.034°E), and transferred to Edith Cowan University (ECU) Joondalup Campus in Perth, Western Australia (31.751°S 115.772°E) . Initially 22 *C. cainii* were transferred for the purpose of a pilot study (during June – August [winter] 2017) to test the set-up of the aquaria and to establish maintenance regimes, practice handing the animals, and see if changing temperature could induce breeding. The transfer occurred on the 14th of June, which is earlier in the year than *C. cainii* would spawn in a natural system (Lawrence, 2007). Then, on the 5th of September 2017, 44 *C. cainii* and 44 *C. tenuimanus* were transferred for the main study (September – December [Spring] 2017). The *C. tenuimanus* were from stock originally sourced from the Margaret River two generations prior (DPIRD unpub.). A subset of *C. tenuimanus* males and females were genetically screened (16S), to authenticate species identification of the animals to ensure they were not hybrids, all identified as *C. tenuimanus* (K. Dawkins, pers.comm.).

On arrival at ECU marron were bathed in salt water (30g/L NaCl) for 2 minutes (as per Langdon, 1991) to ensure the animals were healthy by removing external parasites as is standard practice for handling and transporting crayfish (Jones, 1998). Marron were weighed (Mettler Toledo PB3002-S) and occipital carapace length (OCL) was measured using Vernier callipers to the nearest 0.01mm. They were allocated to a numbered aquarium using a random-number generator, and aquaria either held one individual or a mixed-sex pair; experimental design and specific protocols for each part of the project are described in Chapters 3–5. Each marron was given a unique marking with coloured nail polish on the dorsal surface of the second abdominal segment to distinguish sex, aquaria, and pilot/main study.

All marron were tail clipped upon arrival at ECU to assess moult stage according to Burton & Mitchell (1987). A small v-shaped fragment was clipped from a uropod of each marron and placed into an Eppendorf tube with water from the aquarium and stored at 4°C. Moult status was assessed by viewing the tail fragment via a compound microscope within 7 days of sampling. Marron in a pre-moult phase would have been excluded from the study, but all animals were in intermoult (*Figure 2.1*).

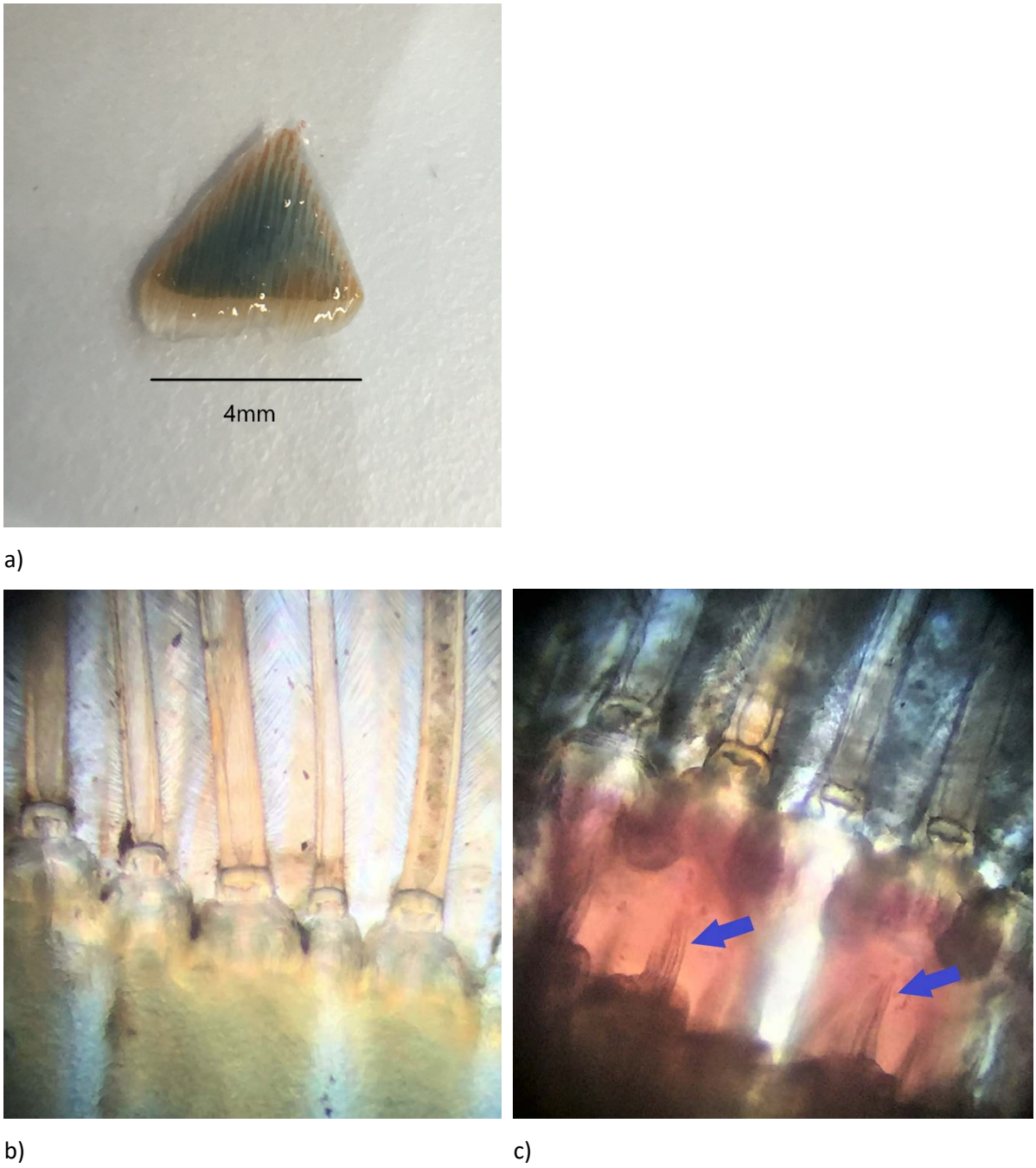


Figure 2.1. a) tail clip from *Cherax cainii* for the purpose of assessing moult stage. b) marron tail clip identified as in intermoult - Stage C_1 according to Burton & Mitchell (1987). c) tail clip of *C. cainii*, taken later in the study after haemolymph collection, identified as pre-moult Stage D_1 – D_2 according to Burton & Mitchell (1987), with new setae buds marked by blue arrows. Observed at low power. Images: E. Lette.

2.2.2 Housing

Marron were housed in a climate-controlled aquarium room at ECU, with water temperature at 15°C and a photoperiod of 10 hours light: 14 hours darkness (corresponding to the late winter daylight hours to which they were acclimatised in Pemberton). Two sizes of glass aquaria were used for the duration of the experiments: small for single marron (dimensions 350mm (L) x 200mm (W) x 230mm

(H), total volume ~16L); and large for pairs of marron (dimensions 600mm (L) x 300mm (W) x 300mm (H), total volume ~ 50L). Initially aquaria were scrubbed with water, sprayed with ethanol, and allowed to dry by exposure to sunlight, then rinsed with a vinegar and water solution before drying in sunlight again. Each aquarium included a glass lid to prevent marron from escaping, with a bead of silicone around the edge of the lids to prevent them from being slid out of place by the marron. Aquaria were kept only on the middle wire shelf of three-tiered units to ensure even light to all aquaria, where they were placed on plastic-wrapped wooden boards on top of 10mm thick sheets of polystyrene to relieve any pressure points on the bottom panes of the glass aquaria and minimise vibration. Shelves were arranged with either five large or ten small aquaria. All electrical equipment such as power bars for water pumps were kept on the upper shelf and enclosed in outdoor safety boxes. The lower shelf was kept clear for plumbing and access.

Aquaria substrate was small gravel and shell grit spread evenly across the bottom at an average depth of 20mm (Viau & Rodríguez, 2010). The gravel consisted of sub-angular to well-rounded pebbles 3-10mm in diameter of silica rich material such as granite and assorted quartz, which would not impact water quality. Prior to use, the substrate was washed and steamed, using a Simons SB Series Electric Steam Boiler for pasteurising soils to remove potential pathogens. Artificial habitat in each aquarium consisted of 200mm sections of 90mm Australian standard stormwater grade polyvinyl chloride (PVC) tubes; aquaria with individual marron contained one such hide and aquaria with two marron had two hides. Opaque laminated sheets covered three sides of each aquarium, visually isolating marron in different aquaria from each other but still allowing for observations to be made. Photoperiod was controlled using overhead fluorescent lights, with cool daylight 36W triphosphor tubes set on a timer.

Water used during the study was from the Perth Integrated Water Supply Scheme (conductivity 108-590 µS/cm) that was de-chlorinated with API® Tap Water Conditioner (dosage 1mL/20L) and aerated for at least 24 hours prior to use. Aeration and filtration of water in the aquaria was provided by airlift bio-filters using air delivered to each by a 4mm clear flexible silicone aquarium tube. Bio-filters were enclosed in hard mesh to prevent marron from accessing the spongy filter medium. As the filters were new, a bacterial starter was added to the water; the pilot study used API Stresszyme+ ® at start-up and weekly for two weeks, and the main study used Seachem Stability ® at start-up and then daily for 7 days. Once set-up, the aquaria were cycled for four weeks prior to introducing the marron.

To regulate water temperature, the water in each aquarium was circulated in a closed system through a coiled segment of poly tubing in a chilled sump, which was temperature controlled by a TECO2000 water chilling unit, and then returned to the same aquarium. This was driven by small pumps (Aquapro MK3) in each aquarium that moved the water at a maximum rate of 550L/hr through 13mm black poly irrigation tubes. This system ensured temperature control was achieved without mixing water between aquaria, preventing the transmission of possible chemical signals (i.e. hormones) or parasites/diseases. With the use of multiple sumps different temperatures could be achieved in various aquaria in a single climate-controlled room. Water temperatures were monitored daily with thermometers and eight Thermochron TCS temperature loggers were placed in aquaria throughout the room to record water temperatures, while two more were used to record air temperature. Both water and air temperatures were recorded at hourly intervals throughout the experimental period. The electric cable of the water pump was covered with a section of 20mm PVC tubing to prevent marron from chewing through the insulation.

2.2.3 Maintenance

Daily observations noted animal condition and behaviour, as well as water temperature. General cleaning of aquaria, including siphoning waste from the gravel and changing 10-25% water, was undertaken weekly. A solution of white vinegar and hot water was used to clean all equipment involved (aquarium dip nets, gravel siphons, etc.) as it is non-toxic and would not be absorbed into silicon or plastics in any of the materials used. Equipment was washed between use in different aquaria to prevent both the transmission of parasites and the transfer of water that may have carried pheromones. Weekly water quality monitoring was undertaken to measure pH and nitrogenous wastes (NO_2, NO_3, and NH_3/NH_4). The pH was measured using either a pH meter (WTW pH330) or API® pH test kit and remained within recommended guidelines. For this study NO_2, NO_3, and NH_3/NH_4 were measured using test kits (API® Freshwater Master test kit); in freshwater aquaria any reading above 0ppm (ammonia and nitrite) or 40ppm (nitrate) was considered to represent high levels necessitating a larger portion of the water being changed (25%) (Johnston & Jungalwalla, 2005; Langdon, 1991). Measurements were also taken periodically for hardness of water (including general hardness (GH), carbonate hardness (KH)) and calcium (Ca+) with API® aquarium test kits.

To ensure optimal nutrition, all marron were fed a combination of food including Skrettings Nova ME 3mm marine Fish Pellet, New Life Optimum Freshwater Flakes + garlic and Tropical® Spirulina Super Forte Granulat. Marron were fed at a rate of 3% of their body weight per week (Lawrence, 2007), split evenly across three feeds per week (i.e. Monday, Wednesday, Friday) to minimise aquarium fouling. They were fed late in the day, approximately 0.5-2 hours before the artificial lights would turn off. Unconsumed food was siphoned up as part of daily maintenance to ensure good water quality standards. Marron were regularly weighed to ensure that they were maintaining condition (initially weekly and then later monthly).

2.3 RESULTS

2.3.1 Pilot Study

In late July, an attempt was made to induce mating by replicating seasonal cues; specifically raising water temperature from 15°C to 18-20°C in conjunction with increasing the hours of lighting from 10:14hr light-dark photoperiod to a 12:12 light-dark photoperiod (Beatty et al., 2003; Huner, 1994; Lawrence, 2007; Morrissy, 1992). After these changes were made, one pair (out of five) mated. A spermatophore was found on the ventral surface of the female and she had extruded eggs attached to the pleopods on the ventral side of the abdomen. Successful maintenance of the aquatic environment conditions within desired levels and animal health, along with the instigation of reproductive activity, suggested that the conditions in the aquarium were suitable for housing and breeding marron, thus the project advanced to the main study.

2.3.2 Main Study

Staff at the DPIRD Pemberton Freshwater Research Centre reported finding several female *C. cainii* with eggs or spermatophores attached to the underside of their thorax on the 5th of September 2017 as they were being collected from their ponds for this experiment. These females were not selected for this study. Upon inspection after arrival at ECU, some *C. cainii* males displayed a bluish tint to the membranous extensions from the male gonopores (penes) (Figure *2.2*) which indicates reproductive readiness. This state did not develop in *C. tenuimanus* males until several weeks later.

Figure 2.2. Ventral view of male *Cherax cainii* illustrating the blueish tint to the extension of the male gonopores (penes) which are not modified for sperm transfer but instead to place a sperm packet on the female sternal area which is typical of Parastacidae males (McLay & van den Brink, 2016).

After the marron arrived for the main study, there was an acclimation period to the aquarium room of 21 days, after which water temperatures were decreased to induce the maturation of ovaries in the females outside of their normal breeding season. This artificial induction consisted of dropping the water temperature from 16°C to 10°C for a 24 hour period (following McRae & Mitchell, 1996) before warming it to either 16°C (temperature 1 – slow change and cooler) or 20°C (temperature 2 – fast change and warmer) (described in detail in Chapter 3). These two temperatures were chosen to replicate the natural springtime water temperatures of the Margaret River (the natural habitat of *C. tenuimanus)* and the approximate temperature of water in the Pemberton region where DPIRD are attempting to breed the marron in ponds, respectively.

During the acclimation period three pairs of *C. cainii* mated (water temperature at 15°C) and during the induction period (as the water temperature was in the process of being lowered to 10°C over a 24-hour period) a fourth pair mated (Table 2.1); this likely occurred at 12°C when artificial day length was at 10 hours light: 14 hours dark. Following cooling of the water to 10°C, no more *C. cainii* mated. The first *C. tenuimanus* mating event occurred 21 days after the change in temperature (i.e. the induction). The *C. tenuimanus* pairs mated over a six-week period, between the 11th of October and 18th of November (75 days after arrival at ECU) (Table 2.1). There was more success with *C. tenuimanus* mating than *C. cainii*, as the cold water disruption during breeding may have prevented further spawning in *C. cainii*, and there were separate breeding times for each species with no overlap between species (*Table 2.1)*.

Table 2.1. Mating events of *C. cainii* and *C. tenuimanus* in aquaria at ECU from September to December 2017. The induction (cooling water temperature to 10°C for 24 hours) occurred on day 15-16 marked by * (19-20th of September).

Days at ECU	Date	Species	Water (°C)	Air (°C)
6	10-Sep-17	*C. cainii*	15	16
8	12-Sep-17	*C. cainii*	15	16
13	17-Sep-17	*C. cainii*	15	16
15*	19-Sep-17	*C. cainii*	12	16
37	11-Oct-17	*C. tenuimanus*	18	16
37	11-Oct-17	*C. tenuimanus*	18	16
43	17-Oct-17	*C. tenuimanus*	20	16
43	17-Oct-17	*C. tenuimanus*	15	16
60	03-Nov-17	*C. tenuimanus*	16	18
72	15-Nov-17	*C. tenuimanus*	20	18
75	18-Nov-17	*C. tenuimanus*	19	18

2.3.3 Physical observations

Mating was confirmed by the presence of a spermatophore and eggs on the ventral side of females. Once mating was confirmed, disturbance of the incubating female was minimized by removing the male and covering all surfaces of the aquaria, and whilst berried females would eat very little, careful feeding and cleaning continued to ensure consistency and maintain water quality. The number of eggs held by the berried females of either species was not recorded to avoid disturbance, however *C. tenuimanus* appeared to be carrying a greatly reduced number of eggs compared to records and observations of *C. cainii*. (i.e. in the region of 50 eggs compared to 150+ eggs). Similar observations of eggs from both species were made by J. Bunn (unpub. data) from the wild populations in the Margaret River.

Marron were removed from aquaria on day 2 or 3 after a spermatophore was observed during an examination to confirm that eggs were present. The eggs from both species were dark grey in colour, approximately 2.5mm and oval in shape, as shown in the photo of the berried female *C. cainii* (*Figure 2.3*). This is consistent with other descriptions of marron eggs (Lawrence & Jones, 2002). Although a high proportion of females extruded eggs during the trials (70% of paired *C. tenuimanus*), no female of either species carried eggs for longer than 3 weeks. Often by the end of the first week no eggs or only a few eggs (i.e. <20) remained. One *C. tenuimanus* female kept eggs for 3 weeks and, though the number of eggs declined over this period, she was observed fanning and grooming until there were no eggs left.

Figure 2.3. Berried female *Cherax cainii*, three days after spermatophore was first found on the ventral surface of the thoracic segment, with approximately 150 eggs dark-grey in colour.

2.3.4 Behavioural observations

The mating behaviours observed in the aquaria during the trials were very similar for both species. On multiple occasions, 24-48 hours prior to a mating event, female marron were observed in a 'preening posture' with legs extended to raise the body above the substrate. The carapace and part of the abdominal segment were flat, and the posterior part of the body and tail were at a sharp angle to allow for preening of the ventral side of the abdominal surface and pleopods, using the 4th and 5th walking legs (*Figure 2.4a*). This action would occur continuously for a few hours. Observations of the ventral side of the abdomen during this time showed a very clean surface, similar to what would be expected of the carapace after moulting.

A female *C. tenuimanus* was observed in a 'post-mating posture' where she was positioned on her dorsal surface with both chelipeds anterior to the head and against the substrate, forming a 'Y' shape (*Figure 2.4b*). This posture has also been described in *C. destructor* (R. Duffy, pers. comm.), *Austropotamobius pallipes* (McLay & van den Brink, 2016) and *Homarus americanus* (Talbot & Helluy, 1995). The uropods were extended up to the thoracic segment covering the genital pore (at the base of the 3rd walking leg) and were in this position for at least 30 minutes; it is likely that the female was depositing eggs at this time. The female then rolled onto her ventral surface with the telson tucked up into the joint between the thoracic and abdominal segments and the uropods wrapped closely to create an incubating pouch. From this angle it was confirmed that a spermatophore was attached to the sternal area.

a)

b)

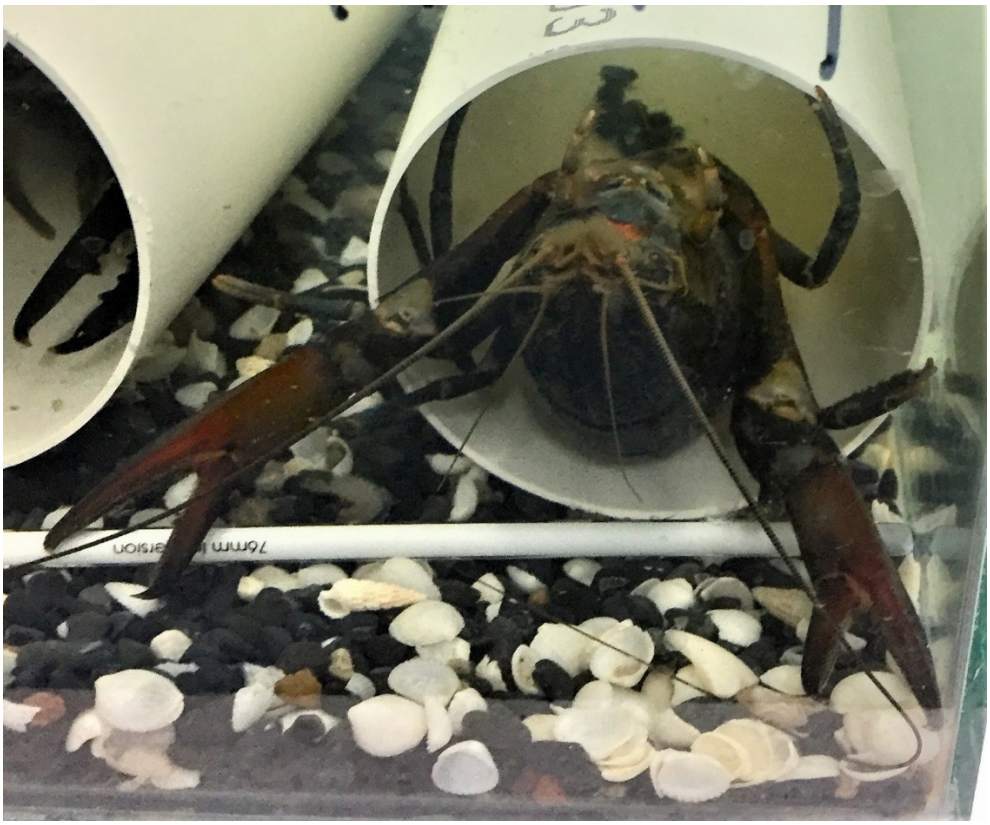


Figure 2.4 a) Female *Cherax tenuimanus* preening ventral side of abdomen using the 4th and 5th walking legs, 24-48 hours prior to mating. This position was referred to as a 'preening posture'. b) *C. tenuimanus* female in post-copulation position. Although difficult to see in the image, at the time the photo was taken the position of the uropod was anterior the join of the thoracic and abdominal segments, and it appeared that she was depositing eggs.

After a spermatophore was present (*Figure 2.5*) and eggs had been deposited on the ventral side of the abdomen the females would curl the uropod and take on an 'incubation position' (*Figure 2.6)* which is different to the normal resting position (Figure *2.7*). During regular observations in the first few days of the incubation phase, females were occasionally observed resting laterally (on their side) in one of the hides with the uropod forming an incubation pouch. After a few days the females would change body position so that they were upright but still with the uropod tucked in a ball shape. After the first week, the females remained in this protective pose and then opened the tail away from the thoracic segment and fan the pleopods to which the eggs were attached.

Figure 2.5. Spermatophore present on the ventral surface of a female *Cherax tenuimanus* (indicated by the blue arrow); note also the ball-shape of the tail as it forms an incubating pouch.

Figure 2.6. A female *Cherax cainii* in incubation position, with the posterior edge of uropod tucked up into the join between abdominal and thoracic segments forming an incubating pouch.

Figure 2.7 *Cherax tenuimaus* in resting position where the uropod is relaxed under the marron, sometimes the abdominal segment is flat rather than curled as above.

2.4 DISCUSSION

Both *C. cainii* and *C. tenuimanus* mated in aquaria in this study and all crayfish that mated spawned, showing that they will produce eggs if provided with appropriate conditions. It was unknown if all the eggs were fertilised as most eggs were not held long enough to show any larval development. Although *C. cainii* have a long history of captive breeding (as evidenced by a commercial aquaculture industry), it is important to note that the critically endangered *C. tenuimanus* can successfully mate and spawn in captivity, and can successfully attach their eggs to pleopods and tend to the eggs. However, for currently unknown reasons incubation in this study did not progress to the hatching stage.

2.4.1 Mating and fertilization of eggs

Ovarian maturation is influenced by seasonal changes in day length and water temperature in many species of crayfish (McLay & van den Brink, 2016) including *C. cainii* (Beatty et al., 2003), however, preening behaviours that appear to be a precursor to mating were only observed for female marron in mixed sex aquaria during the study. Therefore, the presence of a male may be important for females to fully prepare for reproduction. In a captive breeding program one strategy to improve success could be to collect eggs for artificial incubation so it would be beneficial to know when a female is preparing to mate. McLay & van den Brink (2016) state that females of the congeneric *C. quadricarinatus* do not undergo any external changes to indicate reproductive receptivity, however observations of both *C. cainii* and *C. tenuimanus* females preening 24-48 hours prior to the mating event suggest this is a visual cue for readiness that could be utilised.

In addition to behavioural observations this study has highlighted, in a later experiment, another indicator of reproduction that warrants further investigation. Freshly drawn haemolymph was clear and colourless with a blueish hue in all marron, however, a slight orange hue was also noted for some females. After proteins were precipitated out of the haemolymph by the addition of acetonitrile and centrifugation, samples from some female marron had a very distinct orange colour (*Figure 2.8*) (methods discussed further in Chapters 3 and 4). Wainwright, Prescott, Rees, & Webster (1996) describe an orange colouration of haemolymph and their interpretation was that it was most likely due to the presence of vitellogenin. Vitellogenin is a glycolipoprotein found in female crustaceans that increases in concentration as the ovaries become more reproductively mature (the stage is known as

vitellogenesis), and is a precursor to egg yolk production (D'agaro et al., 2003; Girish & Swetha, 2015; Sukumaran et al., 2017). Further work is required to demonstrate a correlation between intensity of colour (as a proxy for vitellogenin levels) and different reproductive stages, as this would be a simple and cheap tool to assess reproductive readiness in female marron (and probably all crayfish at least, if not crustaceans).

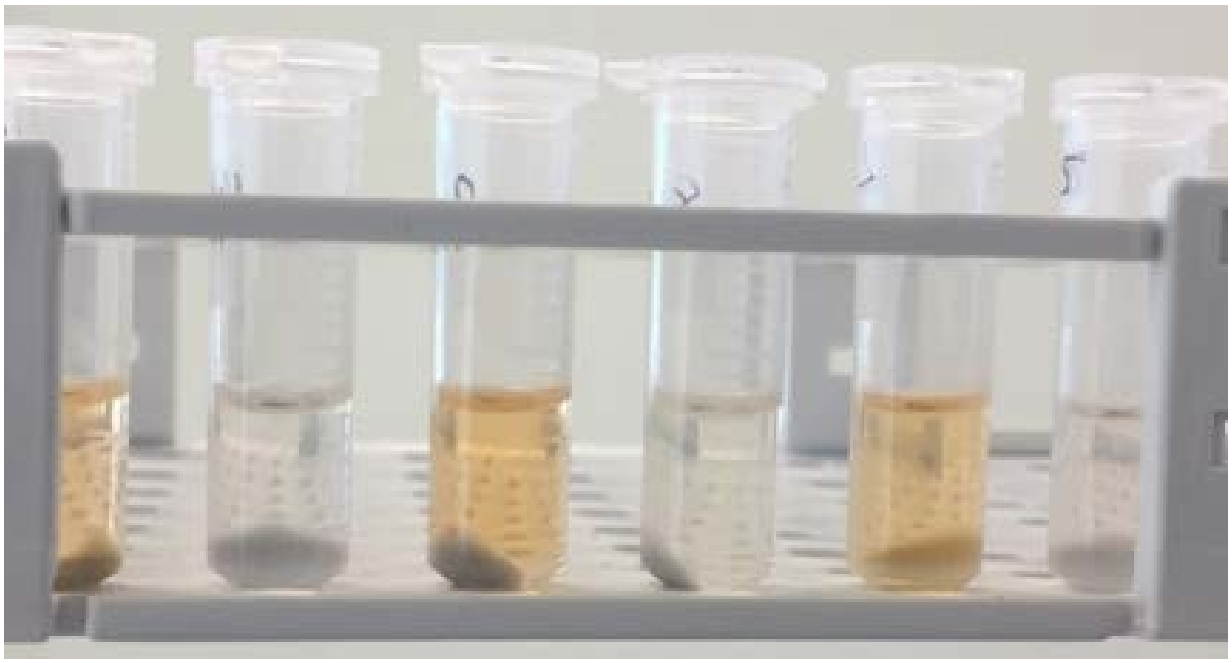


Figure 2.8. Marron haemolymph sample preparation for LC-MS analysis. This step is after the addition of acetonitrile and centrifugation where the proteins precipitated out of the haemolymph solution and formed a pellet at the bottom of the 2 mL Eppendorf tube. Haemolymph collected from female marron is orange and male marron haemolymph is colourless.

For both marron species, eggs were deposited shortly after the male had placed the spermatophore on the female and it is suggested that the sperm was released within hours, not days as suggested by López Greco & Lo Nostro (2008), as the spermatophores in this study had broken down within 3-4 days. The timing of depositing eggs suggests that in both species of marron the sperm is not stored for fertilisation later as for some decapod crustaceans (e.g. *Orconectes limosus*, *Austropotamobius pallipes*), where multiple mating may occur and egg-laying may be delayed for several days or weeks (McLay & van den Brink, 2016).

2.4.2 Incubation of eggs

The reproductive activity throughout the trial demonstrates that the conditions and techniques were suitable for housing and breeding marron in captivity, however some challenges regarding incubation may need to be addressed for future studies. Aquaria conditions were suitable for adult marron, as they maintained or gained weight and appeared healthy throughout the trial but due to the lack of success with females keeping their egg clutches, it is possible that the aquaria were unsuitable for egg development and incubation purposes. Any or all of nutrition, light, water quality, temperature, aquarium size or disturbance may have played a role in eggs not being carried to hatching, whether by directly affecting the eggs or the adult female, or indirectly through stress-related effects, or it may have been the result of an infection or disease (although there were no obvious signs).

Nutrition before and during the trial may have played a factor in the success of reproduction in both species of marron as it is important that female broodstock in particular have adequate nutrient intake during ovarian development and maturation for egg quality and larval survival and growth (Duffy, Godwin, Nolan, & Purvis, 2011; Ghanawi & Saoud, 2012). The marron wintered in earthen ponds where they were fed pellets and supplemented their diets from naturally occurring pond organic material (DPIRD staff, pers. comm.) and during the trial were fed a recommended combination of pellets and flakes providing protein and plant material to meet nutritional requirements (R. Duffy and B. Roennfeldt, pers. comm.).

The bright lighting in the room may have played a role in the loss of eggs prior to hatching, as marron would normally breed in the murkiness of a pond or the dark tannin coloured water of the WA rivers (Morrissy, 1970, 1992). However, as hides were supplied and used by the females with all sides of the aquaria covered when females were berried, it is not clear how light would impact their ability to carry eggs to hatching but not affect mating and spawning.

Water quality remained within recommended guidelines but there may be parameters that were not ideal for crustacean reproduction and incubation which may need to be investigated. As the water was from the Perth Integrated Water Supply Scheme, which treats and conditions the water, some essential ions may have been removed in the process. As stated earlier the water temperatures used in this trial were based on the temperature in the pond in Pemberton and the pools of the Margaret

River, the natural environment for marron, and remained within the recommended guidelines of 12.5°C to 24°C for optimal marron growth (Lawrence, 2007; Morrissy, 1992). Henryon & Purvis (2003) suggest that temperatures of 20°C to 24°C are best for the artificial incubation of eggs but the water temperature of our aquaria did not rise above 20°C. This may be important for future work if artificial incubation of eggs as part of captive breeding for species recovery is to be considered.

Stress from aquaria confinement may contribute to difficulties with reproduction and the incubation of eggs and juveniles (Luckens, 2015). The size of the aquarium in relation to the body size of the animal may have stressed females if they felt vulnerable whilst berried and unable to flee potential danger. On the other hand, it is known that crayfish shelter when in berry for protection, in small spaces such as burrows (Huner, 1994; Morrissy, 1992). It is also likely that despite our best intentions to keep a quiet laboratory and precautions taken to reduce disruptions, the females were disturbed by movement or noise in the lab. All of these factors may have stressed the female and led to her dropping the eggs. In this scenario stress is difficult to measure, which is why techniques such as the metabolomics approach being used in this study (to be discussed later) are so powerful.

An alternative explanation to a deficiency or stress cause is that there may have been a bacterial or fungal infection of the eggs. It was noted that some eggs within a clutch changed colour to bright orange and were surrounded by a white substance that was cotton-like in appearance *(Figure 2.9*). The orange colour of the egg suggests that the egg was unviable, and it may be due to a fungus such as *Saprolegnia* that occurs in water and affects fish eggs and juvenile fish in hatcheries worldwide (Lone & Manohar, 2018). Normally, a female would pick abnormal eggs off and clean the rest; perhaps this behavior was impacted in some way. A salt bath was used when introducing the marron to the aquarium but perhaps it should have been repeated once females were berried. Excess handling of berried females was avoided to reduce disturbance but a preventative salt bath or other treatment once they were berried may have produced a better outcome. It is unknown whether stress or handling were the issues but either way there is a fine balance between handling for precautionary measures and causing unnecessary distress to the animal making it difficult to determine which disturbance was worse.

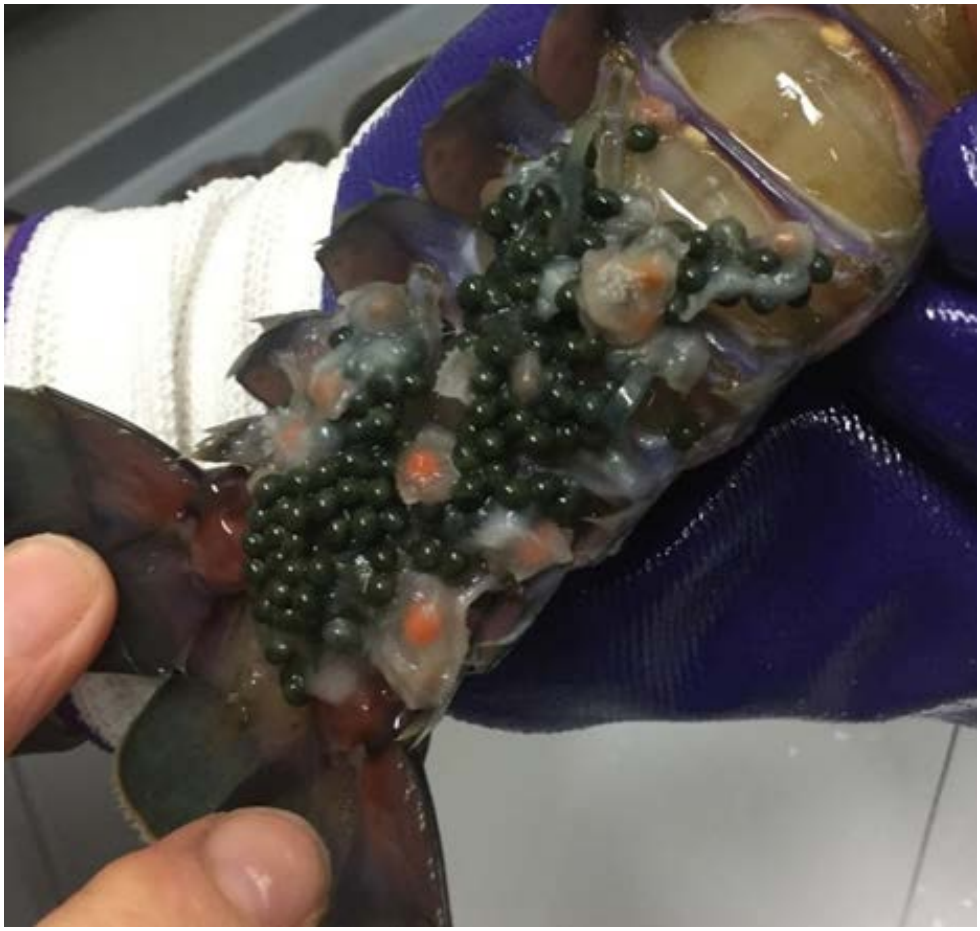


Figure 2.9. Berried female marron (*Cherax cainii*), eggs at day 16. Most eggs colour change to dark greenish but some changed completely to a bright orange and became fuzzy (cotton-like) in appearance, possibly due to *Saprolegnia* sp.

2.4.3 Conservation implications

The results of this study demonstrate that *C. cainii* are likely to be reproductively active earlier in the year than *C. tenuimanus*, which aligns with anecdotal reports of timing in the wild and at Pemberton (i.e. August to October for *C. cainii*; October to December for *C. tenuimanus* (Bunn, unpub)). The change of colour and external appearance of male marron gonopores indicated that *C. cainii* may be reproductively ready as early as June (as indicated by the marron collected for the June pilot study) and still active in September (when marron were collected for the main study), whereas *C. tenuimanus* males did not show these characteristics until early October. The difference was evident in females too as when *C. cainii* were collected from ponds for the main trial some were found with a spermatophore present on their ventral surface (DPIRD, pers. comm.). This information agrees with observations of marron in the Margaret River by DPIRD researchers and volunteers (pers. comm.) as well as research conducted by J Bunn (unpub. data 2006-2008) and is important to acknowledge for future work.

Spawning of *C. cainii* in the pilot study confirms that they can be induced into mating in captivity earlier than in the wild, as has been suggested previously (Huner, 1994; Morrissy, 1970). The increase in temperature between Pemberton and the aquaria at ECU in the main study may have cued reproduction in this species as four pairs of *C. cainii* mated within the first week at ECU. These actions indicate that late August/early September when water temperatures are rising with longer day lengths falls within the natural timing and occurrence for such breeding behaviours (Beatty et al., 2003). In the days and weeks following the cooling of the water to 10^{o}C in the lab no more *C. cainii* mated, thus the unnatural cool water temperature change appeared to be a considerable disruption to their breeding activity.

Another interesting finding was that mate selection does not appear to have a significant effect on reproduction in *C. tenuimanus* as seven out of ten pairs of *C. tenuimanus* mated successfully to produce eggs. Although the marron were all approximately size matched upon collection from the Pemberton Freshwater Research Centre they were selected randomly from the containers after transport and placed into aquaria using a random number generator. Only one randomly selected pair was changed as one of the smallest female *C. tenuimanus* (105g, 54mm OCL) was matched with a large male (238g, 72mm OCL). This pair were left for a week together in the aquarium but as the female was observed to avoid the male, she was removed from the aquarium and replaced with another female *C. tenuimanus* (142g, 59mm OCL). The new pairing of size matched individuals resulted in successful mating, which may indicate that whilst mate selection is not strong, approximate size matching is important.

The apparent lack of mate selection suggests that marron that are reproductively ready at the same time are likely to mate which, when combined with the difference in timing of reproductive readiness in the males of the two species, may shed some light on the displacement and hybridization occurring in the Margaret River (Guildea et al., 2015; Kennington et al., 2014). It seems reasonable to suggest *C. cainii* pairs will mate earlier than *C. tenuminaus* pairs and that *C. cainii* males that are reproductively active earlier in the season than *C. tenuimanus* males may encounter female *C. tenuimanus* that are reproductively receptive and produce hybrids. The offspring of *C. cainii* pairs and hybrid mating will have earlier access to resources and potentially outcompete *C. tenuimanus* juveniles that are younger and less developed.

2.5 CONCLUSION

The sub-hypothesis being tested in this chapter was that marron can be housed in aquaria under conditions that facilitate reproduction; this has been supported at least in part. It was demonstrated that it is possible to breed large freshwater crayfish in glass aquaria but highlighted a potential issue with them carrying their eggs to hatching. The laboratory observations of mating and associated behaviours contribute to the knowledge for the target species, and the outcome of *C. tenuimanus* mating in aquaria provides a glimmer of hope for a species on the brink of extinction. This has provided evidence that despite the two species being so closely related there are significant differences in fecundity and timing of reproduction, and this mechanism should therefore be considered in other cases of species displacement/hybridisation. The remainder of this thesis will be testing whether these differences can be detected by metabolomic approaches and if these data can be used to inform conservation efforts.

3 Targeted metabolomics: (non)detection of the crustacean reproductive hormone methyl farnesoate in haemolymph.

3.1 INTRODUCTION

One of the ways metabolomics may be able to contribute to conservation is via a targeted approach. It was hypothesised in this study (sub-hypothesis 2) that the reproductive hormone (methyl farnesoate) can be detected in marron haemolymph and used as a non-lethal, low stress tool to predict reproductive success (i.e. as a targeted metabolomic approach). Methyl farnesoate (MF) was chosen as it plays a role in crustacean reproduction for both sexes (Borst et al., 1987; Laufer et al., 1993; Nagaraju, 2007). MF is known to increase in concentration during ovarian development in females and is present in higher levels in the more reproductively aggressive males (Borst et al., 1987; Nagaraju, 2011). It is a sesquiterpenoid compound that is produced by the mandibular organ in crustaceans (Homola & Chang, 1997; Laufer et al., 1987, 1993) and has been identified in more than 30 species of crustacean, including the congeneric *C. quadricarinatus* (Abdu et al., 2001; Jo et al., 1999; Laufer & Biggers, 2001; Rotllant et al., 2001; Xie et al., 2015). In some crustaceans, higher levels of MF may be correlated with more aggressive mating attempts and behaviour, with the greatest levels found in larger, sexually mature males (Laufer & Ahl, 1995). Very low levels of MF are found in immature males and females that have not reached reproductive maturity (Laufer et al., 1993). MF is detected in females during ovarian development, with the highest rates observed close to the end of the ovarian cycle when vitellogenesis and oocyte growth are greatest (Laufer et al., 1987), before declining once she is ready to deposit eggs (Laufer & Biggers, 2001).

It has been suggested that the cues for reproductive readiness in these crayfish are initially environmental (an increase in day length and water temperature), with the final reproductive cue being the presence of the opposite sex (Huner, 1994; Morrissy, 1996). One hypothesis to explain the limited success of captive breeding of *Cherax tenuimanus* at the PFRC is that the water temperature differences between the PFRC (where the marron captive breeding program is housed) and the Margaret River (where *C. tenuimanus* naturally occur), is stunting their reproductive development. There are differences in both the peak water temperatures and the rate at which the temperature increases in spring between the two locations. Water temperature in the pools of the Margaret River in late winter/early spring ~8^{o}C and it warms slowly, reaching ~15^{o}C in October and then plateauing

for several weeks at ~15-16°C before increasing to peak surface temperatures in December at ~21°C (DPIRD unpub.). Water temperature at the PFRC increases steadily from ~10°C in winter to reach ~15°C in late august, peaking in December at ~27.5°C (in December) (Lawrence, 2007).

This chapter tests sub-hypothesis 2; that a reproductive hormone (MF) can be detected in marron haemolymph and used as a non-lethal, low stress tool to predict reproductive success (i.e. as a targeted metabolomic approach). To test this statement, an assay to detect MF in the haemolymph was adapted and employed using gas-chromatography-mass spectrometry (GC-MS). Marron (both *C. tenuimanus* and *C. cainii*) from which haemolymph was collected were kept as singles and in pairs and were exposed to one of two temperature treatments to determine whether temperature would produce a detectable impact on the production of MF in these crayfish.

3.2 METHODS

3.2.1 Experimental design

Eighty marron (20 of each sex for both *C. cainii* and *C. tenuimanus* (Table 3.1)) were housed in forty small aquaria (individual marron) and 20 large aquaria (a male/female pair) (as per the housing details described in Chapter 2). Two water temperature treatment regimes were used (Table 3.2). To begin with, the water temperature for both treatments was maintained at 16°C for a three week acclimation period, then cooled to 10°C for 24 hours (as an environmental cue to induce reproduction) (McRae & Mitchell, 1996). The temperature was then raised across the two treatments as follows:

- The 'cool' temperature treatment had a slower increase in temperature and lower maximum temperature. It was raised from 10°C to 14°C over 21 days, then held at 14-15°C for three weeks before finally increased at an average rate of 0.5°C/day until 20°C was reached.
- The 'warm' temperature treatment was increased at a faster rate and to a higher temperature. The temperature was increased from 10°C to 16°C at an average rate of 0.5°C/day for the first seven days, then increased by 1°C every three to five days until 20°C was reached where it remained for the duration of the experiment.

Day length was controlled for both treatments: it was gradually increased from winter hours (10hr light: 14hr dark) to spring hours (12hr light: 12hr dark) in conjunction with the water temperature treatments being manipulated (to mimic changing seasons).

Table 3.1 Sizes of marron in the trial testing methyl farnesoate. Mean wet weight in grams and mean occipital carapace length (OCL) in millimetres (+/- the standard deviation (SD)).

Species	Sex	OCL (mm)	+/- SD (mm)	Weight (g)	+/- SD (g)
C. tenuimanus	Female	59.39	4.65	142.68	30.20
C. tenuimanus	Male	66.81	6.62	196.76	48.02
C. cainii	Female	62.81	5.58	172.93	46.28
C. cainii	Male	61.82	6.51	165.82	50.53

Table 3.2 Experimental design for testing methyl farnesoate levels in marron with two water temperature treatments and five replicates for each species of marron. The total number of marron = 80 animals. *C. cainii* = 40 (20 ♂ + 20 ♀); *C. tenuimanus* = 40 (20 ♂ + 20 ♀).

Temperature treatment	*C. cainii* replicates	*C. tenuimanus* replicates	♂ : ♀
Cool	5	5	1:0
Cool	5	5	0:1
Cool	5	5	1:1
Warm	5	5	1:0
Warm	5	5	0:1
Warm	5	5	1:1

3.2.2 Chemicals and standard solutions

The standards of 10mg (E,E) methyl farnesoate and 500mg nonadecanoic acid methyl ester (NDAME), a non-biological isomer, were purchased from Sapphire Bioscience Pty. Ltd. (Redfern, NSW). A 1mg/mL stock solution was made for each standard with serial dilutions to 1µg/mL using n-hexane 96% (HPLC Basic hexane). These standards were run on the GC-MS and standard peaks were identified. A stock solution (1 mg/mL) of the internal standard (NDAME) was prepared in 50:50 acetonitrile:crab saline buffer from which the working internal standard solution was prepared by serial dilution with acetonitrile to 1µg/mL.

3.2.3 Haemolymph collection and storage

Haemolymph was collected from both *C. cainii* and *C. tenuimanus*. Marron haemolymph samples were collected in a 1mL syringe from the ventral sinus with a 21G needle inserted into the soft tissue at the base of the 5th pereopod (according to Leland and Furse, 2012). Haemolymph (1mL) was drawn and added to 10mL Eppendorf tubes (chilled on ice) containing either 2.5mL acetonitrile (CH_3CN) plus 1mL crab saline buffer (Duan & Cooke, 1999) at a ratio of 5:2:2 ACN:saline buffer:haemolymph (Abdu et al., 2001), or 1:1:1 acetonitrile:saline buffer:haemolymph (Xie et al., 2015). These two extraction solvents were trialled to identify the optimal solvent. Tubes were shaken and placed on ice to prevent clotting.

Internal standard (NDAME 100ng) was added to each haemolymph sample and the mixture was vortexed for 2 minutes prior to adding 500µL hexane and centrifuging for 10 minutes at 1500 x g (4000rpm). The upper hexane layer was collected using glass Pasteur pipettes and transferred to labelled amber GC vials. Another 500µL of hexane was added to the tube and the hexane extraction was repeated with the hexane fractions combined and dried overnight in a fume hood or under a stream of nitrogen (Xie et al., 2015). Samples were then stored at -20°C until GC-MS analysis.

3.2.4 Gas chromatography-mass spectrometry analysis

Dried hexane extractions were removed from -20°C storage and reconstituted in 100µL of GC-MS grade hexane and mixed well by vortexing for 1 minute. Samples were then processed either individually or, in order to boost the MF signal, as pooled samples (where the contents of ten vials were combined after reconstitution). All samples were dried under a stream of nitrogen, reconstituted in 50µL of hexane, vortexed to mix well then transferred into low volume inserts and analysed using a Thermo Scientific Trace 1310 gas chromatograph paired with a Thermo Scientific ISQ LT single quadrupole mass spectrometer (Australia). Separation was achieved on a Thermo Scientific DB-5 capillary column (SGE BP5MS-UI, 30m x 0.25mm internal diameter x 0.25µm film thickness) with the MS operated in electron impact ionisation mode. The mass spectrometry transfer line temperature was 300^{o}C, ion source temperature 320^{o}C, with solvent delay of 5 minutes and mass range 50-550amu (in scan mode) and dwell or scan time of 0.2 seconds. Temperature of the GC column was held at 100^{o}C for 2 minutes, then increased to 280^{o}C at a rate of 10^{o}C/min, held 5 minutes then increased further to 300^{o}C at a rate of 100^{o}C/min, and held for 2 minutes. The sample injection volume was 1µL and the injector port, operated in split-less mode, was maintained at 250^{o}C. In selected ion monitoring (SIM) mode, mass-to-charge ratios (m/z) of 69, 81, 114, 121, 207 and 250 were chosen to detect MF and 55, 74, 87, 143, 269 and 312 were selected to detect the internal standard NDAME (Rotllant et al., 2001; Xie et al., 2015).

3.3 RESULTS

The MF and NDAME standards were well separated by GC and resulted in well-defined sharp peaks with retention times of 12.480 min (MF) and 16.864 min (NDAME). The MS detector was operated in both scan and selected ion monitoring (SIM) modes, with SIM being more sensitive. In SIM mode MF had a limit of detection of 13ng/mL and NDAME a limit of detection 31ng/mL.

Haemolymph samples for *C. cainii* and *C. tenuimanus* were analysed by GC, but neither MF nor the internal standard NDAME was identified in any sample. The experiment was repeated, and the internal standard spike increased 10-fold, and while the standard was now detected, the recovery was not acceptable at less than 20 %. Pooled marron haemolymph (10 samples combined) was also analysed, as it could potentially increase the MF concentration 10 times, but MF was still not detected. These results were obtained regardless of species, sex or temperature.

3.4 DISCUSSION

The targeted metabolomic approach of this study was not able to provide the expected results (i.e. the detection of MF in the haemolymph of *C. tenuimanus* and/or *C. cainii*). This is in spite of the GC-MS method successfully separating and detecting standard solutions of MF and NDAME and the limits of detection reported for this method being suitable for the detection of MF in haemolymph. For example, Xie et al. (2015) detected MF in the haemolymph of *Portunus trituberculatus,* a swimming crab, in the range of 5.5-32ng/mL, which are within the detection limits of the instrument in the present study. Some published studies have pooled samples to improve detection of MF (Rotllant et al., 2001; Xie et al., 2015), however, even though the methods of these studies were followed, MF was not detected in our pooled haemolymph samples. Possible explanations for non-detection fall into one of three categories: biological, experimental, or analytical.

Methyl farnesoate has been detected in over 30 crustacean species including other *Cherax*, hence the absence of this compound is considered an unlikely biological explanation for non-detection. A more plausible explanation could be that the timing of collection of haemolymph samples was unsuitable, as other studies of crustaceans (e.g. Laufer et al., 1987; Laufer & Ahl, 1995; Rotllant et al., 2001; Sagi et al., 1994) including the congeneric *Cherax quadricarinatus* (Abdu et al., 2001) have indicated that levels of haemolymph MF can vary depending on stage in life cycle, size and sex (Nagajaru, 2007).

However, levels of haemolymph MF are highest at later stages of ovarian development in females (Xie et al., 2015) and males (Laufer & Ahl, 1995) and marron sampled in this study were at a reproductively active stage in their life cycles (i.e. marron were successfully mating during this period), so it is expected that the hormone should have been present in the haemolymph. It is possible that an aspect of the experimental design, rather than a biological phenomenon could explain the non-result. This experiment was designed to test the hypothesis that the different temperature regimes at Margaret River and Pemberton may be affecting reproductive success of the marron. However, as previously mentioned, crayfish in the aquaria did mate under both temperature regimes (see Chapter 2), so this is not considered as the cause of non-detection. Stress due to sampling via needles could have resulted in altered biochemistry, but the same methods of haemolymph collection have been successfully used in other studies (D'agaro et al., 2003; Leland & Furse, 2012).

As biological and experimental explanations seem unlikely, the most likely is an analytical issue as the poor extraction efficiency for the internal standard indicates an issue with the extraction method. Extraction method modifications were attempted (including using two different extraction solvents), and even the addition of a 10-fold internal standard spike to the haemolymph samples still resulted in an unacceptable recovery level. While other issues (i.e. potentially of a biological or experimental nature as previously discussed) cannot be completely discounted, it seems clear that despite a series of troubleshooting attempts the method failed, making it impossible to know. The result (or more specifically non-result) of this method highlights the potential issues with using this type of targeted approach for investigating issues such as reproduction for conservation purposes. It is debatable if the amount of work required to optimise the extraction method for this metabolite would be a good use of research resources for this project. One conclusion that we cannot state is whether the work to optimise extraction would have been successful, perhaps MF would have been a perfect biomarker for reproductive success, but further work is required. Although there are substantial benefits to having a single (or few) biomarkers to follow, it also highlights why, rather than focusing on a single targeted analyte, using untargeted metabolomics to create a complete metabolomic profile for marron haemolymph could be more effective and informative for conservation. In the next chapter, sub-hypothesis 3 (untargeted metabolomics can detect differences in the metabolome between sexes and species of marron) will be tested to see if the data it produces supports this supposition.

4 Metabolomic profiling of crayfish haemolymph: an investigation of differences between marron species and sexes.

This chapter is a manuscript that has been anonymously peer-reviewed and published in the journal Freshwater Crayfish (April 2020) with the citation as follows:

Lette ED, Lawler NG, Burnham QF, Boyce MC, Duffy R, Koenders A and Broadhurst DI. (2020). Metabolomic profiling of crayfish haemolymph distinguishes sister species and sex: implications for conservation, aquaculture and physiological studies. *Freshwater Crayfish* 25(1):89-101.
doi: 10.5869/fc.2020.v25-1.089

4.1 ABSTRACT

Hairy marron (*Cherax tenuimanus* Smith) are critically endangered freshwater crayfish found only in a single river in south-west Australia. Conservation efforts have included a captive breeding program, which has been largely unsuccessful, despite the closely related smooth marron (*Cherax cainii* Austin) being successfully bred for aquaculture. Using an untargeted liquid chromatography-mass spectrometry (LC-MS) metabolomic approach we created a profile of the metabolites in the haemolymph for males and females of the two species of marron. A non-lethal method was used to collect haemolymph and 84 reproducible annotated metabolites were identified. Variation in the levels of some metabolites were detected between species and between sexes within species. Multivariate analyses clearly differentiated the congeneric species and univariate analyses identified differences between species, sex and for some metabolite interactions between species and sex. This study created a baseline metabolome dataset for the two species and began to investigate the biological significance of metabolites that varied between species. We have shown metabolomic profiling could be used for targeted studies to potentially assist reproductive success. This approach will be beneficial for conservation and aquaculture practices with potential applications for other aquatic taxa worldwide.

Keywords: *Cherax cainii, Cherax tenuimanus*, LC-MS, marron, metabolome, metabolomics, species conservation.

Page 37 -55 are not included in this version of the thesis.

5 A case study: Metabolomics and hairy marron.

5.1 INTRODUCTION

In the previous chapter, untargeted liquid chromatography-mass spectrometry (LC-MS) was used to create a metabolomic profile (i.e. the metabolome at a single time point) for *Cherax cainii* and *Cherax tenuimanus,* held in isolation. Many of the metabolites identified in the metabolome of the two sister species were amino acids or their derivatives, lipids, nucleosides and nucleotides, and neurotransmitters. Differences in metabolites between species and between sexes were attributed to stages in the crustacean life cycle, as some were considered to influence, or be influenced by, high-energy processes such as reproduction and moulting. At the time of haemolymph collection as *C. cainii* had finished breeding and moulting was about to commence, whereas *C. tenuimanus* were still mating. The study showed that untargeted metabolomics can detect differences in the metabolome between sexes and species of marron (thus supporting sub-hypothesis 3) with the subsequent step in the hypothesis testing approach was to determine whether untargeted metabolomics can detect changes between sexes of marron when they are placed in breeding pairs (sub-hypothesis 4).

It is believed that temperature and day length are environmental cues required to prepare for mating, with the final cue being the presence of the opposite sex (Huner, 1994; Morrissy, 1970). Therefore, the metabolome of male and female *C. tenuimanus* were analysed over five weeks after the introduction of an individual of the opposite sex during their reproductive period to determine whether this produced different physiological changes from animals held individually. Detecting changes in their metabolomic profiles whether between the sexes, over time, or due to sex and time interactions and linking these to processes occurring in the animals will allow us to identify biomarkers that are potentially useful for *ex situ* conservation (Pukazhenthi & Wildt, 2004). These can later be tested by manipulating the environment of the animals (i.e. altering factors that may affect reproductive success). For example, if increased levels of a stress biomarker such as inosine were seen in *C. tenuimanus* females this would shed light on how they are affected by the presence of males around the breeding season, which could then be tested experimentally to identify optimal stocking densities. Once an attempt to identify potential biomarkers through untargeted metabolomics has been completed, sub-hypothesis 4 will have been tested, and the overall hypothesis (i.e. that the

metabolome of *C. cainii* and *C. tenuimanus* provides potential bioindicators related to reproduction and stress) can be addressed.

5.2 MATERIALS and METHODS

Methods for haemolymph collection and storage, sample preparation for metabolomic analysis, liquid chromatography-mass spectrometry (LC-MS), data pre-processing, metabolite identification, data modelling, and statistical analysis for this current chapter generally followed the same methods as Chapter 4, with any changes and/or additional methods noted below.

5.2.1 Study Organisms

The data for this chapter is derived from the same ten *C. tenuimanus* (five male and five females) utilised in Chapter 4. There was no control group for this experiment (no animals that were maintained without a partner for the entire experiment) due to the number of animals that were available

5.2.2 Experimental design

Five aquaria (approx. 50L) with one male and female crayfish in each were used for this experiment, with conditions described in the methods of Chapter 2. Water temperature was maintained at 19-20^{o}C before and during the trial and haemolymph was collected from the marron four times over the five-week experimental period. The haemolymph collected was used for methyl farnesoate (MF) analysis (Chapter 3) and an untargeted metabolomics analysis (this chapter).

Day 0 marked the first day of the experiment and the first date haemolymph was collected. Until this time point the marron had been held as individuals in separate aquaria before placing them into pairs. Over the next few days the males and females were progressively paired with a similar sized individual of the opposite sex so that each aquarium contained a mixed-sex pair. Haemolymph was collected for the second time from all marron on day 12 of the experiment. The third collection was at day 18 and the last date of haemolymph collection was on day 34 where the marron had been housed as pairs for an average of 30 days.

5.2.3 Haemolymph Sample Collection and Storage

Haemolymph was collected over a 5-week period from 27th of October to the 30th of November 2017. On each occasion 1200µL of haemolymph was collected per animal (200µL for metabolomics, 1000µL for MF). All haemolymph collections for all animals occurred at the same time of day and the animals were handled in the same order on each of the four collection dates. Haemolymph samples for metabolomic analysis were prepared following the methods in Chapter 4 and stored at -80°C until required for analysis. All dried extractions (40) were later reconstituted on the same date for metabolomic analysis.

5.2.4 Data pre-processing and metabolite identification

Data pre-processing and metabolite identification proceeded as described in Chapter 4 using the standard untargeted metabolomics workflow by Compound Discoverer 3.0 software (Thermo Scientific). Compounds that were detected in the blank samples were removed from the final data matrices. Metabolite data from both ionization modes (positive and negative) were combined into a single data matrix. Several metabolites appear twice in *Table 5.1* (indole-3-lactic-acid, kynurenic acid, and inosine) as they were identified using both the positive and negative C18 columns: this provides additional support for their presence.

Prior to statistical analyses, as with the previous chapter, metabolites were annotated by matching the exact molecular mass data, retention time and peak grouping using databases such as the in-house MS/MS Thermo Scientific mzVault and mzCloud online (https://www.mzcloud.org/) spectral libraries and recorded following the Metabolomics Standards Initiative (MSI) reporting protocol (Sumner et al., 2007). In order to maximise rigour a conservative approach was adopted where the majority of metabolites identified in this chapter were matched at MSI level 1. Further pathway enrichment analysis was done using the reference metabolome from Metaboanalyst 4.0 (Chong et al., 2019).

5.2.5 Quality Assurance

Principal component analysis (PCA) *(Figure 5.1*) showed significant clustering of multivariate covariance in the first two principal components explaining 48.35% of the total observed variance. The tight grouping of blue circles in the scores plot shows the comparative variance of the pooled QC samples, which gives a measure of precision for each of the samples, indicating very high-quality reproducibility data.

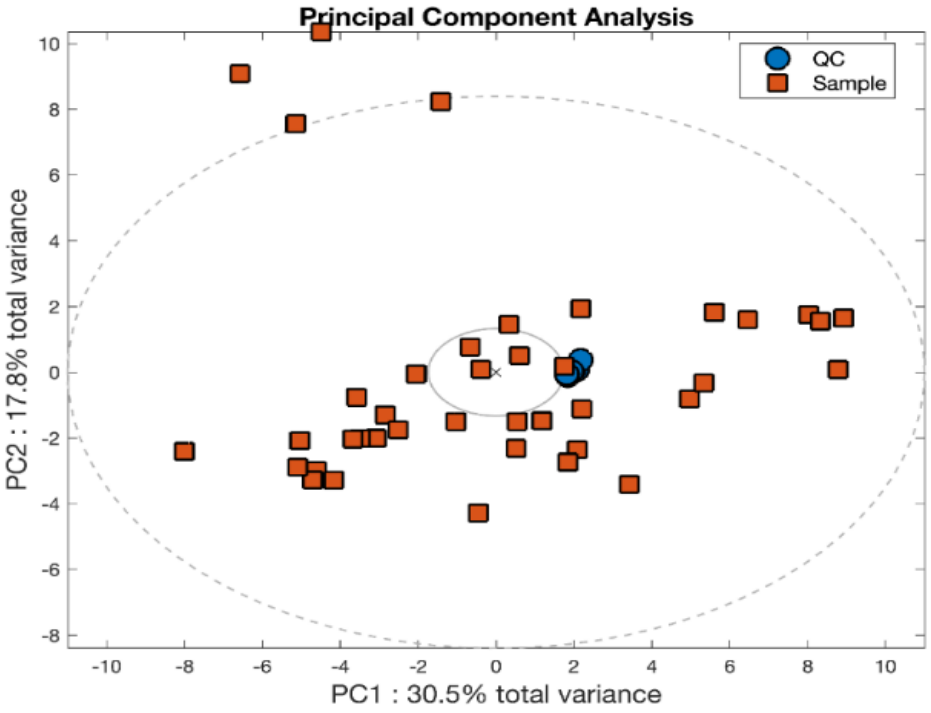


Figure 5.1. **Principal component analysis (PCA) plot presenting PC1 (sex) against PC2 (time) for the metabolites detected in the haemolymph of *Cherax tenuimanus*.** Each point represents a single sample: orange squares are marron haemolymph samples including both male and females; blue circles, quality control (QC). The mean (x) and 95% confidence interval (CI) of the population (dotted line) is shown.

5.2.6 Data Modelling and Statistical analysis

For each identified metabolite a two-way repeated measures ANOVA was conducted to examine the effects of sex and time on metabolite concentration (p-values, *Table 5.1)*. If there was a significant interaction between sex and time, then an analysis of simple main effects was performed. Correction for multiple comparisons was performed using the method described by Benjamini & Hochberg (1995) and corrected p-values (q-values) are also reported (*Table 5.1*). All identified metabolites were then combined into a single data matrix and the multivariate covariance analysed using PCA (Jolliffe, 2002). Hierarchical cluster analysis (HCA) (Hastie et al., 2009; Kaufman & Rousseeuw, 1990) was then performed to assess the similarities between individual metabolite concentrations, both PCA and HCA as described in Chapter 4. For this study, a Principal Component-Canonical Variate Analysis (PC-CVA) was conducted using a five principal components projection to illustrate multivariate discrimination between the clusters. Data was log-transformed prior to univariate and multivariate analyses. All statistical analyses were performed using IBM SPSS® Statistics 25 software package and Matlab® scripting language version R2018a (Mathworks®, Natick, MA).

5.2.7 Oxidative Stress

Oxidative stress was calculated using the Nernst equation for half-cell reduction potential (E_{hc}) mV of the ratio for reduced glutathione to oxidised glutathione metabolite peak areas measured from the C18 positive column (Schafer & Buettner, 2001).

5.3 RESULTS

In this study, the LC-MS analysis of 40 haemolymph samples collected from ten *C. tenuimanus* (five males, five females) at four time points yielded 54 reproducible annotated metabolites (*Table 5.1*), including amino acids, fatty acids, biogenic amines, purine and pyrimidine metabolites, and excretion metabolites.

5.3.1 Univariate analysis

A two-way repeated measures ANOVA corrected for multiple comparisons revealed significant differences between the effects of sex, time, and time × sex interactions in a number of metabolites over the four time points (*Table 5.1*). Univariate analysis showed significant differences between sexes for 19 metabolites (q-values <0.05, FDR-adjusted p-values); 25 metabolites showed significant differences over time; and 11 metabolites showed a significant interaction effect between sex and time. The trajectories (plots) from the two-way repeated measures ANOVA for each metabolite are found in Appendix B.

Table 5.1. Metabolites identified in *Cherax tenuimanus* haemolymph. Statistically significant differences between factors, based on p value <0.05 and q value <0.05 (FDR-adjusted p values) from a two-way repeated measures ANOVA examining the effects of sex, time, and time × sex interactions on metabolite concentration, highlighted in bold. Metabolites that are repeated twice (indole-3-lactic-acid, kynurenic acid, inosine, glutathione, oxidised glutathione, and tryptophan are marked with a superscript number) were identified using both the positive and negative C18 columns. Molecular weight (MW); Retention time in minutes (Rt_min); C18 column either negative or positive (Mode); metabolomics standards initiative level (MSI Level); relative standard deviations calculated for the pooled quality control injections (%RSD); false discovery rate (%D-Ratio). Clusters indicated are based on hierarchical cluster analysis (see *Figure 5.2*).

Metabolite name	***Formula***	***MW (g/mol)***	***RT (min)***	***Mode (C18)***	***MSI level***	***% RSD***	***% D-Ratio***	***Sex***		***Time***		***Time × Sex***		***Cluster***
								p-value	***q-value***	***p-value***	***q-value***	***p-value***	***q-value***	
urocanic acid	$C_6 H_6 N_2 O_2$	138.0	1.28	Pos	ms1	3.17	2.65	**0.01**	**0.05**	**0.01**	**0.02**	0.10	0.27	**A**
hypoxanthine	$C_5 H_4 N_4 O$	136.0	1.28	Pos	ms1	3.51	2.46	**0.01**	**0.05**	**0.01**	**0.02**	0.09	0.25	**A**
acetyl-L-methionine	$C_7 H_{13} NO_3 S$	191.1	3.27	Pos	ms1	2.71	2.20	**0.04**	0.09	0.18	0.24	0.06	0.18	**A**
indole-3-acetic acid	$C_{10} H_9 NO_2$	175.1	3.84	Pos	ms1	2.45	2.51	**0.03**	0.08	0.07	0.10	0.16	0.39	**A**
2-piperidinone	$C_5 H_9 NO$	99.1	3.04	Pos	ms1	4.47	2.31	0.12	0.21	0.97	0.97	0.57	0.76	**A**
indole-3-lactic acid[1]	$C_{11} H_{11} NO_3$	205.1	3.67	Pos	ms1	1.11	1.17	0.47	0.61	**<0.01**	**<0.01**	**0.02**	0.09	**A**
indole-3-lactic acid[1]	$C_{11} H_{11} NO_3$	205.1	3.67	Neg	ms1	4.13	7.44	0.46	0.60	**<0.01**	**<0.01**	**<0.01**	**0.01**	**A**
arginine	$C_6 H_{14} N_4 O_2$	174.1	0.83	Pos	ms1	4.23	5.80	0.68	0.75	**<0.01**	**<0.01**	0.17	0.40	**A**
p-hydroxyphenyllactic acid	$C_9 H_{10} O_4$	182.1	3.19	Neg	ms1	1.49	2.78	0.13	0.23	**<0.01**	**<0.01**	0.51	0.74	**A**
glutathione reduced[2]	$C_{10} H_{17} N_3 O_6 S$	307.1	1.27	Pos	ms1	8.24	7.40	0.83	0.86	**<0.01**	**<0.01**	0.18	0.40	**A**
glutathione[2]	$C_{10} H_{17} N_3 O_6 S$	307.1	1.29	Neg	ms1	7.28	5.34	0.61	0.70	0.20	0.24	0.86	0.87	**A**
thymine	$C_5 H_6 N_2 O_2$	126.0	2.97	Pos	ms1	6.16	9.90	0.49	0.62	**0.01**	**0.03**	0.06	0.18	**B**
pantothenic acid	$C_9 H_{17} NO_5$	219.1	3.05	Neg	ms1	3.93	4.75	0.11	0.20	**0.05**	0.09	0.55	0.76	**B**
5-aminosalicylic acid	$C_7 H_7 NO_3$	153.0	3.13	Pos	ms1	3.30	2.63	0.28	0.43	0.07	0.11	0.12	0.31	**B**
uridine-5'-phosphoric acid	$C_9 H_{13} N_2 O_9 P$	324.0	0.95	Neg	ms1	4.12	3.73	**0.05**	0.11	**0.01**	**0.03**	**0.01**	0.06	**B**
oxidized glutathione[3]	$C_{20} H_{32} N_6 O_{12} S_2$	612.2	1.62	Neg	ms1	2.97	2.00	0.76	0.80	0.05	0.09	0.86	0.87	**B**
citric acid	$C_6 H_8 O_7$	192.0	1.29	Neg	ms1	8.52	6.40	0.51	0.62	**0.01**	**0.03**	0.67	0.81	**B**
betaine	$C_5 H_{11} NO_2$	117.1	0.87	Pos	ms1	6.46	15.25	0.25	0.40	**0.02**	**0.04**	0.87	0.87	**C**
adenosine	$C_{10} H_{13} N_5 O_4$	267.1	2.19	Pos	ms1	1.08	1.03	0.08	0.16	**<0.01**	**<0.01**	0.52	0.74	**C**
tyrosol	$C_8 H_{10} O_2$	138.1	3.30	Pos	ms1	8.46	18.29	0.06	0.12	0.21	0.25	0.32	0.58	**C**
sucrose	$C_{12} H_{22} O_{11}$	342.1	0.95	Neg	ms1	0.94	1.03	0.38	0.51	**0.01**	**0.02**	0.75	0.84	**C**
azelaic acid	$C_9 H_{16} O_4$	188.1	3.74	Neg	ms1	2.32	2.31	0.69	0.75	**<0.01**	**<0.01**	0.70	0.81	**C**

Metabolite name	*Formula*	*MW (g/mol)*	*RT (min)*	*Mode (C18)*	*MSI level*	*% RSD*	*% D-Ratio*	*Sex*		*Time*		*Time × Sex*		*Cluster*
								p-value	*q-value*	*p-value*	*q-value*	*p-value*	*q-value*	
uridine	$C_9 H_{12} N_2 O_6$	244.1	1.54	Neg	ms1	2.71	13.37	0.94	0.94	**0.01**	**0.03**	**0.03**	0.12	**C**
nicotinic acid	$C_6 H_5 NO_2$	123.0	1.28	Pos	ms1	10.27	18.76	0.29	0.44	0.25	0.29	0.29	0.56	**C**
phthalic acid	$C_8 H_6 O_4$	148.0	3.76	Pos	ms3	5.13	6.99	**0.05**	0.11	**0.04**	0.09	0.68	0.81	**C**
7-methylguanosine	$C_{11} H_{15} N_5 O_5$	297.1	2.08	Pos	ms1	2.02	2.20	**0.02**	0.06	**<0.01**	**0.01**	**<0.01**	**0.02**	**C**
nicotinamide	$C_6 H_6 N_2 O$	122.0	1.36	Pos	ms1	8.24	10.50	0.76	0.80	**0.04**	0.09	0.27	0.56	**C**
glutathione disulfide[3]	$C_{20} H_{32} N_6 O_{12} S_2$	612.2	1.77	Pos	ms1	16.87	12.18	0.34	0.48	0.09	0.13	**0.01**	**0.05**	**C**
proline	$C_5 H_9 NO_2$	115.1	0.91	Pos	ms1	2.12	2.26	0.92	0.94	0.21	0.25	0.05	0.17	**C**
spermidine	$C_7 H_{19} N_3$	145.2	0.71	Pos	ms1	9.78	9.85	0.15	0.26	0.20	0.24	0.79	0.86	**C**
pantothenic acid, salt	$C_9 H_{17} NO_5$	219.1	3.05	Pos	ms1	5.21	7.37	0.14	0.24	**<0.01**	**0.02**	0.65	0.81	**C**
N-acetyl-L-aspartic acid	$C_6 H_9 NO_5$	175.0	1.27	Pos	ms1	13.91	13.86	0.35	0.48	0.19	0.24	0.85	0.87	**C**
norspermidine	$C_6 H_{17} N_3$	131.1	0.71	Pos	ms1	15.58	16.53	0.50	0.62	0.09	0.13	0.52	0.74	**C**
uric acid	$C_5 H_4 N_4 O_3$	168.0	1.27	Pos	ms1	1.50	1.40	0.24	0.38	0.60	0.67	0.50	0.74	**C**
phenylalanine	$C_9 H_{11} NO_2$	165.1	2.99	Pos	ms1	1.83	2.40	0.61	0.70	**0.05**	0.09	0.63	0.79	**C**
histidine	$C_6 H_9 N_3 O_2$	155.1	0.83	Pos	ms1	5.01	8.97	**0.01**	**0.05**	0.70	0.75	0.62	0.79	**C**
carnitine	$C_7 H_{15} NO_3$	161.1	0.89	Pos	ms1	3.13	4.65	**<0.01**	**0.01**	0.35	0.40	0.41	0.66	**D**
acetylcholine	$C_7 H_{15} NO_2$	145.1	0.95	Pos	ms3	2.22	2.32	**0.02**	**0.05**	0.16	0.22	0.40	0.66	**D**
propionylcarnitine	$C_{10} H_{19} NO_4$	217.1	2.53	Pos	ms1	1.16	0.97	**<0.01**	**0.01**	0.11	0.15	0.33	0.58	**D**
decanoylcarnitine	$C_{17} H_{33} NO_4$	315.2	5.03	Pos	ms1	2.80	4.01	**<0.01**	**<0.01**	**0.01**	**0.03**	0.44	0.69	**D**
kynurenic acid[4]	$C_{10} H_7 NO_3$	189.0	3.23	Pos	ms1	3.57	1.76	**<0.01**	**<0.01**	0.90	0.94	0.75	0.84	**D**
kynurenic acid[4]	$C_{10} H_7 NO_3$	189.0	3.22	Neg	ms1	1.97	0.98	**<0.01**	**<0.01**	0.73	0.77	0.58	0.76	**D**
kynurenine	$C_{10} H_{12} N_2 O_3$	208.1	2.96	Pos	ms1	3.24	2.20	**<0.01**	**<0.01**	0.97	0.97	0.85	0.87	**D**
guanosine	$C_{10} H_{13} N_5 O_5$	283.1	2.25	Pos	ms1	2.97	2.85	**<0.01**	**<0.01**	**0.04**	0.09	0.35	0.59	**D**
guanine	$C_5 H_5 N_5 O$	151.0	2.25	Pos	ms1	3.27	3.48	**<0.01**	**<0.01**	0.08	0.11	0.24	0.51	**D**
inosine[5]	$C_{10} H_{12} N_4 O_5$	268.1	2.23	Pos	ms1	0.96	1.02	**0.01**	**0.03**	0.06	0.09	0.32	0.58	**D**
inosine[5]	$C_{10} H_{12} N_4 O_5$	268.1	2.25	Neg	ms1	2.27	3.74	**<0.01**	**<0.01**	0.62	0.68	0.29	0.56	**D**
glutamine	$C_5 H_{10} N_2 O_3$	146.1	0.86	Pos	ms1	1.70	3.02	0.09	0.17	0.05	0.09	**<0.01**	**0.01**	**E**
O-acetyl-L-carnitine	$C_9 H_{17} NO_4$	203.1	1.29	Pos	ms1	4.30	5.18	**0.01**	**0.05**	**<0.01**	**<0.01**	0.06	0.18	**E**

Metabolite name	*Formula*	*MW (g/mol)*	*RT (min)*	*Mode (C18)*	*MSI level*	*% RSD*	*% D-Ratio*	*Sex*		*Time*		*Time × Sex*		*Cluster*
								p-value	*q-value*	*p-value*	*q-value*	*p-value*	*q-value*	
hydroxyproline	$C_5 H_9 NO_3$	131.1	0.87	Pos	ms1	1.70	3.49	0.31	0.45	**0.01**	**0.03**	**<0.01**	**0.02**	**E**
pyroglutamic acid	$C_5 H_7 NO_3$	129.0	0.88	Pos	ms1	2.25	4.12	0.65	0.74	**0.04**	0.08	**<0.01**	**0.02**	**E**
cytosine	$C_4 H_5 N_3 O$	111.0	1.21	Pos	ms1	10.52	10.17	**<0.01**	**0.01**	**<0.01**	**0.01**	**<0.01**	**0.01**	**E**
cytidine	$C_9 H_{13} N_3 O_5$	243.1	1.21	Pos	ms1	5.50	5.04	**<0.01**	**0.02**	**0.03**	0.07	**0.04**	0.15	**E**
tyrosine	$C_9 H_{11} NO_3$	181.1	1.67	Pos	ms1	0.75	0.39	**<0.01**	**0.05**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**E**
2-hydroxycinnamic acid	$C_9 H_8 O_3$	164.0	1.67	Pos	ms1	0.76	0.42	**0.01**	**0.05**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**E**
deoxyinosine	$C_{10} H_{12} N_4 O_4$	252.1	2.76	Neg	ms1	2.29	2.06	**0.02**	0.05	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**E**
tryptophan[6]	$C_{11} H_{12} N_2 O_2$	204.1	3.20	Pos	ms1	2.51	3.31	0.08	0.17	0.05	0.09	**0.03**	0.13	**E**
tryptophan[6]	$C_{11} H_{12} N_2 O_2$	204.1	3.19	Neg	ms1	3.93	11.30	**0.03**	0.08	**0.01**	**0.03**	**<0.01**	**0.01**	**E**

Univariate analysis (*Table 5.1*) indicated 19 metabolites contributed to the differences between sexes (q-values <0.05), including amino acids, their intermediates and derivatives (histidine, tyrosine, urocanic acid, kynurenic acid, kynurenine), nucleotides and nucleosides and their derivatives (guanosine, cytosine, cytidine, guanine, hypoxanthine, inosine), fatty acids (carnitine, propionylcarnitine, decanoylcarnitine, O-acetyl-L-carnitine), and other organic compounds (acetylcholine (a neurotransmitter) and 2-hydroxycinnamic acid).

Metabolites showing significant changes over time (n=24) were: amino acids and their derivatives (arginine, tyrosine, tryptophan, urocanic acid, indole-3-lactic acid, betaine, hydroxyproline, hydroxyphenyllactic acid, and glutathione reduced), nucleotides and nucleosides and their derivatives (cytosine, adenosine, hypoxanthine, thymine, deoxyinosine, uridine, uridine-5'-phosphoric acid disodium salt, and 7-methylguanosine), fatty acids (azelaic acid, decanoylcarnitine, O-acetyl-L-carnitine; vitamin: pantothenic acid), and other organic compounds (2-hydroxycinnamic acid, citric acid, and sucrose).

Eleven metabolites showed significantly different interactions between time × sex (q-values <0.05), namely amino acid derivatives (tyrosine, tryptophan, glutamine, hydroxyproline, pyroglutamic acid, indole-3-lactic acid, and glutathione disulphide) and nucleotides and nucleosides and their derivatives (deoxyinosine, 7-methylguanosine, cytosine, and 2-hydroxycinnamic acid).

Some metabolites indicated significant differences for more than one factor: urocanic acid (sex, time), hypoxanthine (sex, time), indole-3-lactic acid (time, time × sex interaction), 7-methylguanosine (time, time × sex interaction), decanoylcarnitine (sex, time), O-acetyl-L-carnitine (sex, time), hydroxyproline (time, time × sex interaction), cytosine (sex, time, time × sex interaction), tyrosine (sex, time, time × sex interaction), 2-hydroxycinnamic acid (sex, time, time × sex interaction), deoxyinosine (time, time × sex interaction), and tryptophan (time, time × sex interaction).

5.3.2 Heirarchical Cluster Analysis (HCA)

Based on phenotypic similarity, five metabolite clusters (labelled A-E) were identified in the metabolite profile of male and female *C. tenuimanus* haemolymph in this study using a circular hierarchical cluster analysis (HCA) dendrogram (Figure *5.2*). The statistical significance from univariate analyses is illustrated on the HCA dendrogram with factors for time, sex, time & sex, and time × sex interaction. ANOVA plots for each cluster are in appendix B.

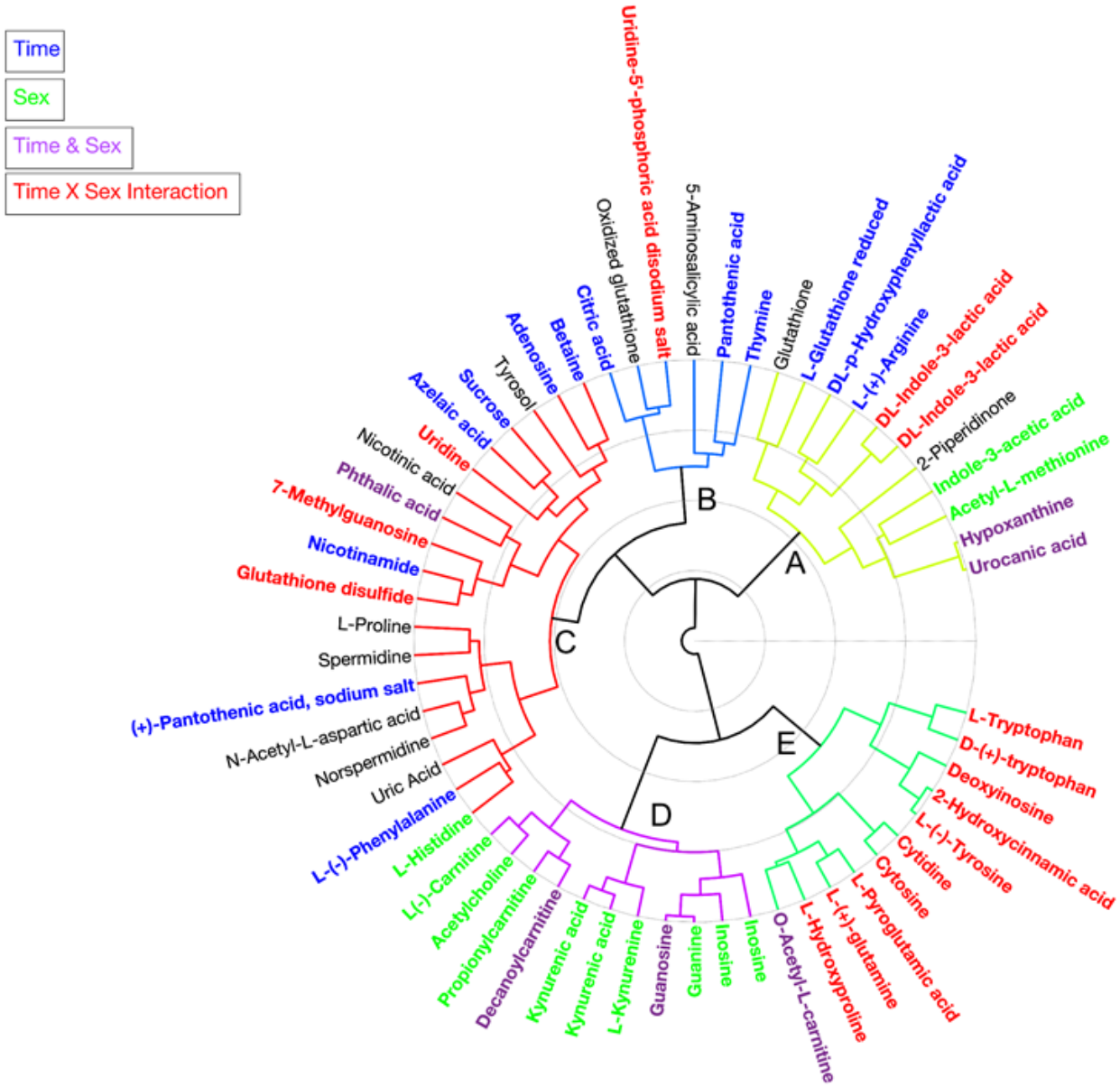
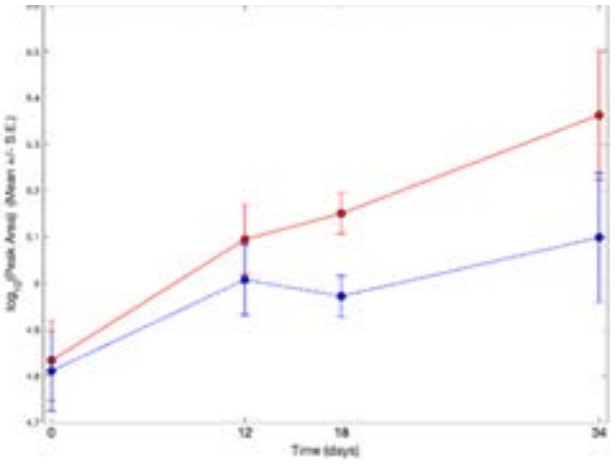


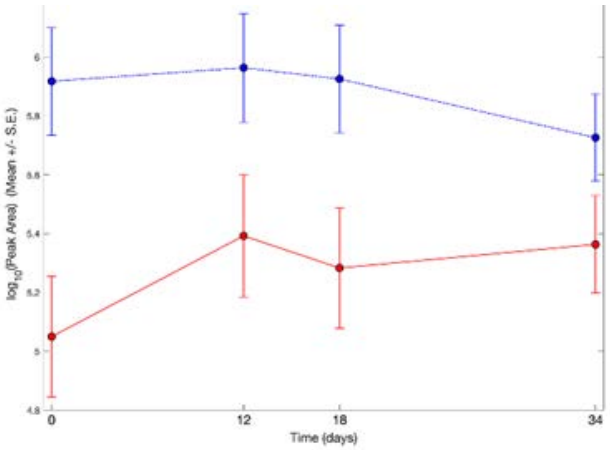
Figure 5.2. Circular hierarchical cluster analysis (HCA) dendrogram grouping individual metabolites identified in the haemolymph of *Cherax tenuimanus* into five clusters (A-E) based on phenotypic similarity. Colours of the metabolite name are based on the univariate analysis (q-value <0.05), metabolites that are significant over time (blue), between sexes (green), time and sex (purple), time × sex interaction (red), and not significant for any factor (black). Duplicate metabolites identified in positive and negative modes including L- and D-Trp.

Cluster A

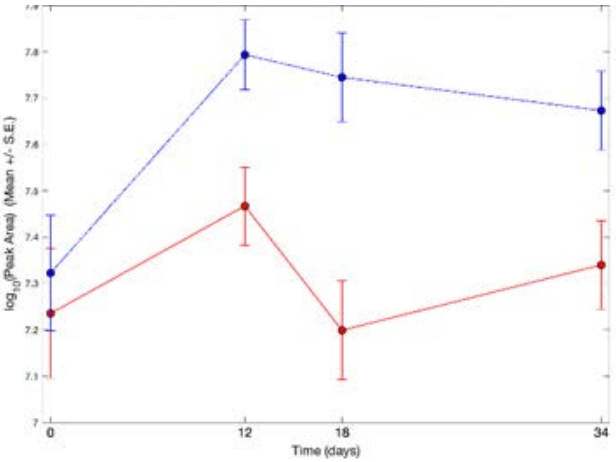
Cluster A consisted of ten metabolites, which were amino acids and their derivatives or products of energy metabolism. Tryptophan metabolism was the dominant pathway for this cluster; however, the metabolites were also involved in pathways for histidine metabolism, methionine metabolism, arginine and proline metabolism, tyrosine metabolism, and glutathione metabolism. Cluster A was a varied group in terms of the patterns of metabolite responses and significant factors but the general trend shown in eight of the ten metabolites was that levels had increased from day 0 (when marron were held as individuals) through to the end of the trial (after animals were put together). Changes in metabolite levels occurred in both sexes but at different rates (Figure *5.3*). Three metabolites had significant changes over time, two metabolites had significant changes between sexes, two metabolites were significant for both time and sex, one metabolite had a time x sex interaction, and two were not significant for any factor (*Table 5.2*).



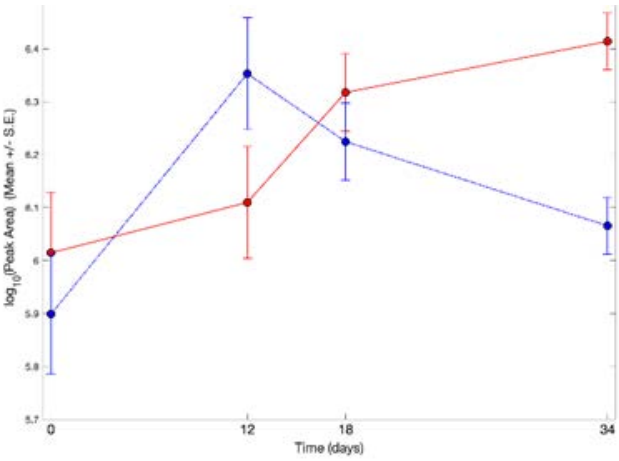
a) Time (n = 3)
p-hydroxyphenyllactic acid (p <0.01)



b) Sex (n = 2),
acetyl-L-methionine (p = 0.04)



c) Time and Sex (n = 2),
urocanic acid (time p = 0.01, sex p = 0.01)



d) Time × Sex interaction (n = 1),
indole-3-lactic acid (time p <0.01, time × sex p <0.01)

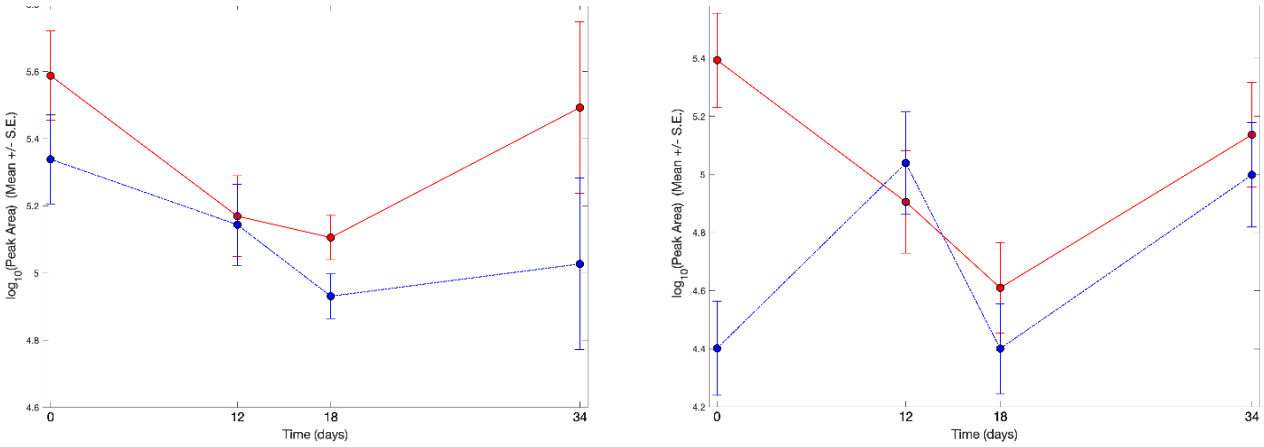
Figure 5.3 Examples of metabolite phenotypic behaviour for cluster A. The plots are the result of a two-way repeated measures ANOVA with four time points on the x axis (days) and the mean peak area of each metabolite on y axis; point values are log 10 mean metabolite peak area ± standard error bars of five females (red) and five males (blue). Significant factors (q values <0.05) are: a) Time, b) Sex, c) Time and Sex, d) Time × Sex interaction; n = the number of metabolites with this significant factor in this cluster.

Table 5.2 Cluster A metabolites from HCA which display significance over time, between sexes, time and sex, time x sex interaction or not significant for any factors based on q-values <0.05.

Time	Sex	Time & Sex	Time × Sex Interaction	Not significant
arginine	indole-3-acetic acid	urocanic acid	indole-3-lactic acid	2-piperidinone
p-hydroxyphenyllactic acid	acetyl-L-methionine	hypoxanthine		glutathione
glutathione reduced				

Cluster B

Cluster B was composed of six metabolites, mainly pyrimidine nucleotide and nucleosides which are part of the pathways for pyrimidine metabolism, beta-alanine metabolism, pantothenate, and CoA biosynthesis. There was some variation in metabolite levels over time where levels were higher at day 0, as individuals, then after potential pairs were put together the metabolite levels dropped to their lowest point at day 18 in both sexes before increasing by day 34 to levels similar to initial levels (Figure *5.4*). Cluster B concentrations of metabolites were elevated in females for most compounds. Three metabolites had significant differences over time (thymine, pantothenic acid, citric acid) and one metabolite had a significant time × sex interaction (uridine 5'-phosphate). There were no metabolites with a significant factor for sex, or time and sex. Two metabolites were statistically not significant for any factor but followed the same trends with similar initial and finals levels and the lowest point at day 18 as observed for the other metabolites in the cluster (*Table 5.3)*.



a) Time (n=3), pantothenic acid (p=0.049)

b) Time × Sex Interaction (n=1), uridine-5'-phosphoric acid (p=0.01)

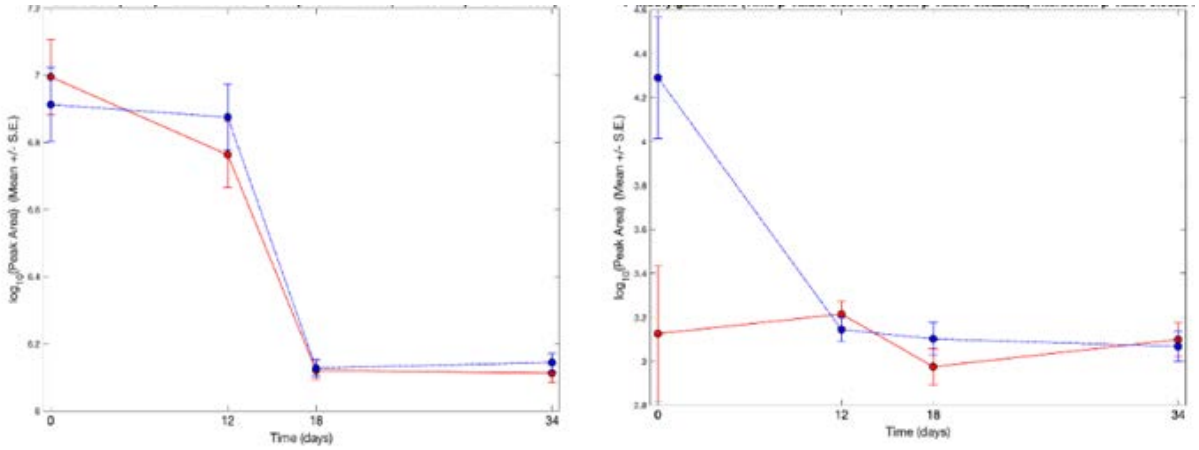
Figure 5.4. Examples of cluster B metabolite patterns with significant factors for a) time and b) time × sex interaction. The plots are the result of a two-way repeated measures ANOVA with four time points on the x axis (days) and the mean peak area of each metabolite on y axis; point values are $\log_{10}$ mean metabolite peak area ± standard error bars of five females (red) and five males (blue). (n= number of metabolites from this cluster for each significant factor). The patterns displayed in this cluster were similar with a change after day 0, lowest levels at day 18 and then recovered back to initial levels (in females) by day 34.

Table 5.3 Cluster B metabolites from HCA which display significance over time, between sexes, time and sex, time × sex interaction or not significant for any factors based on q-values <0.05.

Time	Sex	Time & Sex	Time × Sex Interaction	Not significant
Thymine pantothenic acid citric acid	None	None	uridine-5'-phosphoric acid	5-aminosalicylic acid oxidised glutathione

Cluster C

Cluster C was the largest cluster (19 metabolites) and included alpha amino acids, purine and pyrimidine nucleosides, a sugar, vitamin, and a fatty acid. These compounds were involved in nicotinate and nicotinamide metabolism, glutamate metabolism, glutathione metabolism, arginine and proline metabolism, methionine metabolism, aspartate metabolism, phenylalanine metabolism, histidine metabolism, and tyrosine and derivatives. The overall trend for this cluster shows males and females responding in a similar manner, with a decrease in metabolite levels over time. In general, the levels of most metabolites were higher at day 0 (as individuals) and then decreased after paired with a potential mate over the next two time points with the final levels at day 34 much lower than on the first date of collection. Of the 19 metabolites in this cluster, seven metabolites displayed a significant difference over time (betaine, adenosine, sucrose, azelaic acid, nicotinamide, pantothenic acid, and phenylalanine (*Figure 5.5*a). L-histidine was significantly different between sexes with higher levels present in females. Phthalic acid (presumably a plasticizer and not an endogenous metabolite), was significantly different in both time and sex, with higher levels in males but still followed the trend with highest levels at day 0 and decreased so that the lowest was at day 34. Three metabolites had a significant time × sex interaction (uridine, 7-methylguanosine, and glutathione disulphide (*Figure 5.5*b) where the males had higher levels as individuals but then it decreased when they were paired with a potential mate so that males and females displayed a similar level.



a) Time (n=7), azelaic acid (p <0.01)

b) Time × Sex interaction (n=3), 7-methylguanosine (time p = 0.02, sex p <0.01, time × sex p<0.01)

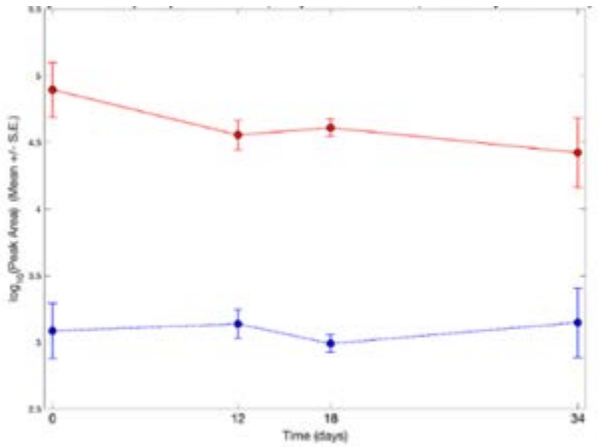
Figure 5.5. Cluster C examples of metabolites with significant factor of time, time × sex interaction; n = the number of metabolites with this significant factor in this cluster.

Table 5.4 Cluster C metabolites from HCA which display significance over time, between sexes, time and sex, time × sex interaction or not significant for any factors based on q-values <0.05.

Time	Sex	Time & Sex	Time × Sex Interaction	Not significant
betaine	L-histidine	phthalic acid	uridine	tyrosol
adenosine			7-methylguanosine	nicotinic acid
sucrose			glutathione disulphide	L-proline
azelaic acid				spermidine
nicotinamide				N-acetyl-L-aspartic acid
pantothenic acid				norspermidine
L-phenylalanine				uric acid

Cluster D

Cluster D consisted of 11 metabolites, including several carnitines (fatty acids), a neurotransmitter (acetylcholine), amino acid derivatives, kynurenic acid and kynurenine (both play a role in tryptophan and purine metabolism), guanine, guanosine, and inosine. Two metabolites (inosine and kynurenic acid) were detected on both the positive and negative column. All of the metabolites in cluster D had higher levels in females than in males and the metabolite levels for each sex fluctuated little across all time points (Figure *5.6*). Of the eleven metabolites in this cluster, nine showed a statistically significant difference between sexes and two metabolites between time and sex (decanoylcarnitine and guanosine) (*Table 5.5*).



Sex (n=9),
kynurenic acid (p<0.01)

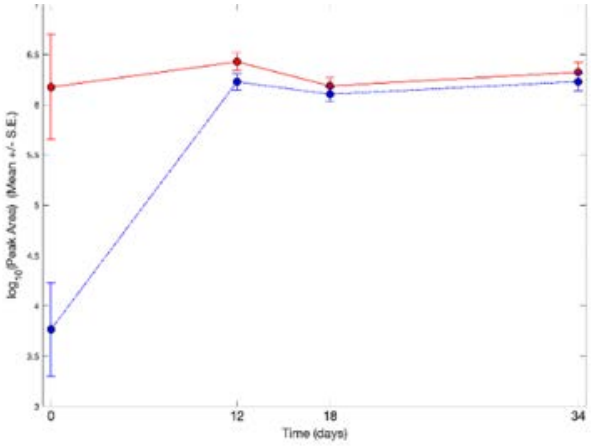
Figure 5.6 Examples of metabolites in cluster D where sex was the significant factor. n = the number of metabolites with this significant factor in this cluster

Table 5.5 Cluster D metabolites from HCA which display significance over time, between sexes, time and sex, time × sex interaction or not significant for any factors based on q-values <0.05.

Time	Sex	Time & Sex	Time x Sex Interaction	Not significant
none	L-carnitine acetylcholine propionlycarnitine kynurenic acid L-kynurenine guanine inosine	decanoylcarnitine guanosine	none	none

Cluster E

Cluster E was composed of ten metabolites, where nine metabolites had a significant difference with a time × sex interaction *(Table 5.6*). The metabolites that make up this cluster were part of the pathways for purine and pyrimidine metabolism, glutathione metabolism, and tryptophan metabolism. In Cluster E the metabolites all had higher levels in females than males, and the female levels remained relatively unchanged throughout all time points. The levels in males began lower while kept as individuals and increased after they were paired with a potential mate, between days 0 and day 12, then synchronised with females for the remaining time points (Figure *5.7*).



Time × Sex interaction (n=9), 2-hydroxycinnamic acid
(time p = 0.01), sex (p <0.01), time × sex (p <0.01)

Figure 5.7 Example of Cluster E metabolites where the significant factor was a time × sex interaction. n = the number of metabolites with this significant factor in this cluster.

Table 5.6 Cluster E metabolites from HCA which display significance over time, between sexes, time and sex, time × sex interaction or not significant for any factors based on q-values <0.05.

Time	Sex	Time & Sex	Time × Sex Interaction	Not significant
none	none	O-acetyl-L-carnitine	L-hydroxyproline	none
			L-glutamine	
			L-pyroglutamic acid	
			cytosine	
			cytidine	
			L-tyrosine	
			2-hydroxycinnamic acid	
			deoxyinosine	
			L-tryptophan	

5.3.3 Principal component-canonical variate analysis (PC-CVA)

Principal component-canonical variate analysis (PC-CVA) illustrated the strong relationship between metabolic profile, sex, and time (Figure *5.8*). The first canonical variate (CV1) clearly separated male and female haemolymph profiles of *C. tenuimanus* at all four time points. Four of the five male marron expressed a difference on CV1 at day 0. These four males also showed a significant shift in metabolite profile after being placed with a female. No females had a similar response to being placed with a male on this axis. The second canonical variate (CV2) indicated some change between time points in each of the sexes, with changes for both sexes occurring in the same direction and at a similar magnitude and with the sexes converging (marginally) over time.

5.3.4 Oxidative stress

Oxidative stress was measured and there was little difference between individuals, sexes and time. The mean of means ± SE for females and males indicating oxidative stress was low and did not change much with the treatment for either sex (*Table 5.7,* calculations in Appendix B).

Table 5.7 Mean of means values ± standard error in mV for oxidative stress (GSSG/ GSH) estimated using Nernst equation for half-cell reduction potential (E_{hc}) in mV of female and male *Cherax tenuimanus*.

Sex	Mean (mV)	±SE (mV)
Female	-353.9	6.02
Male	-353.6	8.16

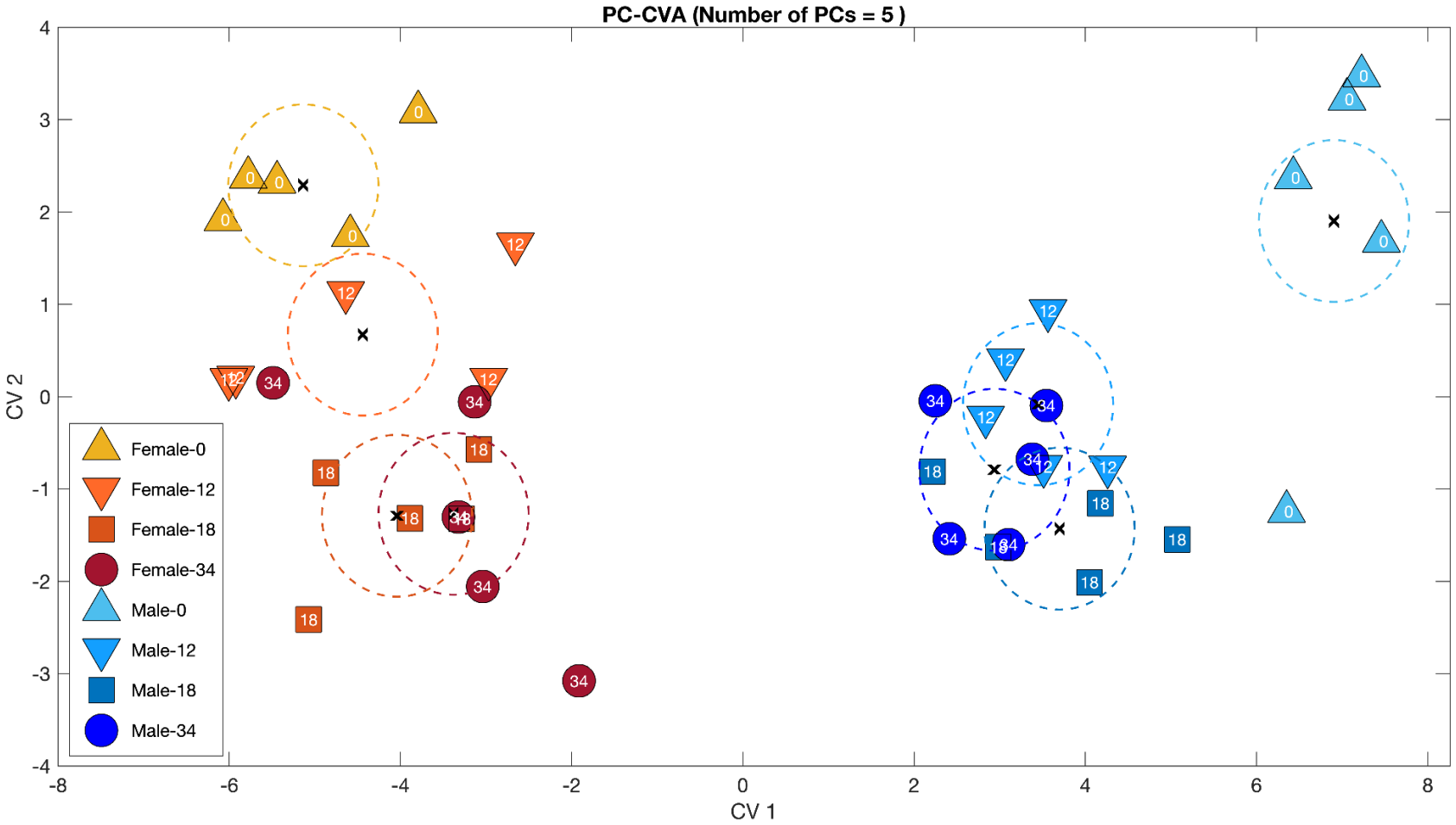


Figure 5.8 Principal component-canonical variate analysis (PC-CVA) of the relationship between metabolomic profile, sex and time from *Cherax tenuimanus* haemolymph samples. CV1 sex; CV2 time. Males in cool colours and females in warm colours. Lightest colour is lowest number of days to darkest colours, greatest numbers of days, from day 0 (as individuals) to (after pairing) day 12, day 18, day 34. X: mean of each group; dashed lines: 95% confidence intervals of the mean of each group. The PC-CVA model was constructed using five principal components.

5.4 DISCUSSION

In the previous chapter, the targeted analysis based on the metabolomic profiles of the haemolymph showed that it was possible to distinguish between the two congeneric *Cherax* species and between sexes of the two species. It is likely that at least some of the differences detected were related to the timing of reproduction and moulting in each sex/species. In this chapter, we report the identification of 54 metabolites in the haemolymph of *C. tenuimanus*, using the same untargeted LC-MS approach and show that changes in the metabolome can be measured in response to environmental conditions such as the presence of a potential mate. The metabolites identified are comprised of the same chemical groups as described in the previous chapter but in this longitudinal study we can see changes occurring in the metabolome highlighting the potential of some metabolites as biomarkers.

A high proportion of the compounds identified in this study have previously been recognised in studies of other decapod crustaceans, many of which used nuclear magnetic resonance (NMR) (a different method of detecting metabolites), as well as biological tissues other than haemolymph (*Callinectes sapidus*, NMR, haemolymph (Schock et al., 2010); *Litopenaeus vannamei*, NMR, whole animal, hepatopancreas, muscle, intestines (Schock et al., 2013); *Astacus leptodactylus*, NMR, haemolymph, muscle, hepatopancreas (Costantini et al., 2018); *Procambarus clarkii*, NMR, hepatopancreas, gill, muscle (Izral et al., 2018); *Paralithodes camtschaticus*, LC-MS, haemolymph, muscle, hepatopancreas, and *Lithodes aequispinus*, LC-MS, haemolymph (Zacher et al., 2018)). However, our ability to detect and identify all metabolites is hampered by the lack of a single global database for crustaceans, which necessitates comparisons to databases created for humans (mammals) to identify metabolites. Although some metabolites have the same function across species, each organism does have its own unique set of metabolites (Kuhlisch & Pohnert, 2015), therefore this needs to be addressed to expand future studies.

A clustering analysis of the data identified five clusters of metabolites based on phenotypic similarity between sexes, time, both time and sex, and time × sex interaction as identified by a two-way repeated measures ANOVA. Differences in the metabolome between sexes (CV1) and over time (CV2) were also supported by principal component-canonical variate analysis (PC-CVA) (Figure 5.5). The metabolites detected across the clusters belong to several biologically important pathways with varying functions (Alfaro & Young, 2016) in energy metabolism, osmoregulation (Rahi et al., 2018), signalling pathways (Hay, 2009), immune functions (Roager & Licht, 2018) and oxidative stress

(Schafer & Buettner, 2001), and some compounds having roles in multiple pathways. The pathways that will be discussed in this chapter have the most metabolites identified in marron haemolymph including, but not limited to, precursors, intermediates and derivatives. Overall, the pathways and metabolites that have been identified involve the metabolism or catabolism of amino acids and nucleotides/nucleosides which was expected with LC-MS and the C18 columns used for analysis. This discussion will report on some aspects of amino acid metabolism, such as tryptophan and kynurenine, phenylalanine, tyrosine and glutathione metabolism as well as purine metabolism and carnitine (lipid) metabolism. We will attempt to describe the biological influence of these metabolites and to which clusters they relate. ***Metabolites in bold italics*** were detected in this study (Chapter 5) as other metabolites from the previous chapter (Chapter 4) may also be discussed. There were some pathways and metabolites detected in this study that may be equally important, but they will not be discussed as relatively little is known about them in relation to crustaceans at present.

The main patterns recognised by the behaviour of the metabolites within the clusters in the HCA (Figure *5.2*) were:

- **Response to disturbance (change)**. Both males and females showed a disturbance in metabolite levels after being placed together. Some metabolites returned to initial levels (transient, Cluster B), others remained changed throughout the experiment (non-transient, Clusters A and C).
- **Differences between sexes**. Some metabolites were significantly different between sexes and remained unchanged whether animals were housed on their own or with a potential mate (Cluster D).
- **Male response to female presence**. Four out of five males had a larger metabolomic response to being placed with a female, whereas females did not show a similar response (Cluster E).

5.4.1 Response to disturbance (change)

Transient response

A transient response to disturbance was identified when a metabolite changed dramatically from its initial level (displaying either a peak or drop) and then recovered back to initial levels by the end of the study (Figure *5.4*b). In cluster B some metabolites for both sexes displayed this response to disturbance as the initial levels as individuals and final levels with a mate were similar, but there was a distinct change after the marron were put together in the same aquaria (occurring around day 18). As the metabolite levels returned to initial levels over the remainder of the study, this may indicate a

transient requirement for elevated levels of certain metabolites. For example, organic acids such as ***pantothenic acid*** (vitamin B5) and ***citric acid***, which are important intermediates in energy metabolism due to their roles in the biosynthesis of coenzyme-A and the metabolism and synthesis of carbohydrates, proteins and lipids through the tricarboxylic acid cycle (Da Poian et al., 2010; Provasoli et al., 1970), were identified within the haemolymph and were transient.

Non-transient response

Some metabolites displayed a non-transient response to disturbance where their levels changed (either increased or decreased) once the animals were housed with a mate and remained at that level for the duration of the experiment. These were seen in both sexes of crayfish in clusters A and C, and to a lesser degree in males in cluster E. The metabolites in clusters A and C all have immune boosting or antioxidant functions and allow an organism to respond to stresses. In cluster A, metabolites were identified that indicated immune responses in an organism such as indoles and glutathione. Indoles are gut metabolites that influence immune functions from the tryptophan pathway (Roager & Licht, 2018), and glutathione is referred to as a master antioxidant in living organisms and important for protection against oxidative stress (Alfaro et al., 2019; Bone et al., 2015; Lavradas et al., 2014; Schafer & Buettner, 2001). In cluster C, betaine and adenosine assist with osmoregulation and fast responses to environmental stress (Polat & Beklevik, 1999; Stegen & Grieshaber, 2001). Cluster E metabolites will be discussed in more detail later.

Indole gut metabolites (cluster A) are intercellular signalling compounds as well as antimicrobial agents inhibiting fungal and bacterial activity in the human gut (Roager & Licht, 2018). ***Indole-3-acetic acid*** and ***indole-3-lactic acid*** (significant for time, $q<0.01$) are derived from the catabolism of tryptophan often by gut bacteria leading to an antioxidant function by stimulation of gut immune cells (Roager & Licht, 2018). As a response to environmental stress, a decrease in indoleacetic acid was found in mice (urine) 15 – 25 days after exposure to the stressor but it then increased after 30 days (Lankadurai et al., 2013). This response is similar to the semi-permanent response to disturbance detected in the marron over the 34-day trial period of this study. ***DL-p-hydroxyphenyllactic acid***, (cluster A) a tyrosine metabolite, was significant over time ($q<0.01$) and is produced by bacteria such as bifidobacteria and lactobacilli and can play a role in production of antioxidants (Wishart et al., 2018).

Glutathione (cluster A) is a strong antioxidant composed of cysteine, glycine and glutamine which is present in reduced (GSH) or oxidised form (GSSG, also known as glutathione disulphide) (Halprin & Ohkawara, 1967; Lu, 2013). GSH is a redox buffer occurring in low amounts in extracellular fluid such as haemolymph and has a role in detoxification (< -100x to -1000x than inside the cell) (Schafer & Buettner, 2001) and protection against oxidative stress (Lavradas et al., 2014; Lu, 2013). The ratio of GSH to GSSG can be used as an indicator or biomarker for cellular oxidative stress (Alfaro et al., 2019) and can be measured in the haemolymph (Bone et al., 2015) where oxidative stress is indicated if GSSG is present at higher levels than GSH. Changes to the half-cell reduction potential (E_{hc}) of the GSSG/2GSH couple correlate with the biological status of the cell (proliferation E_{hc} ∼-240mV; differentiation E_{hc}∼ -200mV; apoptosis E_{hc} ∼-170 mV) with oxidative stress indicated by more positive E_{hc}. The results of the Nernst equation indicate an absence of oxidative stress (*Table 5.7* and Schafer and Buettner, 2001), suggesting laboratory conditions were appropriate and highlighting how these metabolites could be useful biomarkers for oxidative stress. Other studies have used ratios of GSH to GSSG as a measure of oxidative stress caused by thermal changes in the haemolymph of *Crassula aequilatera* with GC-MS metabolomics (Alfaro et al., 2019) and *Cherax quadricarinatus* by using a total glutathione (tGSH) assay kit (Bone et al., 2015, 2017). It was determined that elevated levels in tGSH indicated an increase in protection by antioxidants as the tGSH levels rose with temperatures outside the optimal thermal range (Bone et al., 2015, 2017).

Arginine (cluster A) is an essential amino acid for crustaceans and is known to be involved with salt and ion regulation in decapods (Rahi et al., 2018). Arginine is likely to increase when there is a perceived threat and potential need for an escape response by tail flipping and using arginine phosphate for energy bursts (England & Baldwin, 1983; Morris & Adamczewska, 2002). An alternative explanation for increased and sustained arginine levels could be in response to repeated haemolymph extraction where more handling of the animal was required. Also important are arginine kinase and arginine phosphate (though they were not detected in this study) due to their importance in arginine metabolism in crustaceans. Arginine phosphate is used for the regulation of energy levels in crustaceans whereas other organisms (vertebrates and some invertebrates) rely on creatine phosphate/creatine kinase to buffer ATP levels (Ellington, 2001; England & Baldwin, 1983). Arginine levels increased when the males and females were put together and stayed elevated throughout the study. Changing levels of arginine and arginine phosphate could be indicators of preparation for moulting. Crustacean growth is incremental and therefore different to growth in vertebrates and other invertebrates. Their moult cycles require large swings in their energy metabolism and how they utilise and store fuel (Jimenez & Kinsey, 2015). In the previous study, arginine was detected in

significantly higher levels in *C. cainii* and low levels in *C. tenuimanus* which concurs with the life stage of *C. cainii* at the time, where several males were moulting or preparing to moult.

Purines such as ***adenosine*** (cluster C) can increase ventilation rate, heart rate and haemolymph velocity (Stegen & Grieshaber, 2001) in crustaceans such as *Homarus americanus* where it accumulates in haemolymph during hypoxia and ischaemia. Adenosine is likely to facilitate fast systemic responses to environmental stress (Stegen & Grieshaber, 2001). In crustaceans, adenosine also works with the hormone serotonin as in times of stress serotonin is released from the X-organ sinus gland complex (a neurohaemal organ) (Reddy, 2019) and adenosine from muscle tissue (Stegen & Grieshaber, 2001). Changes in adenosine are difficult to assess as it has a short duration in the haemolymph and was barely detected after two minutes of infusion with adenosine (Stegen & Grieshaber, 2001). However after adenosine has been metabolised by adenosine deaminase, ***inosine*** accumulates in the haemolymph (Stegen & Grieshaber, 2001) which can be detected as a bio-indicator for the fight or flight response that was influenced by adenosine (inosine is also discussed later in regards to differences between sexes). Adenosine was higher in females across all time points in this study. Although adenosine was identified in the haemolymph it should not be used as a biomarker due to the short duration in tissues creating unreliability for detection. Instead inosine is a better marker for stress, as the product of adenosine metabolism and its longer life of the metabolite in tissues which has also been supported by other studies (Schock et al., 2013; Stegen & Grieshaber, 2001). In the previous study, it was the *C. tenuimanus* females that had the highest levels of adenosine which is appropriate as they also had the highest levels of inosine.

Betaine (cluster C) is a modified amino acid that is important for osmoregulation as it has the ability to protect cells against dramatic changes in osmotic pressure in fish and marine invertebrates (Polat & Beklevik, 1999). In tissues it plays a function in protein and energy metabolism as a methyl donor which assists with the synthesis of ***methionine*** and ***carnitine***. Betaine was statistically significant for time in this study and showed significantly higher levels in female *C. tenuimanus*.

Tyrosol (cluster C) is synthesised from ***tyrosine*** and had no statistically significant difference for any factor in this study. However tyrosol is a phenolic compound (Costantini et al., 2018) and this metabolite is known to stimulate a defensive response or signal in crustaceans where the compound is produced by symbiotic bacteria to protect crustacean embryos in *Homarus americanus* and

Palaemon macrodactylus from pathogenic marine fungi (Hay, 2009). Aside from protection, these types of signals are also a form of chemical communication found in conspecific urine, where some signals can stimulate or deter copulation (Hay, 2009). Chemical communication in decapods is important when there is a limited season for mating to occur or it is regulated by another life event, such as for only a short time when females are near moult or recently moulted *Parastacoides tasmanicus* (McLay & van den Brink, 2016). This metabolite was not identified in the previous chapter.

5.4.2 Differences between sexes

There were 19 metabolites (q values ≤0.05) that indicated a significant difference between sexes with females having higher levels in 17 metabolites. This included all the metabolites in cluster D (11), one metabolite in cluster C and five metabolites in cluster E. Cluster E metabolites also had a significant difference over time. The other two metabolites that were significantly different between sexes were found in cluster A where the levels were higher in males. These differences between sexes for all metabolites remained unchanged throughout the entire study whether the marron were on their own or with a potential mate and were the driver for the distinction between sexes illustrated in the PC-CVA (Figure *5.8*).

The females had raised levels of ***carnitines*** (includes L-carnitine***, decanoylcarnitine, propionylcarnitine*** (cluster D) and ***O-acetyl-carnitine*** (cluster E)). The synthesis of lipids in crustaceans are similar to pathways in vertebrates where the lipids accumulate in tissues as an energy source to be used during high energy events such as reproduction and moulting (Jimenez & Kinsey, 2015). Lipids are stored in the hepatopancreas in both sexes and are at their lowest levels during the moult period (Hasek & Felder, 2006). Not only are lipids important as an energy source but they are also stored by females as lipid droplets in ovaries and tissues in preparation for reproduction (Jimenez and Kinsey, 2015) as sufficient stores of lipids are necessary for reproductive success (Li et al., 2010). In crustaceans, ovarian development influences the total lipids in the ovary (Hasek & Felder, 2006). Energy reserves in the hepatopancreas are continually shifted to the ovaries via haemolymph in order to stimulate maturation of the ovaries in *C. quadricarinatus* but lipids from diet are also required to maintain the needs of the ovaries (Hasek & Felder, 2006; Li et al., 2010). As well as indicating greater energy requirements in preparation for reproduction, higher levels of carnitines can also be due to stress as when lipids are used as a source of energy this is a response to manage stress in crustaceans (Schock et al., 2013). Both processes can explain the high levels of carnitines detected in females.

The results from the previous study showed the carnitines listed were all significantly higher in female *C. tenuimanus* where they were likely preparing for reproduction and when *C. cainii* were preparing to moult. Other metabolites detected that are important to carnitine synthesis and metabolism were lysine (detected in Chapter 4) in significantly higher levels in *C. cainii*; ***methionine***, detected in Chapter 4 and significantly higher in *C. cainii* males; and then in the present study, ***acetyl–L–methionine*** was significantly higher in *C. tenuimanus* males; and trimethyl-L-lysine, a carnitine precursor was detected in Chapter 4 with significantly higher levels in both sexes of *C. tenuimanus* over *C. cainii*. In mammals ***L-carnitine*** (cluster D) can be synthesised from lysine and methionine but is also important be included in dietary intake so it is often added to feed as it has a positive effect on growth performance (Costantini et al., 2018).

Higher levels of ***kynurenine*** and ***kynurenic acid*** (cluster D), which are both part of the tryptophan pathway, are indicative of reduced metabolic rate, which could be a result of the demand for high energy metabolites for reproduction. In mammals, the kynurenine pathway is the main route for tryptophan degradation (Wishart et al., 2018) and it is a metabolic pathway leading to the production of nicotinamide adenine dinucleotide (NAD^+) from tryptophan (Wishart et al., 2018). Kynurenine is a neuroprotectant producing vitamins or cofactors such as niacin (as ***nicotinic acid***), ***nicotinamide*** and derivatives nicotinamide adenine dinucleotide (NAD+, detected in Chapter 4), and nicotinamide adenine dinucleotide phosphate (NADP), all of which participate in several energy metabolism pathways (Parthasarathy et al., 2018). Kynurenic acid is formed enzymatically from kynurenine and can be found in the intestine of mammals (Turski et al., 2013); it is an endogenous antagonist for the receptors of some stimulating amino acids such as glutamate in the central nervous system of vertebrates and invertebrates (Janecki & Rakusa-Suszczewski, 2004). The effect of this is a reduction in metabolism. Both kynurenine and kynurenic acid were significantly different between sexes, higher in female marron with metabolite levels fairly constant across all time points (as individuals and paired) ($p<0.01$; $q<0.01$). In the previous experiment, kynurenine was higher in both sexes of *C. tenuimanus* than *C. cainii* and kynurenic acid was significantly higher in *C. tenuimanus* females than males, as well as smooth females. Higher levels of kynurenic acid were found in female marron in both this chapter and the previous chapter.

Higher levels of metabolites such as ***inosine*** (cluster D), provide evidence that female *C. tenuimanus* displayed higher stress levels. Inosine is a purine nucleoside identified as a stress biomarker stress in the shrimp *Litopenaeus vannamei* (Schock et al., 2013) and it relates to adenosine levels which have been shown to increase cardiac activity in *Homarus americanus* (Stegen & Grieshaber, 2001). Increased stress was likely caused by the close presence of males as well as preparing for reproduction. Inosine is metabolised from adenosine in the haemolymph of *Homarus americanus* (Stegen & Grieshaber, 2001), and is further broken down into uric acid which is then excreted. Inosine is a better stress biomarker than adenosine or uric acid as inosine remains in the haemolymph longer than adenosine and uric acid is the end product of several pathways.

Collectively the differences between the sexes suggest that the females were experiencing general stress (as evidenced by higher inosine levels), which can be caused by the presence of another animal in the environment (males) as well as the demands of reproduction. Not only is the reproductive process exhausting but can also be dangerous for females if males are overly aggressive during mating (Huxley, 1906). This is also supported by higher levels of carnitines in the haemolymph, which suggests they are utilising stored lipids; this will happen both in response to stress and for reproductive processes. Furthermore, elevated kynurenine and kynurenic acid indicate females were slowing their metabolism. All of these responses (reduced metabolism, use of lipid stores, elevated stress) could indicate females were (a) spending less time foraging due to the presence of the male, (b) developing eggs, (c) preparing to hide while they care for the eggs after mating, and the compounds identified provide useful biomarkers of these processes.

5.4.3 Response to presence of a mate

A response to presence of a mate was indicated where changes to specific metabolites were not related to stress/immune function and the metabolites were likely to influence reproduction. Metabolite levels between sexes were different when they were kept as individuals but then changed when put together with a potential mate and remained unchanged for the remaining time points. This response describes the metabolites in cluster E, and in particular the four males that were identified in the PC-CVA that were different as individuals. As well as indicating a response to presence of a mate in cluster E, these metabolites also indicate a semi-permanent response to disturbance as mentioned earlier.

Some of the metabolites identified indicate a response by the male marron to female presence, suggesting a response to presence of a mate, as the metabolites have the potential for activation of pathways to release gonad stimulating hormone (tryptophan, serotonin, dopamine) or melatonin (tryptophan stimulation of reproductive cycle). Also, the increased activity in nervous system from tyrosine and glutamine (and increase in neurotransmitters) indicates perhaps an increase in communication between the sexes when they are housed together.

Tryptophan (Cluster E) showed a dramatic increase in males after being placed with a female. As an essential amino acid, tryptophan must be obtained through diet and it is one of the least abundant amino acids (Roager & Licht, 2018). Tryptophan is a precursor to the hormone melatonin (detected and discussed in Chapter 4) and the neurotransmitter serotonin (also known as 5-hydroxytryptamine or 5-HT, not detected) (Parthasarathy et al., 2018). Tryptophan was detected in the previous study (Chapter 4) but there were no significant differences in tryptophan levels between species or sex. In the previous study all animals were kept isolated, indicating that the presence of a potential mate does not necessarily cause a differentiation in tryptophan concentrations. However, the current experiment took place a little later in the reproductive season which could explain why we found significantly lower levels in males at day 0, and they converged to similar levels as females after being introduced to the females.

Serotonin and dopamine are biogenic amines derived from tryptophan that can act as neurotransmitters in crustaceans. Serotonin can play a role in determining mating behaviour in *Homarus americanus* (Kulkarni & Fingerman, 1992; Nagaraju, 2011), promotes ovarian maturation in crustaceans (Reddy, 2019), stimulates the release of gonad-stimulating hormone in females *Procambarus clarkii* (Sarojini et al., 1995), and in males indirectly releases gonad-stimulating-factor, which results in the initiation of testicular development (Sarojini et al. 1993; Nagaruju, 2011). Serotonin was not detected in this study but as a neurotransmitter it may not normally be present in the haemolymph in crayfish. The presence of tryptophan could therefore indicate serotonin production for the release of gonad-stimulating hormone which in males may be in response to the presence of a female, whereas females appear to produce this independently of the presence of males. Dopamine was not detected in the haemolymph this time, but some dopamine metabolites were found in the haemolymph previously (Chapter 4) and were significantly different between species (3-O-methyldopa and phenylalanine), had an interaction between sex and species (homovanillic acid), or were not significantly different (L-dopa – Chapter 4).

Another tryptophan metabolite that is an intermediate of the kynurenine pathway, 3-hydroxykynurenine, has a role in inhibiting moulting in *Callinectus sapidus* (blue crab) and *Procambarus clarkii* (Naya et al., 1989). It is an ecdysone biosynthesis inhibitor that is secreted into the haemolymph from the X-organ sinus gland (Naya et al., 1989). Unlike some crustaceans that will mate while moulting, marron, and other parastacids in general do not mate when moulting, as they are in an inter-moult phase (Burton and Mitchell, 1987). However, male marron do moult soon after mating as observed in our laboratory study and described in Chapter 2. 3-Hydroxykynurenine was not identified in this study as there were no matches with our data and the databases, mzCloud online or the in-house library.

Glutamine (cluster E), an essential amino acid, is a precursor for the neurotransmitters glutamate and gamma-aminobutyric acid (GABA), an inhibitory neurotransmitter (Wishart et al., 2018). Both glutamine and GABA were present in higher levels in female *C. tenuimanus* in Chapter 4. Glutamate (glutamic acid), an excitatory neurotransmitter, is the primary neurotransmitter in arthropods (Smarandache-Wellmann, 2016) (detected in Chapter 4), and it is also known to increase the metabolic rate of some invertebrates (*Abyssorchomene plebs*, an Antarctic amphipod) where glutamic acid becomes a source of energy (Janecki & Rakusa-Suszczewski, 2004). Another metabolite ***tyrosine*** (cluster E), which is part of the signal transduction process, is naturally produced in the body of an organism from the amino acid phenylalanine (Parthasarathy et al., 2018). Some of the secondary metabolites derived from L-tyrosine are neurotransmitters and related compounds (L-dopa identified in Chapter 4; adrenaline and noradrenaline) and animal pigments (Parthasarathy et al., 2018), all of which are important for communication and as a response to the presence of a potential mate.

5.4.4 General overview

Conducted over a five-week experimental period, this study validated and added to the baseline metabolome for *C. tenuimanus* described in the previous chapter. Using untargeted LC-MS metabolomics analysis a variety of compounds and, from these, biochemical pathways were detected in marron haemolymph. Furthermore, we detected differences between sexes and changes in the marron metabolome in response to changing environmental conditions. We verified that the shifts detected in the marron haemolymph could indicate responses to disturbance, whether transient or semi-permanent, or a reproductive response. The method used could be adapted to test a range of

different conditions potentially affecting an organism and highlight stressors from within its environment.

The clustering analysis identified patterns where significant metabolites within each cluster may have been performing different biological functions but were responding in a similar fashion. Cluster A metabolites were responsible for immune functions and the synthesis of antioxidants (indoles, glutathione), cluster B contained organic acids essential for energy metabolism (citric acid, pantothenic acid, and uridine 5'-monophosphate), cluster C had metabolites necessary for osmoregulation, excretion and some communication (betaine, adenosine, uric acid, and tyrosol), cluster D included lipids for energy storage and maintenance in times of stress, and cluster E was comprised of neurotransmitters and other communication metabolites.

The experiment took place during the marron breeding season, and other *C. tenuimanus* mated in the aquarium room at this time. Reproduction is a high energy process, particularly for the females with ongoing care of eggs and larvae after the mating event; however, the females had fewer changes in metabolite levels than the males, perhaps because they had already prepared for reproduction in anticipation of mating (as described in Chapter 2). Male marron on the other hand underwent a sudden shift in their metabolome (specifically the metabolites in cluster E) after being placed with a potential mate, as shown by the PC-CVA. It seems likely that mating would have occurred between these pairs if the marron were not frequently disturbed for collection of haemolymph because marron in other aquaria not part of this experiment did mate.

Whilst collecting haemolymph creates a stress and thus potentially skews the data, haemolymph comprises approximately 25-30% of the biomass of large crustaceans such as crayfish (Leland & Furse, 2012) and the total volume of haemolymph collected (1200uL weekly) should have been rapidly replaced or adjusted through biological processes (Greco et al., 1986; Leland & Furse, 2012). The animals in the trial appeared to fare well as they fed and maintained weight during the 5-week experimental period. According to the Nernst equation of the half-cell reduction potential (E_{hc}) of GSSG/2GSH which was calculated from the metabolite peak areas, the extremely low values indicate that the marron in the experiment were not under any oxidative stress, which is important for maintaining animals in captivity. This metabolite is something that could be further monitored as a biomarker. As well as using glutathione to indicate oxidative stress (Bone et al., 2015), alanine,

glutamate, acetoacetate, succinate and trehalose were found to indicate oxygen stress in laboratory studies (Izral et al., 2018). Of the listed compounds, only glutamate was detected in our study, but the other metabolites may be detectable with other LC-MS columns for future studies.

It was suggested that the amino acids glutamine, isoleucine, leucine, lysine, valine and betaine in tail muscle of the North American freshwater crayfish *Procambarus clarkii* could diagnose stress when food was limited (Izral et al., 2018). In chapter 5, glutamine, betaine and some forms of lysine were identified and changed over time. If this change was due to food stress it may relate to differences in feeding behaviour when males and females are together, or it may represent responses to stress more generally. Therefore, these too warrant consideration as useful biomarkers.

Conclusion

Metabolomic profiling provides a broad sweep of the metabolic state of an organism and how it reacts to its environment. There are thousands of metabolites in any organism with varying degrees of importance and influence over metabolism, defence (in an antibiotic sense), inter- and extracellular signalling. Using LC-MS to create an untargeted metabolomics profile for marron is novel as comparable studies in freshwater crayfish have focused on specific metabolites and whilst some of the metabolites detected in this study have been found in other similar taxa, many have not.

By analysing the metabolome of pairs of *C. tenuimanus* over a 5-week period and detecting differences in metabolites between the sexes, over time, and due to sex and time interactions, potential biomarkers have been identified. This represents a successful attempt at employing untargeted metabolomics to detect changes between sexes of marron when they are placed in breeding pairs and therefore supports sub-hypothesis 4. As the metabolome provides biomarkers that indicate physiological states, we can now generate hypotheses about how environmental conditions may affect the metabolome and investigate this via targeted metabolites. For example, if a researcher wanted to investigate the effect of stocking density or temperature on marron or another *Cherax* species, we now know which metabolites they would expect to find, and which metabolites would be likely to change.

It is achievable to establish cause and effect in a controlled laboratory setting for metabolomic experiments as a laboratory lacks the ecological complexity of *in situ* field experiments in the natural environment. This laboratory study indicated and agrees with other recent and similar studies that the crayfish metabolome is detectable and measurable, and it is capable of potentially diagnosing specific environmental stressors as it is fast responding and sensitive to subtle changes in the organism and its aquatic environment. Understanding the physiology of an animal and having the ability to measure metabolic stress and changes related to reproduction that are otherwise not detectable has the potential to greatly improve *ex situ* conservation practices.

6 Study synthesis

Despite the efforts and knowledge of experienced scientists and aquaculturists, the critically endangered *Cherax tenuimanus* has failed to breed well in captivity. Understanding why is complex, as low fecundity and low breeding success can be linked to a wide variety of environmental or physiological causes (Snyder et al., 1996). These reasons can include poor or inadequate diet, inappropriate housing conditions (e.g. light, temperature, space), various sources of disturbance, pathogens, behavioural incompatibility or changes in natural behaviours, and/or genetic issues such as inbreeding depression, genetic drift and genetic adaptations (Schulte-Hostedde et al., 2015; Snyder et al., 1996). Isolating causes and distinguishing between the impact of these various factors on the regulation of reproduction requires a detailed understanding of the internal and external factors involved (Reddy, 2019), creating a significant challenge for captive breeding programs. For many threatened species successful captive breeding is still elusive even after years of investigations, which means that some of these species may never reach the numbers necessary to be considered self-sustaining captive populations or allow for their reintroduction to the wild (Snyder et al., 1996).

Improving the outcomes of captive breeding programs is difficult, as studying endangered species creates a challenge for researchers; the limited availability of specimens results in working with low sample numbers or few replicates, which affects the reliability of data. Furthermore, *ex situ* conservation is expensive, and it is important to consider the net benefit to conservation and society of money spent. For these reasons, any tools that can quickly and reliably contribute to the knowledge of the complex processes involved in reproduction and captive breeding could be incredibly valuable, which is why modern technologies provide opportunities for time and cost-effective conservation science. Accordingly, in this study we sought to test the hypothesis that the metabolome of *C. tenuimanus* provides bioindicators related to reproduction and stress that will ultimately enhance conservation through captive breeding.

6.1 Reproductive success factors - hairy marron

Only recently recognised as separate species, the highly restricted *C. tenuimanus* and its widespread sister-species *C. cainii* have experienced introgressive hybridisation, with hybrids producing fertile offspring (Austin & Ryan, 2002; Duffy et al., 2014; Guildea et al., 2015); hence, it was assumed that they require similar conditions for successful breeding. *Cherax cainii* has been a popular aquaculture species with a history of almost 50 years of captive breeding in Western Australia (Huner, 1994; Lawrence, 2007; Morrissy et al., 1990), and requirements for captive breeding (such as food, water quality, environmental parameters, stocking densities, etc.) are well-known. However, in captive breeding situations (i.e. in ponds) the reproductive success of *C. tenuimanus* has been much lower compared to *C. cainii*. *Cherax tenuimanus* mate and produce offspring at the PFRC, but numbers of eggs, females with eggs, females with larvae and juveniles are often very low (compared to *C. cainii*), and it is not known whether *C. tenuimanus* are relatively poor spawners with lower fecundity naturally or if there are issues with the captive breeding system. It is clear from this comparison that assumptions about the transferability of methods from one species to another may not be appropriate, despite the phylogenetic and morphological similarity of the species.

What is known about *C. tenuimanus* poses one significant question for their *in situ* conservation, and one for their *ex situ*, specifically:

1) If *C. tenuimanus* numbers are low in the wild primarily due to displacement by *C. cainii* as has been proposed, what are the mechanisms allowing this to occur, and

2) Why has there been so little recruitment success for *C. tenuimanus* in captive breeding programs when the same methods work for *C. cainii*?

This study sought to directly address the second question, however, in doing so it has indirectly contributed to the first. This investigation confirmed in the lab what was suspected but poorly documented; *C. cainii* and *C. tenuimanus* have different breeding periods, with perhaps some overlap. For instance, data collected from the Margaret River populations suggest that *C. tenuimanus* breed later in the spring than *C. cainii* and that *C. tenuimanus* are still mating in November (J. Bunn unpub. data). In our study, *C. cainii* were recorded mating earlier than *C. tenuimanus* with spawning occurring at the onset of increasing water temperature and day length in August and into September, and *C. tenuimanus* breeding in October and into November. These observations were supported by the

metabolomic data as we were able to differentiate between the metabolomic profiles for the two species, which appears to be (at least in part) linked to different timing of life events such as reproduction and moulting. The earlier release of juvenile *C. cainii* would provide them with earlier access to resources such as food and habitat and therefore a competitive size advantage, giving them the ability to outcompete their congeneric and thus affecting annual recruitment of *C. tenuimanus* and possibly explaining the species displacement (Guildea et al., 2015).

Hybridisation between these congeneric species has been confirmed (Austin & Ryan, 2002; Bunn et al., 2008; Guildea et al., 2015; Kennington et al., 2014), but it has been suggested that there are partial reproductive barriers present as levels of introgression were lower than would be predicted under random mating (Guildea et al., 2015). The partial reproductive barriers could be explained by the difference in timing of breeding seasons as indicated in this study. Where hybridisation does occur, it has also been suggested that *C. cainii* males may be larger, due to earlier breeding times and access to resources, than *C. tenuimanus*, therefore preventing the latter access to potential mates and furthering the decline of the wild *C. tenuimanus* population (Guildea et al., 2015). However, this study suggests another mechanism may be operating that requires testing. It is suggested that when female *C. cainii* are physically ready to mate they chemically trigger a reproductive response in the males of both species. The larger, more aggressive, and more abundant *C. cainii* males will generally outcompete *C. tenuimanus* males for access to the available *C. cainii* females; this issue would be exacerbated if the *C. tenuimanus* had not yet completed their own intrinsic preparation when the cue came from the females. For the period where the reproductive receptiveness of both species' females overlap, *C. cainii* will continue to interfere with attempts by the male *C. tenuimanus* to mate and some hybrids would be produced. Particularly if *C. tenuimanus* densities (in the wild) are so low that finding females is problematic, this pseudo-competition may also be taking place indirectly, resulting in fewer conspecific encounters and matings between *C. tenuimanus* individuals (Guildea et al., 2015). By the time the *C. cainii* females are finished their reproductive cycle, and *C. tenuimanus* females have exclusive access to the males, the males may have reached the end of their reproductive cycle.

Evidence for the scenario proposed above can be found in the results of Chapter 5, where a difference was found in the metabolome of *C. tenuimanus* males held individually compared to those with a mate. More specifically, female *C. tenuimanus* maintained constant levels for many metabolites regardless of having a partner where male *C. tenuimanus* profiles changed in response to exposure to a female, hence the suggestion that the presence of a reproductively active female may be required

for the male to become ready to mate. It is hypothesised that *C. cainii* females are cued for final reproductive preparations by environmental conditions as previously described (for females of both species), and once reproductively ready the females provide a chemical signal or chemical-plus-visual signals (Acquistapace et al., 2002) that trigger males to undergo their last stages in preparing to mate. This would also explain why marron can mate at different points in the breeding season as even though some *C. tenuimanus* did mate in aquaria (Chapter 2), those males kept as individuals (Chapter 5) did not seem ready until placed with a female, suggesting some plasticity in their preparation (i.e. the males require a signal).

Another factor limiting the reproductive success of *C. tenuimanus* is that female crayfish in the family Parastacidae appear to mate only once per season (i.e. accept a single spermatophore) (McLay & van den Brink, 2016) as they extrude eggs within hours of mating (as observed in Chapter 2). In contrast, species in other families of freshwater crayfish use a strategy referred to as multiple mating, such as Astacidae (*Austropotamobius italicus* (Galeotti et al., 2007, 2012)) and Cambaridae (*Orconectes placidus*, *Procambarus clarkii* (McLay & van den Brink, 2016)), where females will receive spermatophores from multiple males and extrude eggs days or weeks after mating. This polyandrous strategy does pose a risk of injury to females during mating (Galeotti et al., 2007; Huxley, 1906), but also the benefits in terms of egg fertilisation success with the amount and quality of sperm released (Galeotti et al., 2007). It is assumed, but unconfirmed, that male *Cherax* can have multiple ejaculations (potentially of varying quantity/quality) over a season with potential mates, as seen in *Austropotamobius italicus* (Galeotti et al., 2012; McLay & van den Brink, 2016; Rubolini et al., 2006) and shown in simulation experiments with *Cherax destructor* (Jerry, 2001). This highlights two problems for *C. tenuimanus;* firstly, if there is only one chance for a *C. tenuimanus* female to mate and if *C. cainii* males have an advantage and can mate repeatedly they are likely to get to the her first. The other depends on the quantity and quality (viability) of sperm released while mating, as if a *C. tenuimanus* male has low quality or volume of sperm (which may be connected to being triggered too early by a *C. cainii* female) this could also impact the reproductive success of *C. tenuimanus*.

Due to the problems with *in situ* conservation it was hoped that this study could assist *ex situ* conservation. Specifically, by testing the overall hypothesis (that metabolomics would identify potential biomarkers related to reproduction and stress in two congeneric freshwater crayfish species, which could lead to improved captive breeding success), it was hoped that this study would elucidate potential reasons as to why there has been so little recruitment success for *C. tenuimanus* with captive

breeding when the same methods are employed for *C. cainii*. Captive breeding is often conducted in ponds or large tanks but using glass aquaria in this study provided the opportunity to clearly observe marron and their behaviours in a controlled environment. To further reduce variables, marron were housed both individually and in pairs with a potential mate, whereas most other studies (which focus on *C. cainii*) use higher stocking densities and different ratios of male to females (Lawrence, 2007; Luckens, 2015).

During this project it was confirmed that *C. tenuimanus* will mate while in captivity, even in small aquaria in a laboratory environment, which was a highlight for the initial part of the study. Sub-hypothesis 1, that marron housed as male/female pairs in glass aquaria with controlled lighting and temperature will undertake mating, is therefore accepted. This artificial environment mirrored important features of the natural environment (e.g. water temperature, photoperiod), however many parameters such as turbidity, substrate, groundwater/surface water interactions and other biological and physical factors could not be replicated accurately. It appears that the most important environmental cues were met as the marron mated, a spermatophore was placed on the ventral surface of the female, and eggs were produced, extruded and attached to the pleopods. In this study, seven out of ten pairs of *C. tenuimanus* in one of the trials mated (Chapter 2), and although they were roughly size matched, this suggests that mate selection is not strong in these crayfish which is a difficult concept to investigate in tanks of many individuals.

Through the entire study, two different temperature regimes could be maintained at the same time in the laboratory, which allowed for seasonal environmental conditions required for breeding (i.e. increasing day length and water temperature) to be artificially altered. As described in Chapter 2, *C. cainii* were breeding in the ponds at PFRC in early September and four of the pairs brought to ECU spawned immediately (while the water was at 15^{o}C). *Cherax tenuimanus* mated in both temperature treatments at approximately 15-20^{o}C with the optimal temperature for mating occurring between 18-20^{o}C, and all mating events occurring in October and November. This observation also supports the differences in timing of breeding seasons for the two congeneric species as they may be waiting for different environmental conditions. Observations (both in ponds at PFRC and in the lab at ECU) show that *C. cainii* were mating prior to the natural September equinox (12hr light:12hr dark) whereas *C. tenuimanus* mated afterwards.

In this study, females incubated the eggs for periods from only a few days up to three weeks, which is still well-short of the usual time for eggs to hatch into larvae (around four weeks) and the total incubation period of 12-16 weeks from eggs to juvenile release (Morrissy, 1970). Having berried females during this project is a success; however, further study is needed to determine why the eggs did not develop during this period and no viable young were produced by either species in this environment. This is an example of the type of question that could be investigated through metabolomics. For example, it may be hypothesised that there was an issue with the diet of females; specifically, that there is a relationship between quantity of lipids provided at specific times of year and egg quality. Overwintering female marron require a better diet to improve lipid stores for egg production and good quality eggs (yolk), energy reserves, and to maintain health throughout the breeding season, as the opportunity for food is less available once they have mated and are incubating eggs or young (Gutiérrez-Yurrita & Montes, 1999). However, preparation for reproduction occurs much earlier in the year, with the reproductive cycle for female marron (*C. cainii*) beginning in January (summer) cued by decreasing daylength (Beatty et al., 2016; Huner, 1994) with maturation of oocytes complete by July (winter) (Beatty et al., 2003; Huner, 1994). Nutrition is important throughout this entire preparation for development period as well as directly prior to mating, and Huner (1994) states that a female will reabsorb her eggs in the absence of adequate nutrition and therefore not mate. It is possible that in captivity (ponds or tanks) *C. tenuimanus* were not getting the right nutrition in the pre-breeding phase to sustain them through reproduction. Comparisons between lipid-related biomarkers in the metabolome of crayfish in the Margaret River (where there may be seasonal invertebrate blooms for instance over winter and early spring when the marron are in final preparation for mating) and those in the captive breeding program could test this hypothesis.

6.2 Marron metabolomics

The identification of metabolites often occurs through lethal sampling; however, in this study, profiling was conducted using haemolymph from the crayfish extracted in a non-lethal manner and the same individuals could be sampled repeatedly over time. Equipment required for extraction of haemolymph is standardised, inexpensive and can be sourced easily, and samples can be collected (and preserved) in the field or laboratory. Preparation tubes need to be kept cool both before and after collection of haemolymph which must be a consideration if collection occurs away from the laboratory, and the fast clotting of haemolymph can be an issue; however, this can be overcome by methods such as using heparinised syringes or preloading collection tubes with a solvent to prevent clotting.

The methods for extracting haemolymph are easily replicated, and in some cases may provide quantifiable data almost immediately. As marron shifted into their breeding season the proportion of females displaying orange-tinted haemolymph increased, which is believed to be due to increasing levels of vitellogenin (Chapter 2). Vitellogenin as a glycolipoprotein has a molecular structure of up to 200 000 Da which is too large to be detected using metabolomics (molecules <1500 Da) but could be identified with other methods such as enzyme-linked immunosorbent assays (ELISA)(Sukumaran et al., 2017). This change in haemolymph colour correlated with observations that the ovipores were darker and females were "cleaner" underneath their tail (i.e. their swimmerets) due to their preening behaviour, suggesting they were preparing for egg attachment. Identifying preening and preparation for a mating event was a visual cue that a female is likely ready for the next step in the reproductive process. It was expected at this point that we would be able to identify MF in the marron as is it was hypothesized that females with mature ovaries would have the highest levels of MF as well as the more sexually aggressive males; however, this targeted approach failed.

The (non)result in this study for MF highlighted a potential issue with targeted metabolomics. Although it may have advantages (direct, inexpensive, easy to interpret), its scope is limited and if there are conditions that are not favourable to producing consistent results then the time, effort and money is wasted. Knowledge of which target compounds are found in the organism, when they are likely to be present, at what level, and whether these vary by tissue or biofluid type are all needed prior to sampling. Therefore, a targeted approach to metabolomics will usually require an exploratory search first to see if the compound or metabolite can in fact be detected and measured, or it may fail. For example, it was stated in the literature that MF has been detected in haemolymph of many crustacean species including a congeneric; however, the amounts detected in these studies varied considerably, and the difficulty of detecting MF in the haemolymph of both species of marron despite considerable time spent troubleshooting was an unexpected challenge for this project. Sub-hypothesis 2, that the reproductive hormone MF can be detected in marron haemolymph and used as a non-lethal, low stress tool to predict reproductive success (i.e. as a targeted metabolomic approach), was therefore unable to be tested. Although the methods applied in this study may not have been suitable, the idea is still sound (with the aforementioned caveats noted) and having a simple technique that could indicate reproductive readiness in crayfish would be valuable, especially to assist in species conservation.

Using LC-MS was successful for the untargeted metabolomic analyses of this study as it is sensitive enough to detect subtle changes in the metabolome that occurred in response to a variety of factors and therefore could identify biomarkers. This meant that sub-hypothesis 3 (that untargeted metabolomics can detect differences in the metabolome between sexes and species of marron) and sub-hypothesis 4 (that untargeted metabolomics can detect changes between sexes of marron when they are placed in breeding pairs) were both accepted. This study provides the first example of numerous measured metabolites changing in response to environmental conditions in a critically endangered species whilst captive breeding is attempted. Chapter 4 described 84 metabolites in the haemolymph of two *Cherax* species and Chapter 5 reported the identification of 54 metabolites in *C. tenuimanus*, 31 of which were previously identified in Chapter 4, totalling 107 annotated metabolites recognised by this study in the haemolymph of marron using LC-MS analysis. Many of the identified metabolites in the study (80% of the confirmed compounds) belong to one of the following chemical classes: amino acids or their derivatives, purine and pyrimidine nucleotides and nucleosides, organic acids and lipid-related (*Figure 6.1*). Amino acids and their derivatives contributed nearly 50% of the identified compounds and free amino acids found in crustacean haemolymph have roles in many metabolic processes and biological functions such as osmoregulation in freshwater decapods (Rahi et al., 2018), protein synthesis/catabolism, gluconeogenesis and oxidative pathways (Issartel et al., 2005).

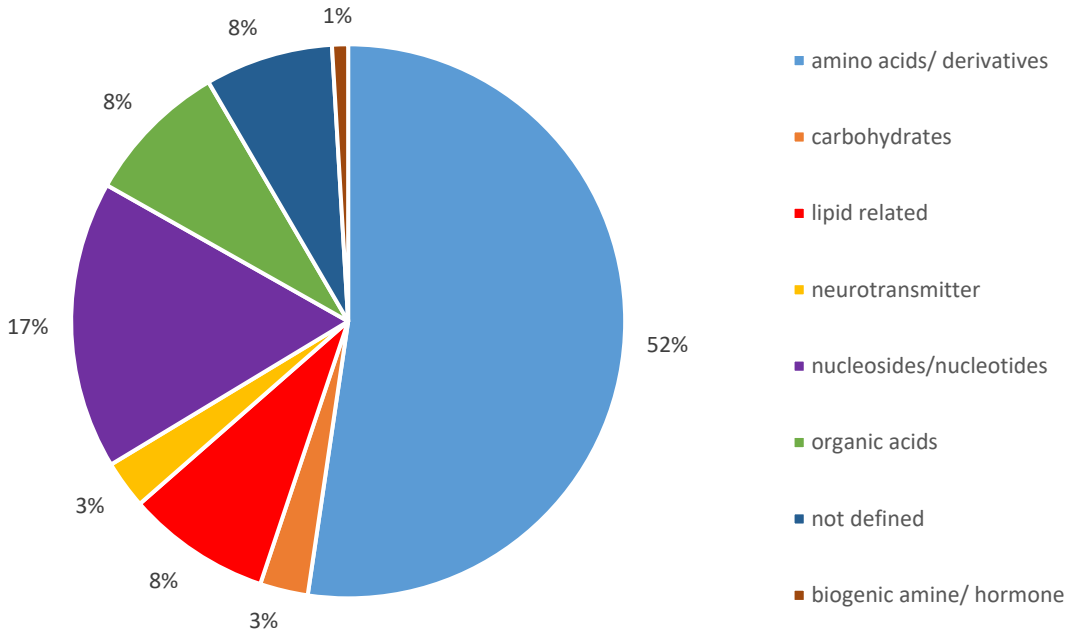


Figure 6.1. Chemical groups of annotated metabolites. Percentage based on the total number of annotated metabolites found in the haemolymph of *Cherax* sp. in this study for both Chapters 4 and 5 (n = 107). (Chapter 4 = 84 metabolites, Chapter 5 = 54 metabolites, where 31 are the same between studies).

While this method as employed in this study may not have directly answered the issue of why there is poor reproductive success in *C. tenuimanus*, this study has shown that metabolomics can provide insights into the problems encountered with captive breeding. Future studies can focus on identifying more crustacean metabolites and linking these to physiological processes, which will allow us to formulate testable hypotheses and to address these using metabolomics. For example, as marron prepare for reproduction with the maturation of oocytes, we would expect to see a change in lipid-related metabolites such as carnitines and other fatty acids. This was illustrated in both *C. tenuimanus* and *C. cainii* females in Chapter 4, where sex was the significant factor as shown by the higher levels of carnitines (lipids), and again in female *C. tenuimanus* in chapter 5. Other important fatty acids are docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) which are essential nutrients important for reproduction and ovarian maturation, fertilisation and hatching (Li et al., 2010). In Chapter 4, the levels of these two compounds were statistically higher in female *C. cainii* over all the other marron tested; this indicates that they were more reproductively mature (i.e. increased ovarian maturation) which agrees with other findings that the two species have different breeding times. These metabolites were not detected in Chapter 5 due to the application of a higher threshold for assigning compound identification, rather than not being present. Another example of useful biomarkers identified in this study are the neurotransmitter acetylcholine and the amino acid arginine, which are also linked to moulting, another highly energetic process in crustaceans (Ghanawi & Saoud, 2012; Raviv et al., 2008). Although in some decapod crustaceans mating is closely linked to moulting, in *Cherax* species a moulting crayfish will not mate (McLay & van den Brink, 2016), although the males will moult soon after the mating season. This was observed and described in Chapter 2 and the males were found to have significantly higher levels of these compounds in the metabolomic analysis.

6.3 Summary

The situation for hairy marron in the wild is dire. This research has improved our understanding of the life history and reproductive physiology of *C. tenuimanus* and the differences between it and *C. cainii*. There is now a starting point for marron metabolomics, and the *Cherax* genus more generally, which can be built upon in future studies. A potentially puzzling finding is the profound differences between *C. cainii* and *C. tenuimanus*, even though the two species are so closely related, reflected by their different reproductive success in captivity and the rapid displacement of one by the other in the Margaret River. The observations and metabolomic data collected during this study confirm a difference in timing of life cycles, including moulting, reproductive maturity and mating season between the two species, and reminds conservationists that what works for one species may not work for the other in spite of their apparent similarity.

The current *ex situ* conservation strategy for hairy marron could be extended to include an artificial incubation program. This would see eggs being collected from female marron soon after extrusion and incubated in an artificial system to (hopefully) improve the success rate of eggs developing into juveniles. Metabolomic studies would be of significant benefit to such an approach; for example, they could be used to identify suitable brood stock, screen eggs for viability and be used to investigate the impacts of rearing conditions of juvenile development including nutrition and food preferences. It is hoped that these tools can be used to identify causes where the breeding techniques are failing and allow for implementation of techniques to improve breeding success of captive *C. tenuimanus*. Ultimately, successful captive breeding of *C. tenuimanus* will produce animals that could be used to restock the population in the permanent pools in the upper reaches of the Margaret River as well as populate a chosen Ark site (an alternate location within the Margaret River watershed that is free of *C. cainii*) (Duffy & Day, 2015). The metabolomic data obtained from this study will not only benefit the conservation of *C. tenuimanus* but can also be applied in conservation of other endangered species as well as the aquaculture industry.

Untargeted metabolomic approaches provide the opportunity to find unexpected, surprising or even novel responses in an organism to environmental stressors (Bundy et al., 2009; Lankadurai et al., 2013). Using a molecular or metabolomic approach can offer valuable information about a species rather than just using visual observation and overall measurements that do not assist with understanding the mechanism of stressors (Bundy et al., 2009). Metabolomics can help us investigate

threats to freshwater crayfish worldwide, which include climate change, habitat loss or modification, overfishing, pollution and environmental toxins, biological invasion, displacement by introduced species, hybridisation, and the spread of pathogen (Richman et al., 2015). For example, metabolomics has already been used to assess how crustaceans are affected by increased water temperatures and dissolved oxygen levels (Izral et al., 2018; Schock et al., 2013), environmental toxins such a metal contaminants and pharmaceuticals in water systems (Izral, 2016), and the spread of pathogens (which could also include bacterial infections in a captive breeding program).

This study highlights the potential to modernise conservation strategies through new technologies and the overall hypothesis that metabolomics would identify potential biomarkers related to reproduction and stress in two congeneric freshwater crayfish species is accepted (while acknowledging that there is much work still to be done). The changes detected in the metabolome of the crayfish during this study represented real-time biochemical changes within the animals and this study will facilitate further development of metabolomic approaches to conservation and captive breeding. This is an exciting area of study and although an increasing amount of research is being published about crustacean metabolomics, metabolite identification, and the roles of various metabolites, we are only scratching the surface for understanding the exact nature of the mechanisms of action for these compounds. Metabolomic approaches can help us understand responses to a range of stressors whether biotic or abiotic, naturally occurring or human induced (Bundy et al., 2009). This study demonstrated that *C. tenuimanus* did mate in captivity and although the reasons for their low fecundity when compared to their sister species remain elusive there is hope that the power of a new approach such as metabolomics can contribute to resolving the puzzle.

7 References

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Appendices

Appendix A - Chapter 4 – Supplemental material

A. 1. Metabolite classes for Chapter 4

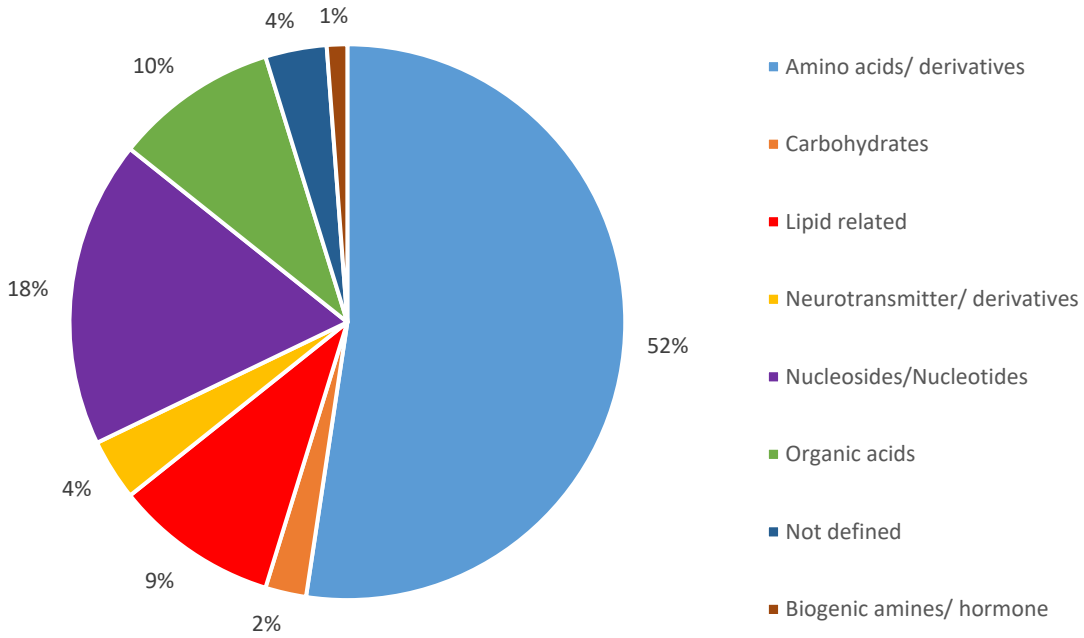


Figure A.1 Metabolite classes for Chapter 4 baseline study comparing C. cainii and C. tenuimanus. Total metabolites 88.

A. 2. HCA Heatmap annotated metabolites for *C. cainii* and *C. tenuimanus*

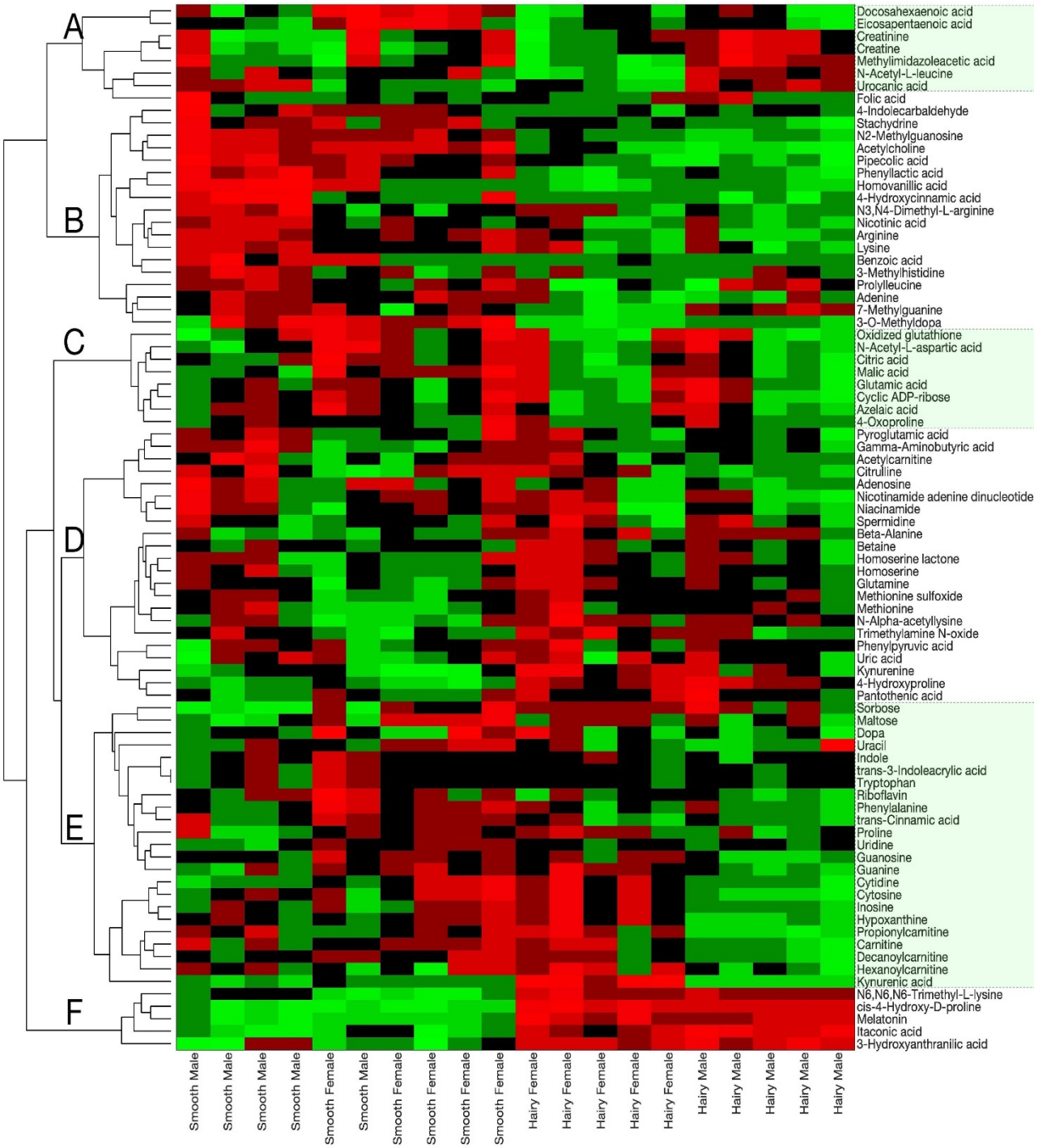


Figure A.2 HCA heatmap for annotated metabolites (Cluster A-F) for *C.cainii* and *C. tenuimanus* males and females. Significantly high metabolite levels (red) low; significantly low levels (green); no significant difference (black).

A. 3. ANOVA plots for Chapter 4

Identified metabolites from *Cherax tenuimanus* and *Cherax cainii* haemolymph, the two-way ANOVA plots below illustrate metabolite phenotypic behaviour displayed by clusters (A-F) based on hierarchical cluster analysis (*Figure 4.3*) and in the order from *Table 4.2*. Species on × axis with *C. cainii* (smooth marron) on the left and *C. tenuimanus* (hairy marron) on the right; the mean peak area of each metabolite on y axis; point values are log mean metabolite peak area ± standard error bars of 10 females (blue) and 10 males (red).

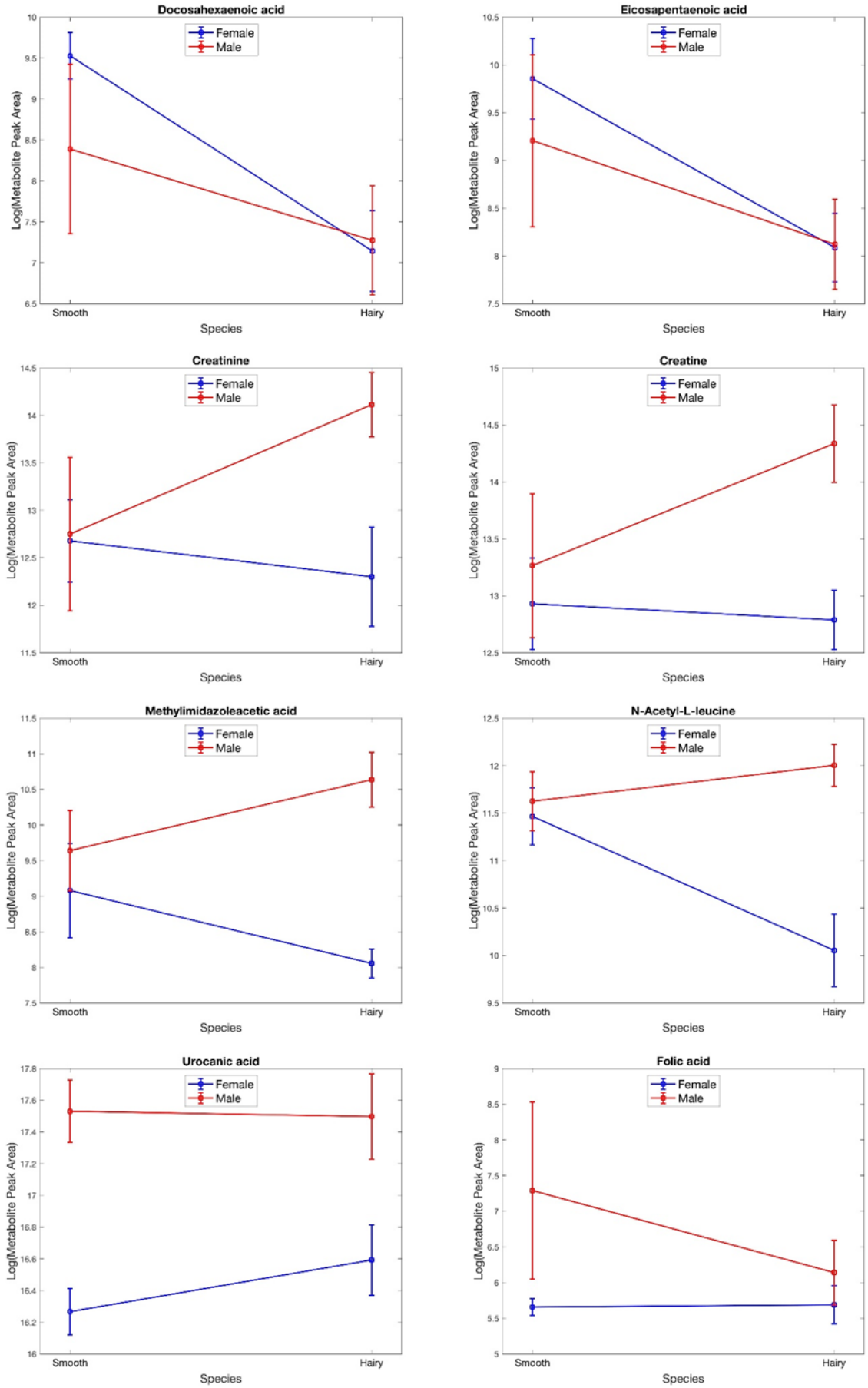
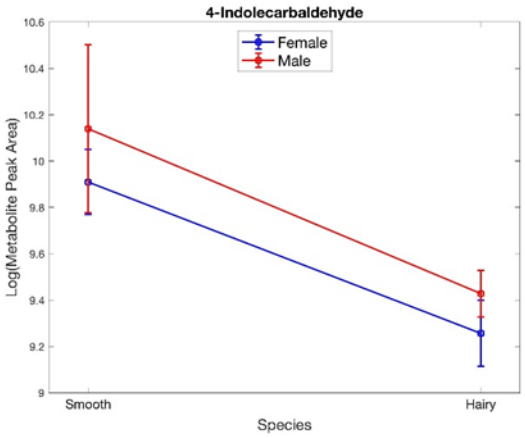
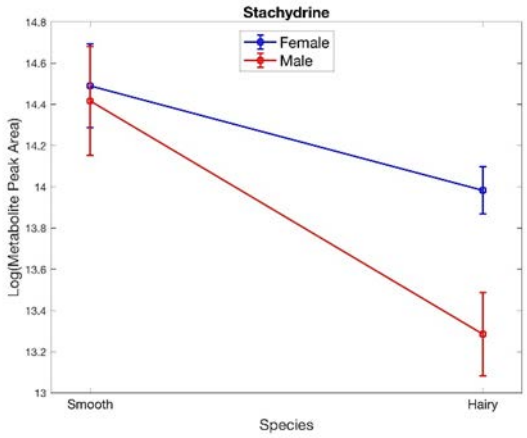
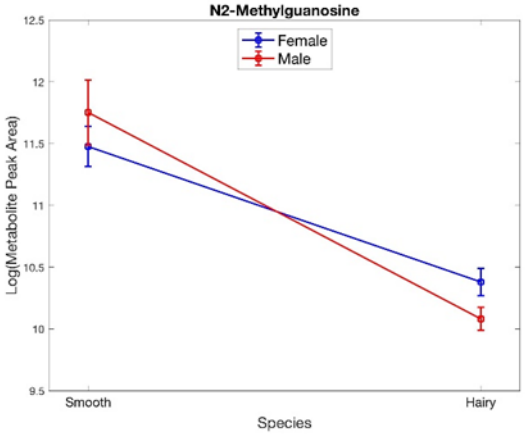
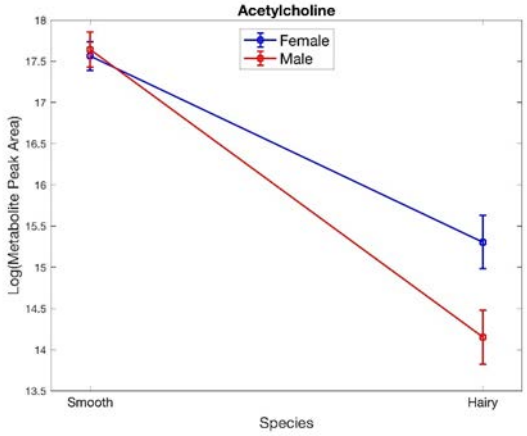


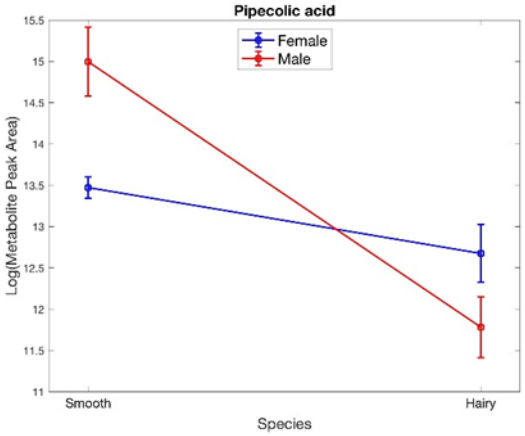
Figure A.3. Identified metabolites from *Cherax tenuimanus* and *Cherax cainii* haemolymph Cluster A (n=8).

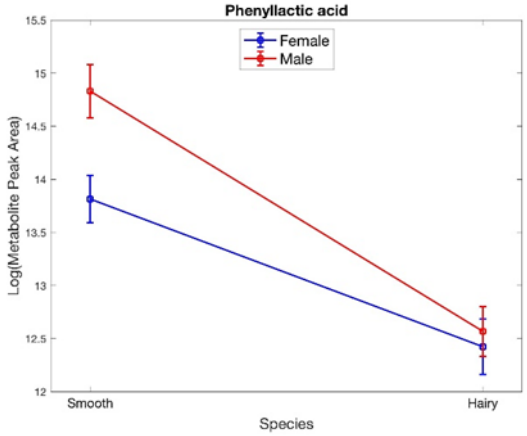

4-Indolecarbaldehyde
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species

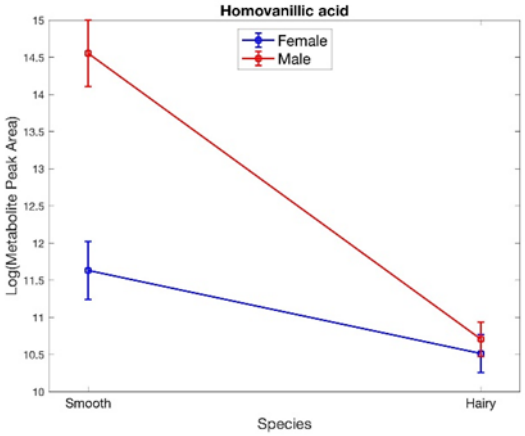

Stachydrine
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species

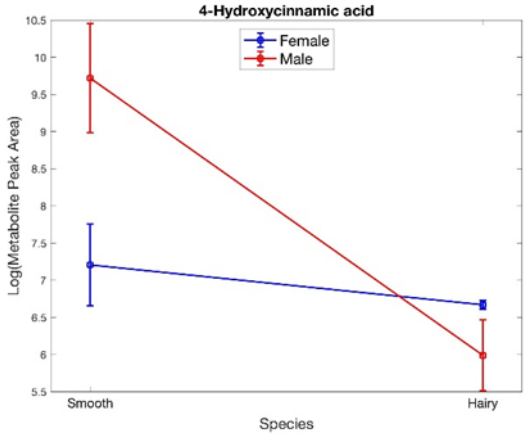

N2-Methylguanosine
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species

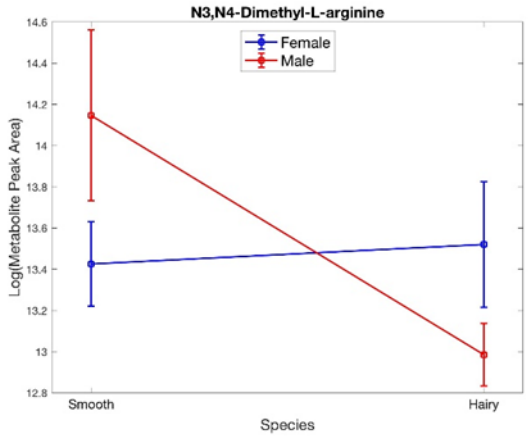

Acetylcholine
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species

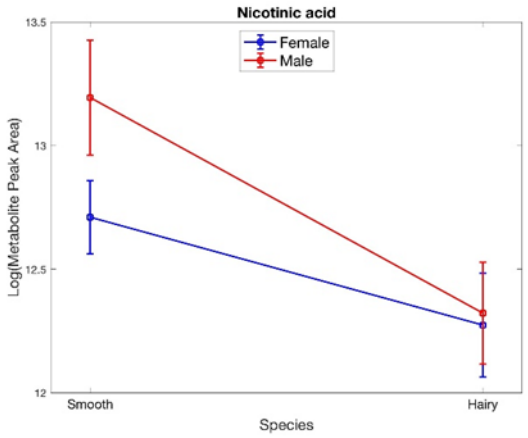

Pipecolic acid
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species

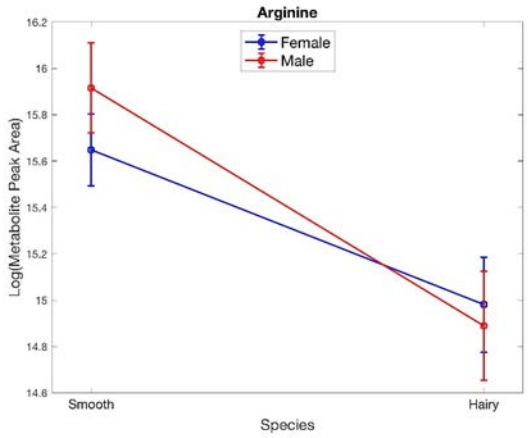

Phenyllactic acid
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species

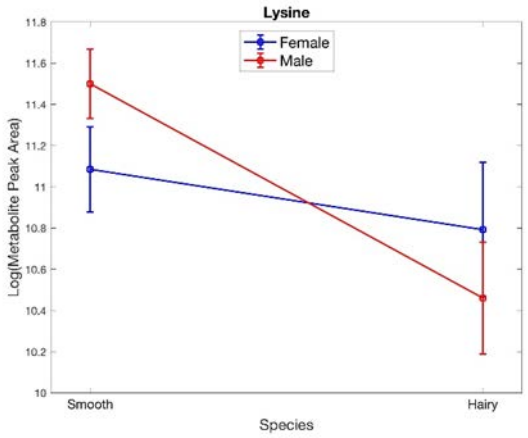

Homovanillic acid
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species

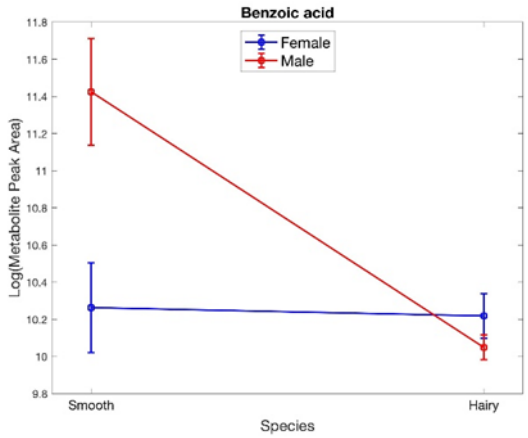

4-Hydroxycinnamic acid
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species

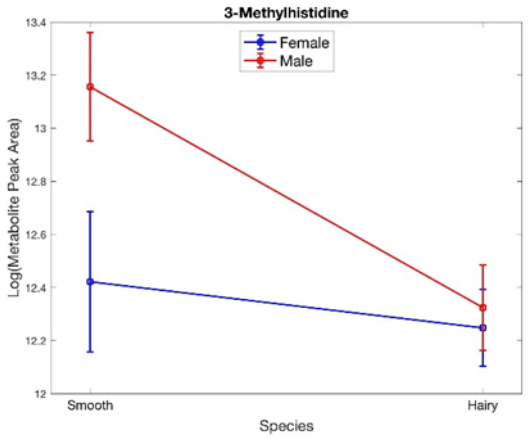

N3,N4-Dimethyl-L-arginine
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species

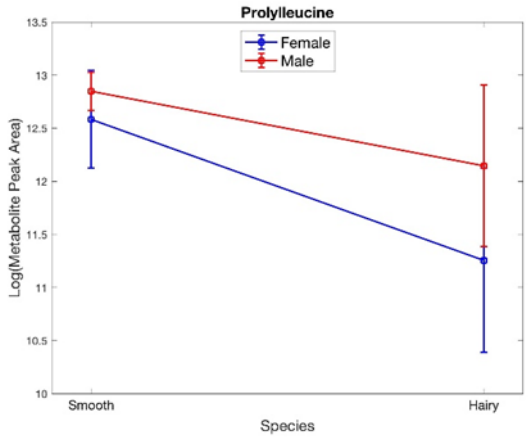

Nicotinic acid
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species

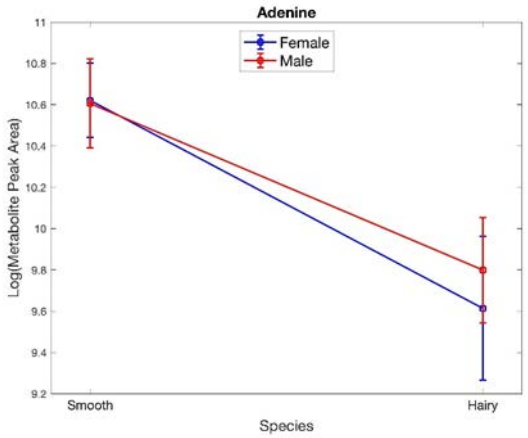

Arginine
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species


Lysine
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species


Benzoic acid
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species


3-Methylhistidine
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species


Prolylleucine
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species


Adenine
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species

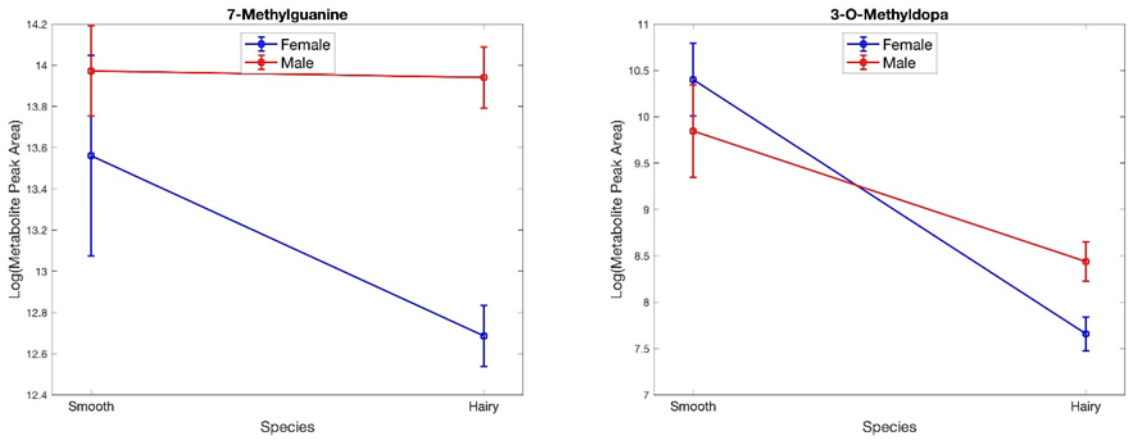


Figure A .3 Cluster B (n=18) Identified metabolites from *Cherax tenuimanus* and *Cherax cainii* haemolymph.

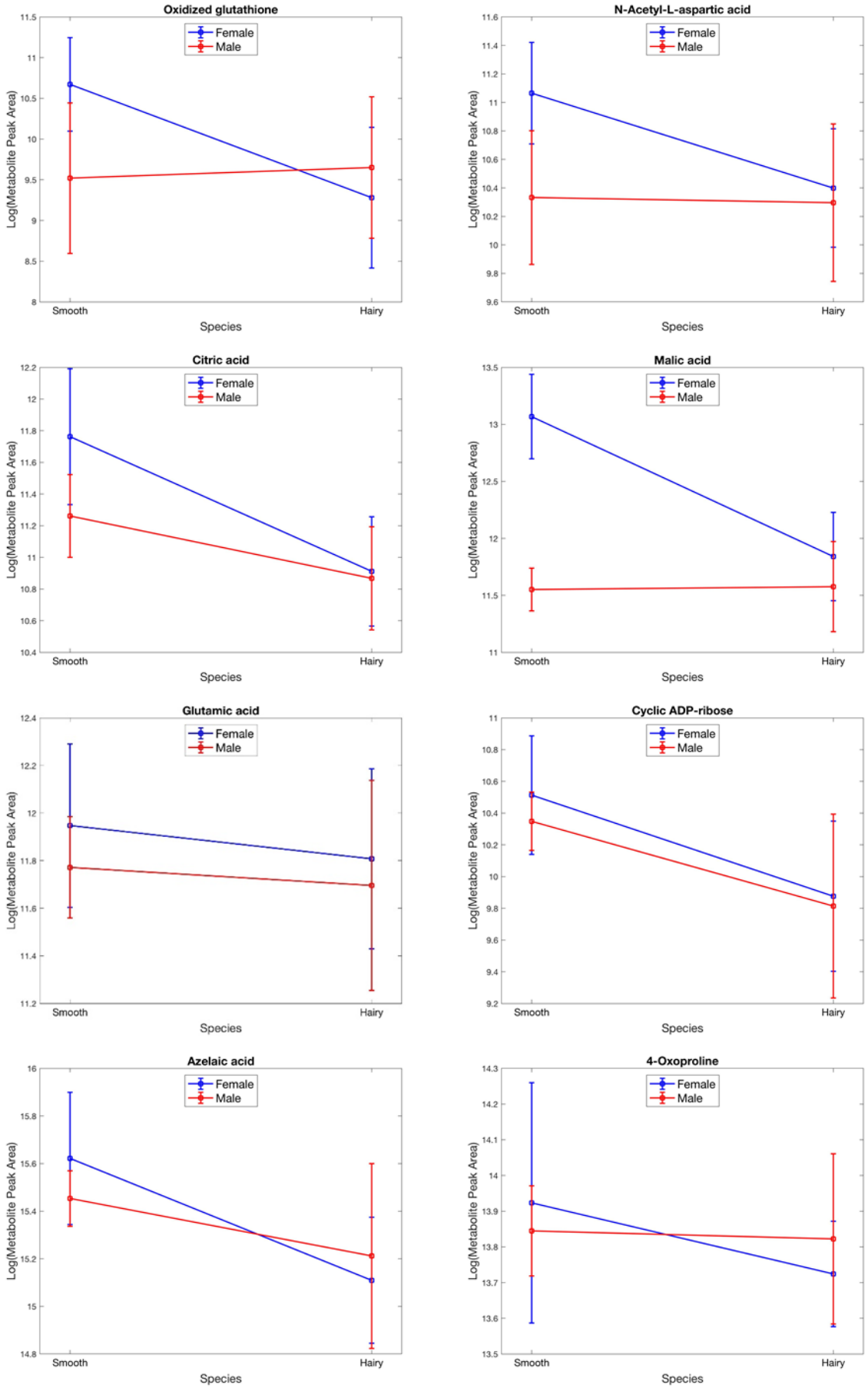
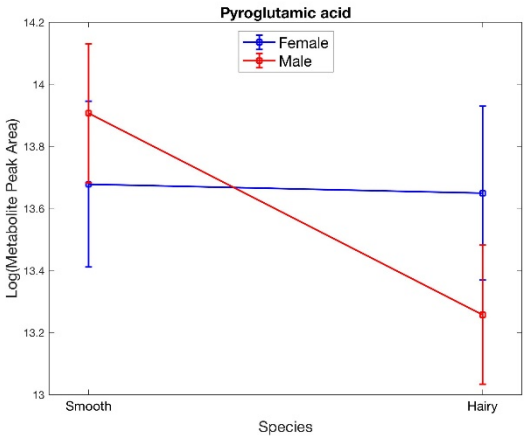
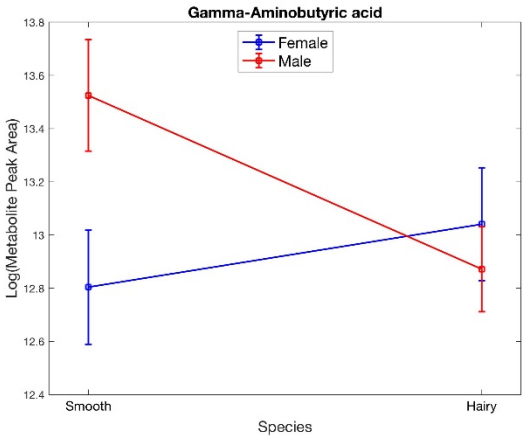
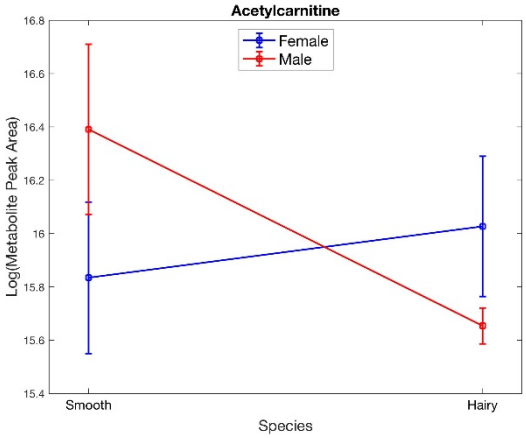
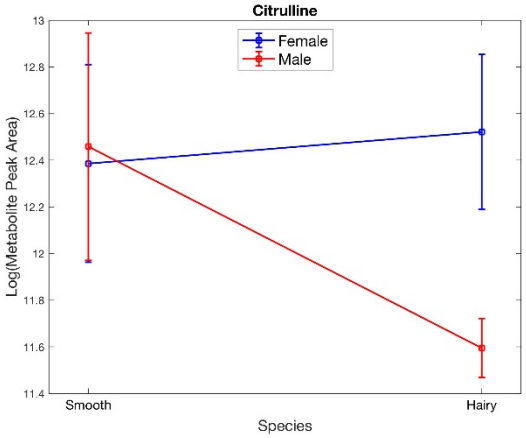


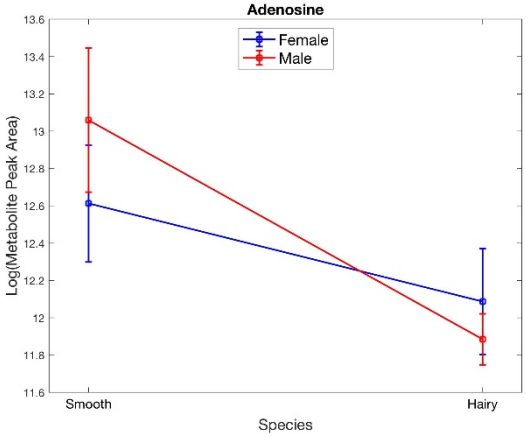
Figure A.3 Cluster C (n=8) Identified metabolites from *Cherax tenuimanus* and *Cherax cainii* haemolymph.

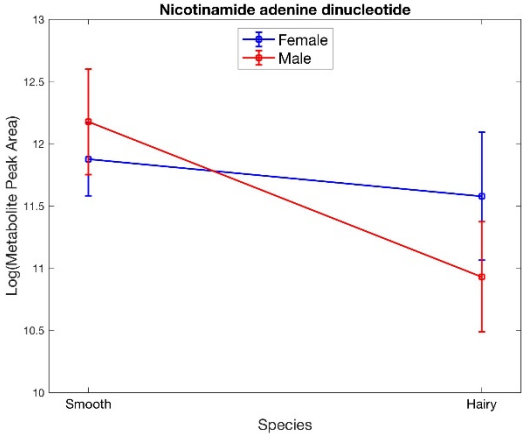

Pyroglutamic acid
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species

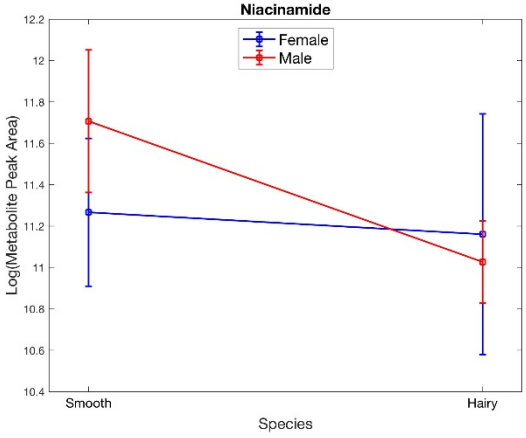

Gamma-Aminobutyric acid
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species

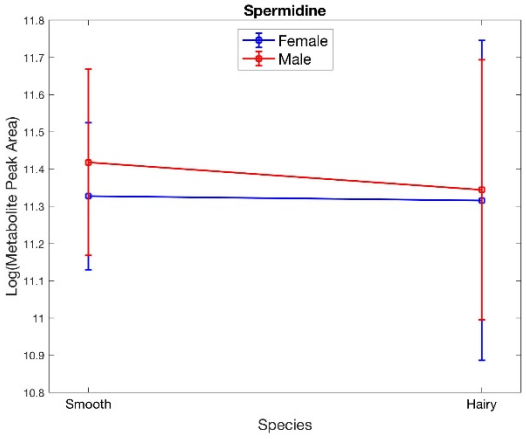

Acetylcarnitine
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species

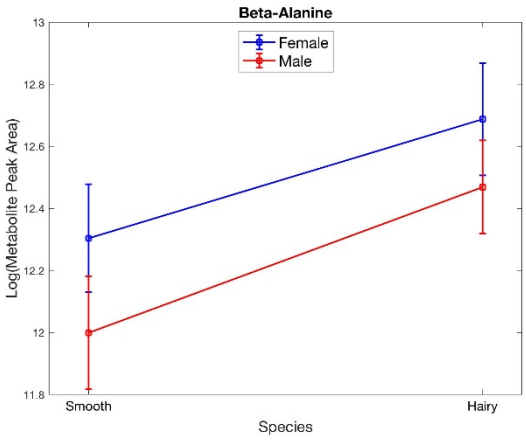

Citrulline
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species

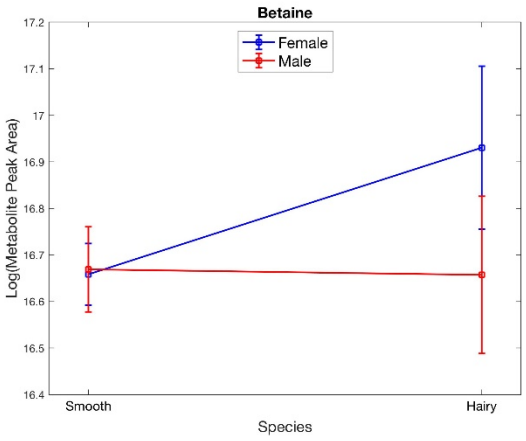

Adenosine
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species

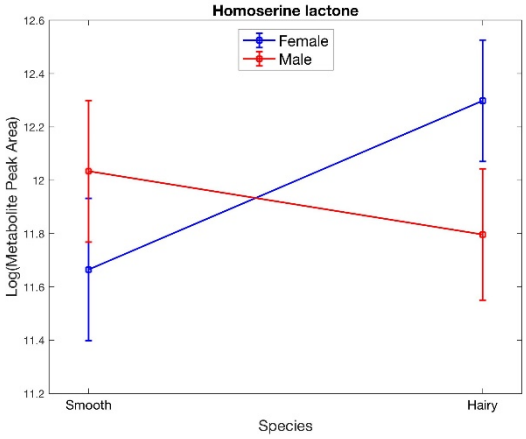

Nicotinamide adenine dinucleotide
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species

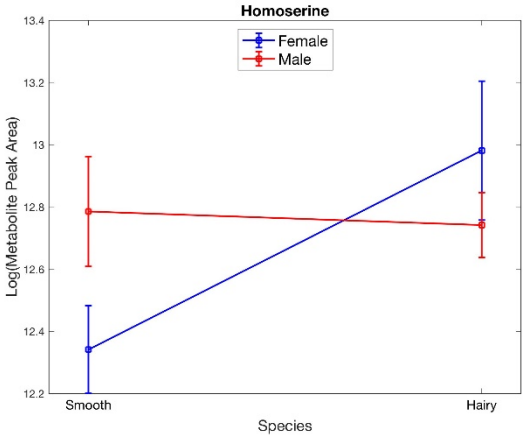

Niacinamide
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species

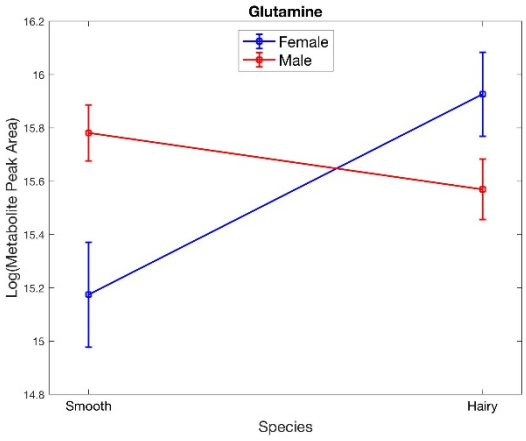

Spermidine
Female
Male
Log(Metabolite Peak Area)
Smooth
Hairy
Species


Beta-Alanine
Female
Male
Log(Metabolite Peak Area)
13
12.8
12.6
12.4
12.2
12
11.8
Smooth
Hairy
Species

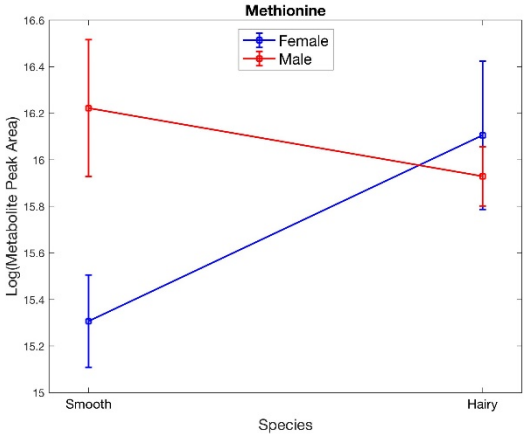

Betaine
Female
Male
Log(Metabolite Peak Area)
17.2
17.1
17
16.9
16.8
16.7
16.6
16.5
16.4
Smooth
Hairy
Species

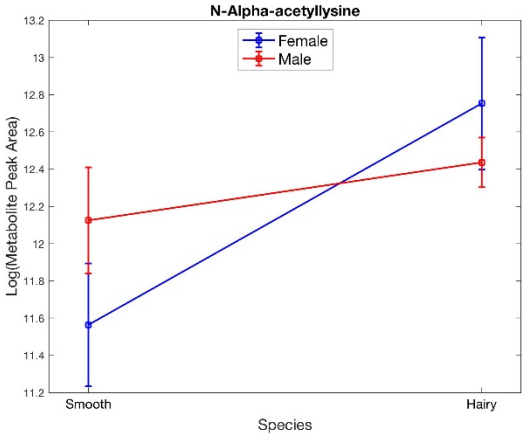

Homoserine lactone
Female
Male
Log(Metabolite Peak Area)
12.6
12.4
12.2
12
11.8
11.6
11.4
11.2
Smooth
Hairy
Species


Homoserine
Female
Male
Log(Metabolite Peak Area)
13.4
13.2
13
12.8
12.6
12.4
12.2
Smooth
Hairy
Species


Glutamine
Female
Male
Log(Metabolite Peak Area)
16.2
16
15.8
15.6
15.4
15.2
15
14.8
Smooth
Hairy
Species


Methionine sulfoxide
Female
Male
Log(Metabolite Peak Area)
13.6
13.5
13.4
13.3
13.2
13.1
13
Smooth
Hairy
Species


Methionine
Female
Male
Log(Metabolite Peak Area)
16.6
16.4
16.2
16
15.8
15.6
15.4
15.2
15
Smooth
Hairy
Species


N-Alpha-acetyllysine
Female
Male
Log(Metabolite Peak Area)
13.2
13
12.8
12.6
12.4
12.2
12
11.8
11.6
11.4
11.2
Smooth
Hairy
Species

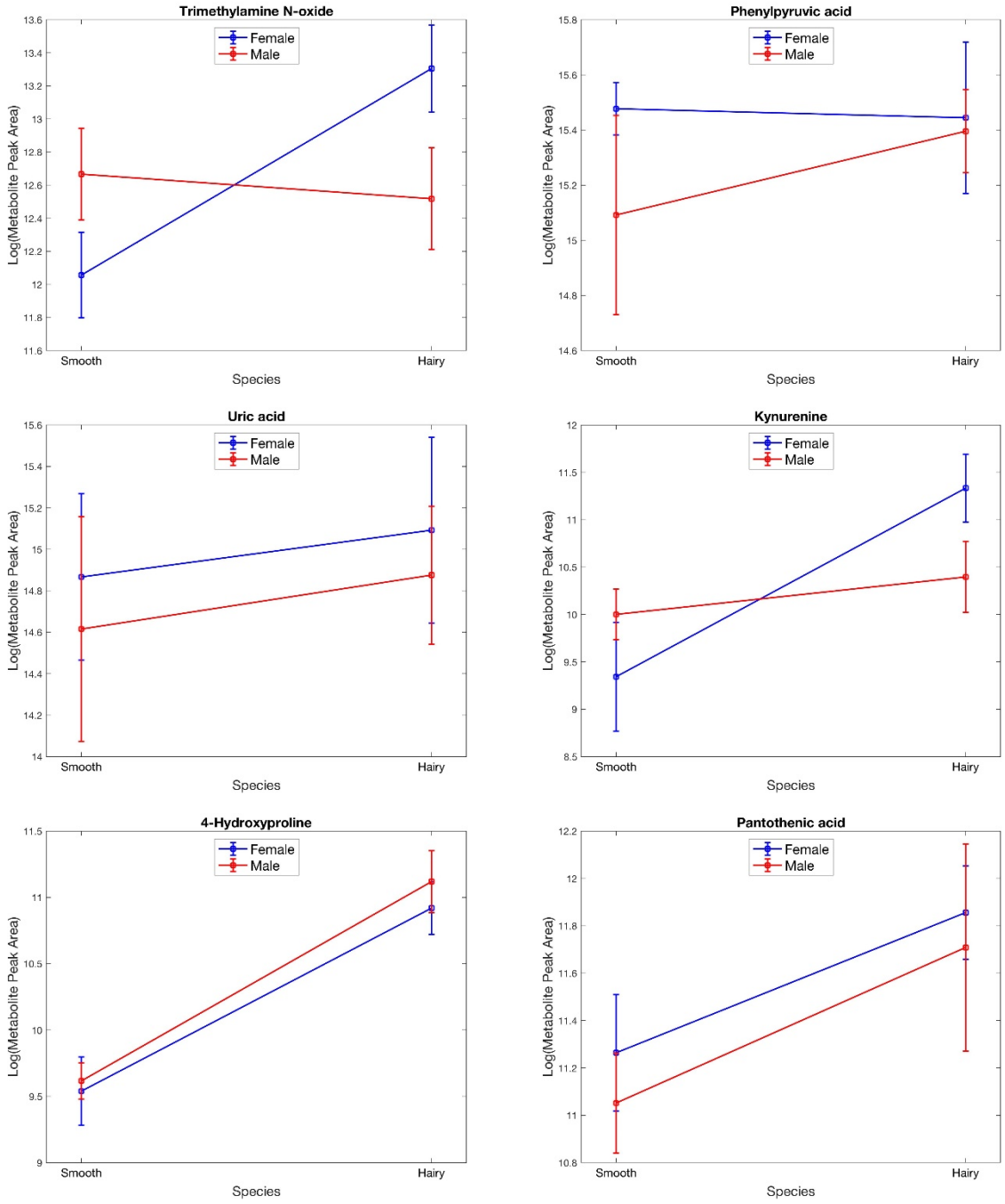
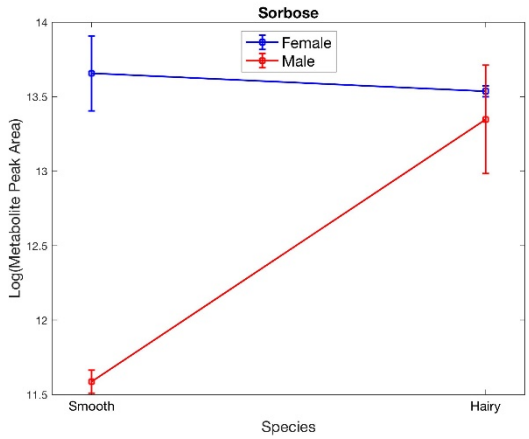
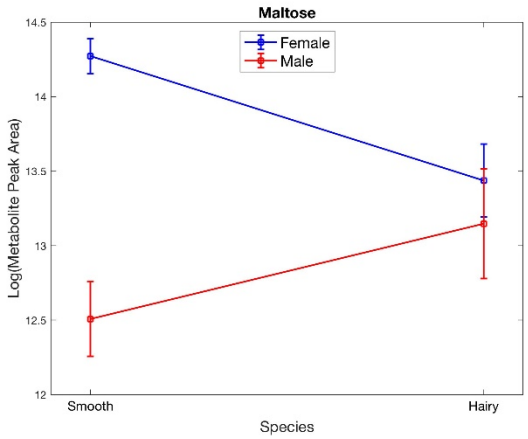
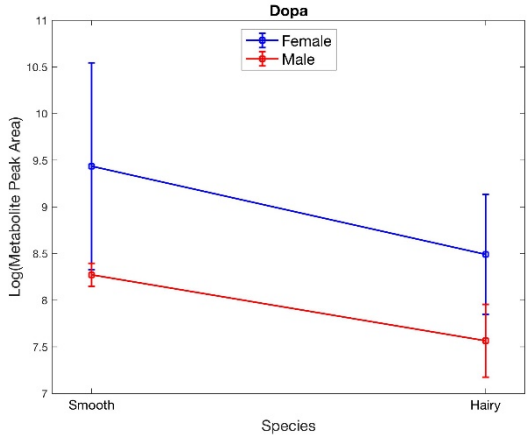
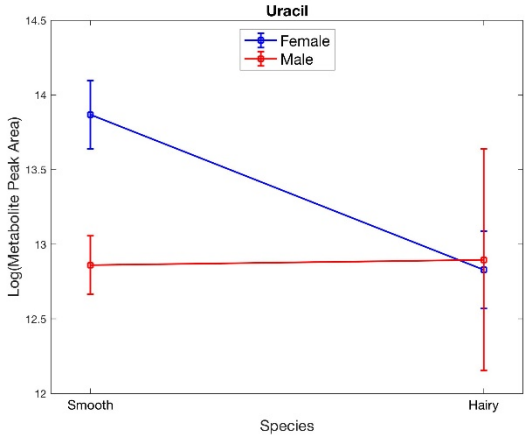


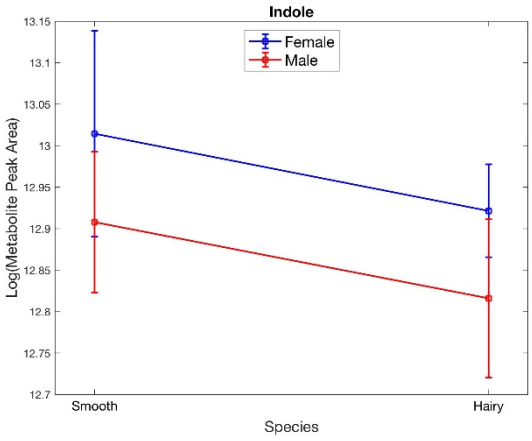
Figure A.3 Cluster D Identified metabolites from *Cherax tenuimanus* and *Cherax cainii* haemolymph.

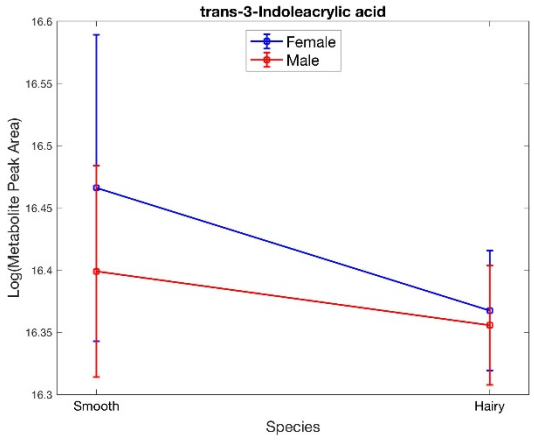

Sorbose
Female
Male
Log(Metabolite Peak Area)
14
13.5
13
12.5
12
11.5
Smooth
Hairy
Species

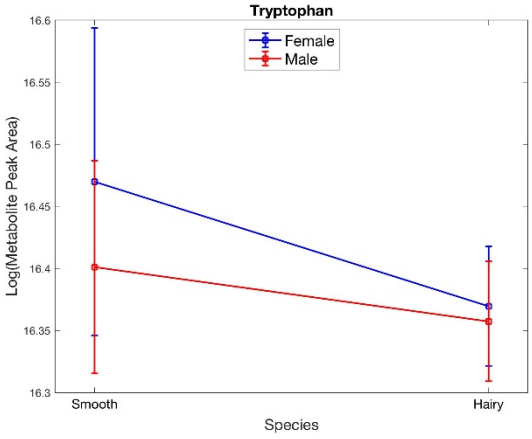

Maltose
Female
Male
Log(Metabolite Peak Area)
14.5
14
13.5
13
12.5
12
Smooth
Hairy
Species

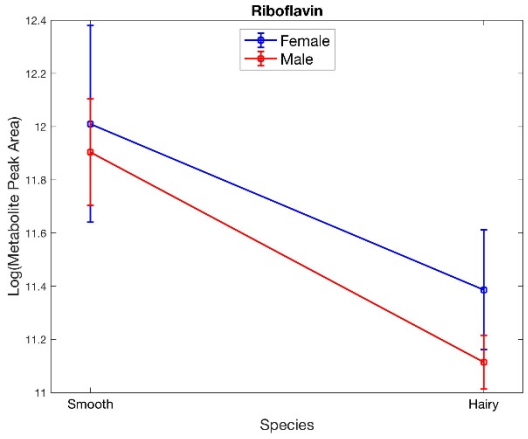

Dopa
Female
Male
Log(Metabolite Peak Area)
11
10.5
10
9.5
9
8.5
8
7.5
7
Smooth
Hairy
Species

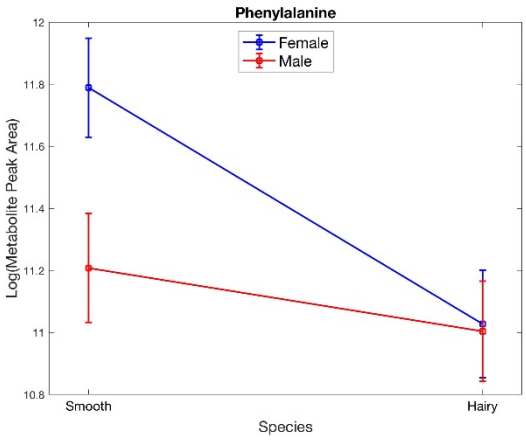

Uracil
Female
Male
Log(Metabolite Peak Area)
14.5
14
13.5
13
12.5
12
Smooth
Hairy
Species

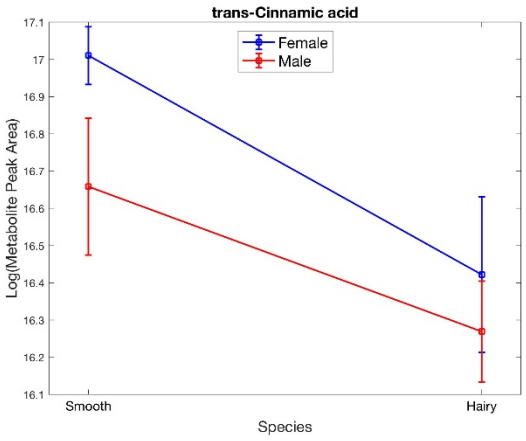

Indole
Female
Male
Log(Metabolite Peak Area)
13.15
13.1
13.05
13
12.95
12.9
12.85
12.8
12.75
12.7
Smooth
Hairy
Species

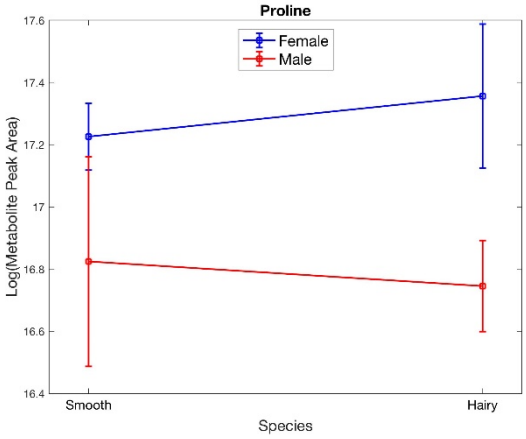

trans-3-Indoleacrylic acid
Female
Male
Log(Metabolite Peak Area)
16.6
16.55
16.5
16.45
16.4
16.35
16.3
Smooth
Hairy
Species

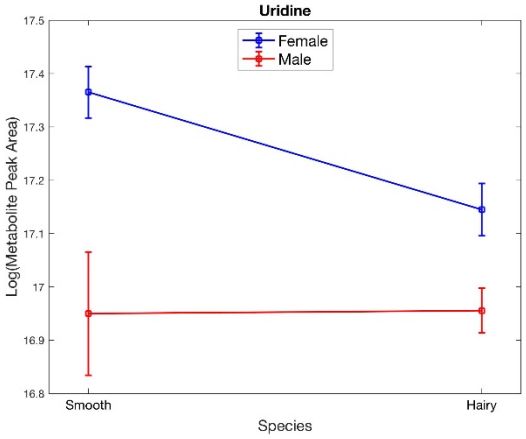

Tryptophan
Female
Male
Log(Metabolite Peak Area)
16.6
16.55
16.5
16.45
16.4
16.35
16.3
Smooth
Hairy
Species

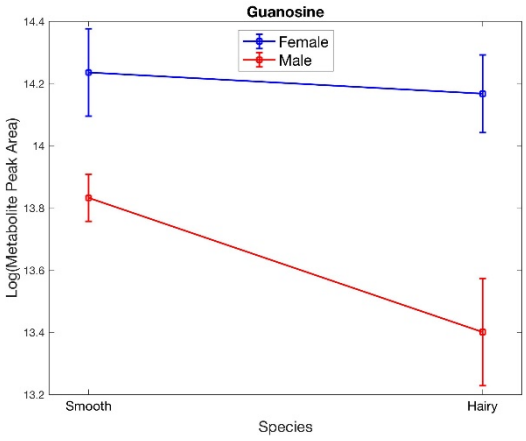

Riboflavin
Female
Male
Log(Metabolite Peak Area)
12.4
12.2
12
11.8
11.6
11.4
11.2
11
Smooth
Hairy
Species

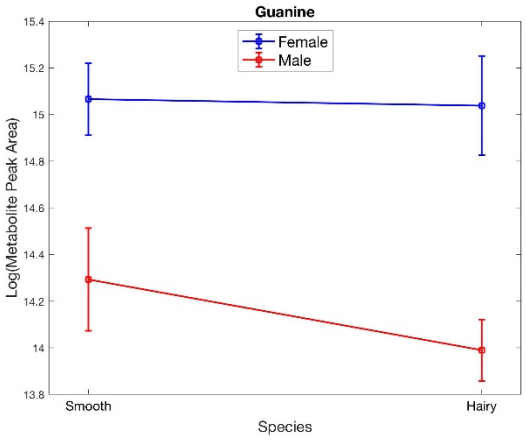

Phenylalanine
Female
Male
Log(Metabolite Peak Area)
12
11.8
11.6
11.4
11.2
11
10.8
Smooth
Hairy
Species

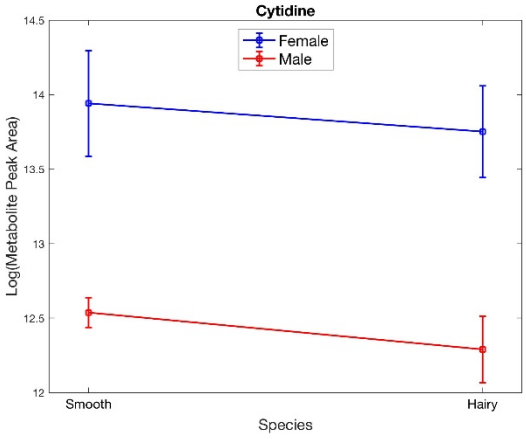

trans-Cinnamic acid
Female
Male
Log(Metabolite Peak Area)
17.1
17
16.9
16.8
16.7
16.6
16.5
16.4
16.3
16.2
16.1
Smooth
Hairy
Species

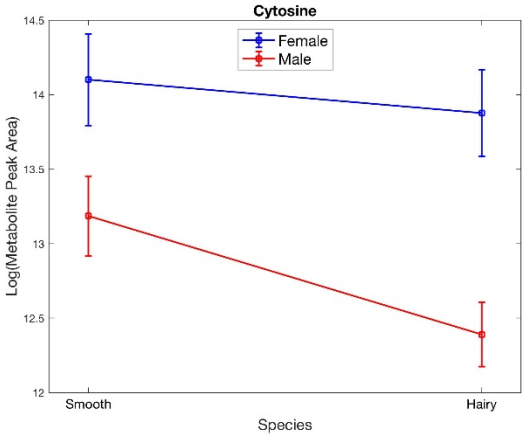

Proline
Female
Male
Log(Metabolite Peak Area)
17.6
17.4
17.2
17
16.8
16.6
16.4
Smooth
Hairy
Species


Uridine
Female
Male
Log(Metabolite Peak Area)
17.5
17.4
17.3
17.2
17.1
17
16.9
16.8
Smooth
Hairy
Species


Guanosine
Female
Male
Log(Metabolite Peak Area)
14.4
14.2
14
13.8
13.6
13.4
13.2
Smooth
Hairy
Species


Guanine
Female
Male
Log(Metabolite Peak Area)
15.4
15.2
15
14.8
14.6
14.4
14.2
14
13.8
Smooth
Hairy
Species


Cytidine
Female
Male
Log(Metabolite Peak Area)
14.5
14
13.5
13
12.5
12
Smooth
Hairy
Species


Cytosine
Female
Male
Log(Metabolite Peak Area)
14.5
14
13.5
13
12.5
12
Smooth
Hairy
Species

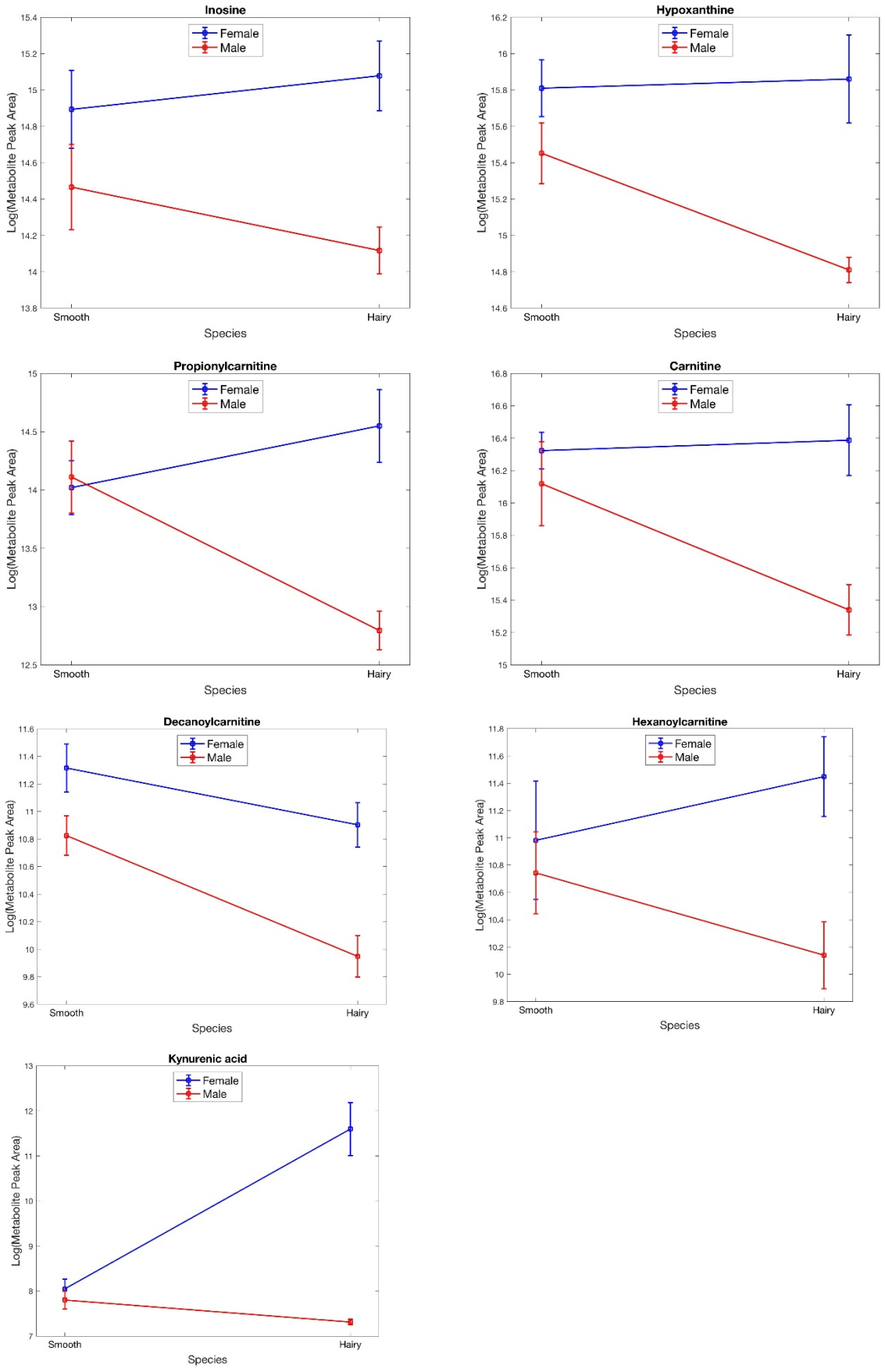


Figure A.3 Cluster E. (n=23) Identified metabolites from *Cherax tenuimanus* and *Cherax cainii* haemolymph.

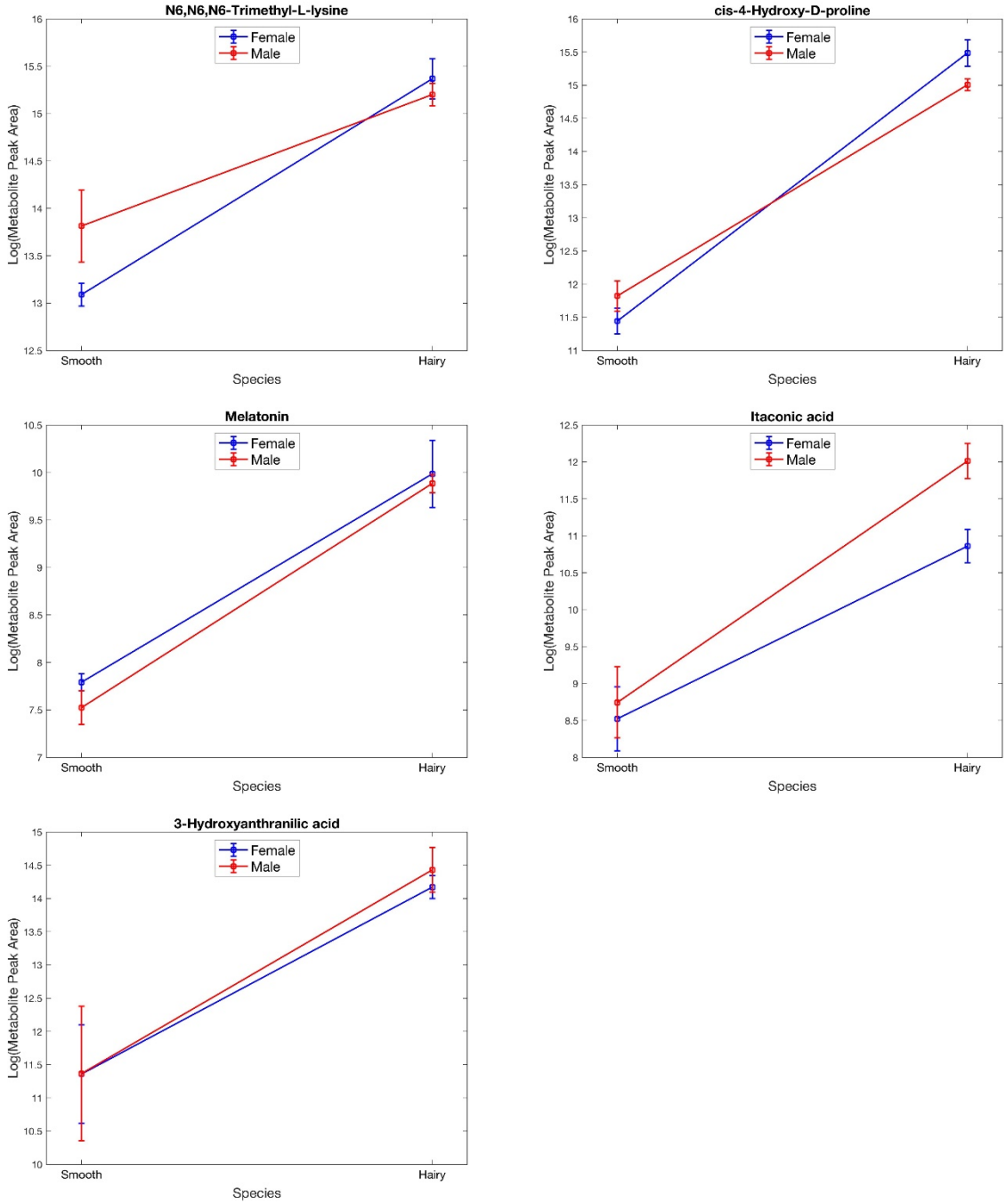


Figure A.3 Cluster F. Identified metabolites from *Cherax tenuimanus* and *Cherax cainii* haemolymph.

Appendix B – Chapter 5 - Supplemental Material.

B.1. Metabolite classes for Chapter 5

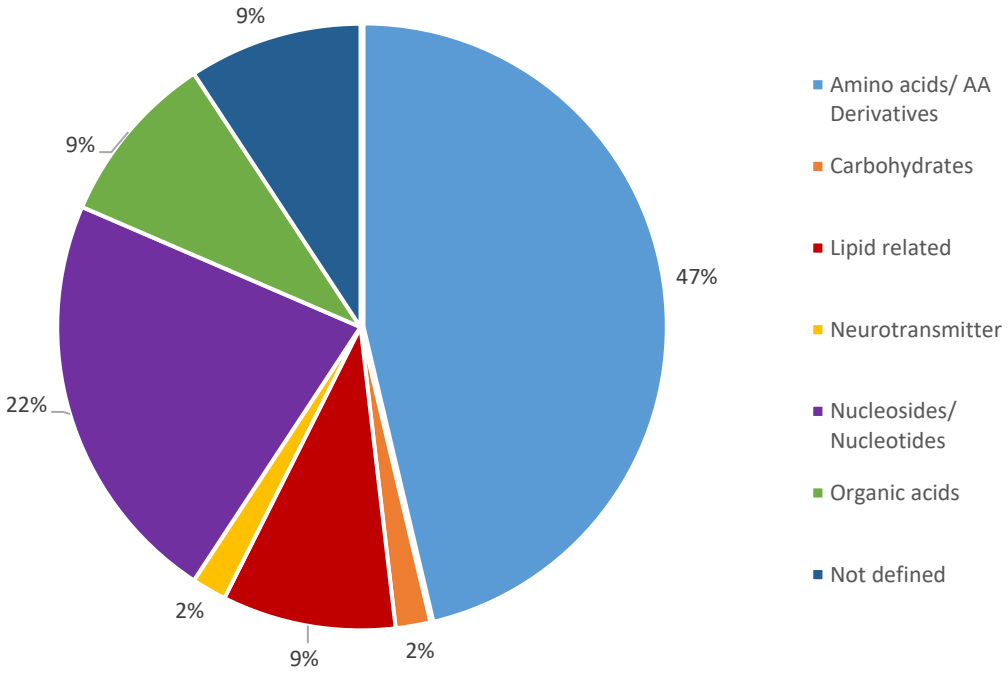


Figure B.1 Chemical groups of annotated metabolites. Percentage based on the total number of annotated metabolites (54) in this study (Chapter 5).

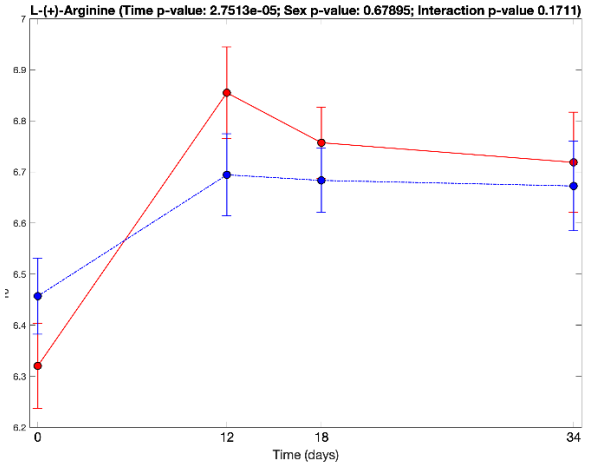
Table B.1 *Cherax tenuimanus* metabolomics trial from 27 October to 30 November 2017. Identification of individual animals (marron ID) FH – female, MH – males; wet weight (in grams) at day 0, which animals were placed together (aquaria), the dates that they were paired and the number of days with a potential mate at each haemolymph collection date.

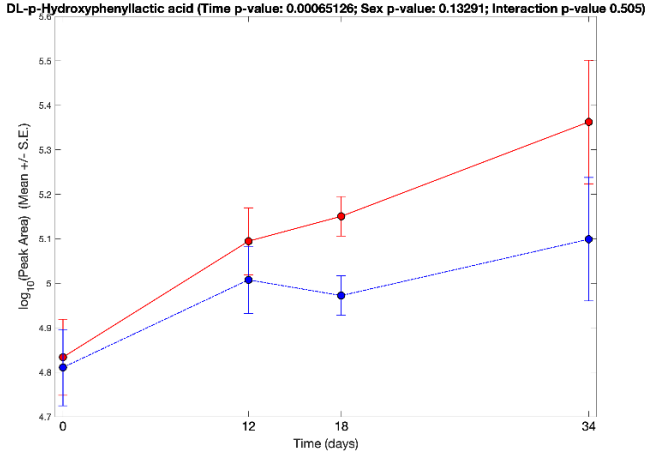
Marron ID	Weight (g)	Aquaria	Date together	8-Nov	14-Nov	30-Nov
FH 3	115.71	A12	2/11/2017	6	12	28
MH 4	199.95	A12	2/11/2017	6	12	28
FH 2	90.02	A19	2/11/2017	6	12	28
FH 5	104.82	A16	1/11/2017	7	13	29
MH 5	123.77	A16	1/11/2017	7	13	29
FH 4	126.55	A11	1/11/2017	7	13	29
MH 1	185.54	A11	1/11/2017	7	13	29
MH 3	239.94	A20	28/10/2017	10	16	32
FH 1	143.53	A18	28/10/2017	10	16	32
MH 2	240.50	A18	28/10/2017	10	16	32

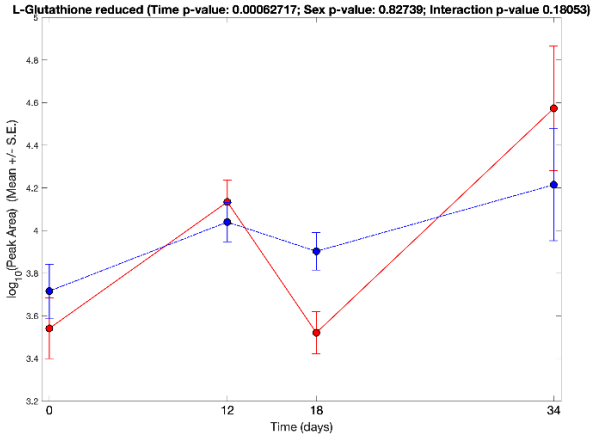
B.2. ANOVA plots for Chapter 5

Plots of two-way repeated measures ANOVA analysis for each metabolite with clusters (A-E) organised by HCA analysis according to phenotypic similarity. The metabolites levels of females (red) and males (blue) over the four time points on the X axis and the mean peak area of the metabolite on the Y axis.

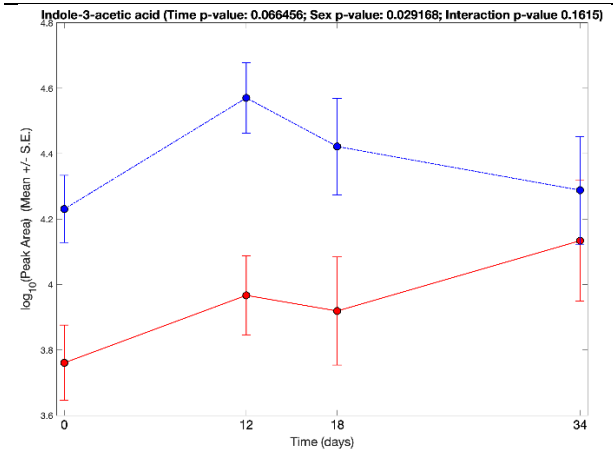
Cluster A

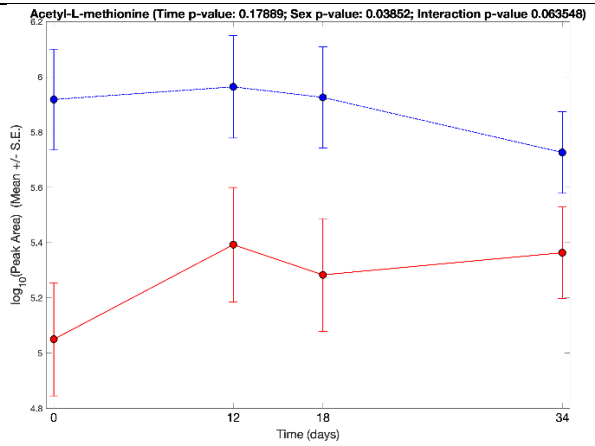




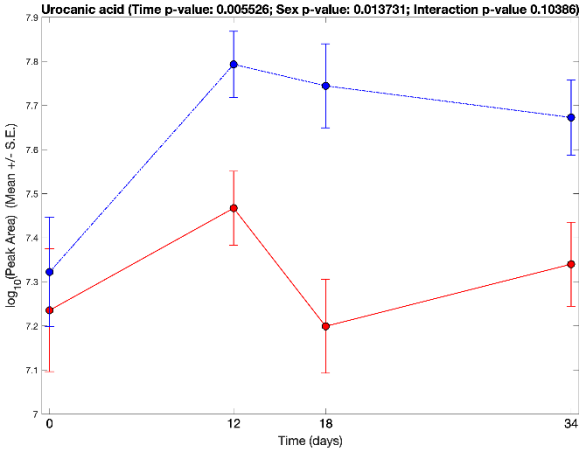


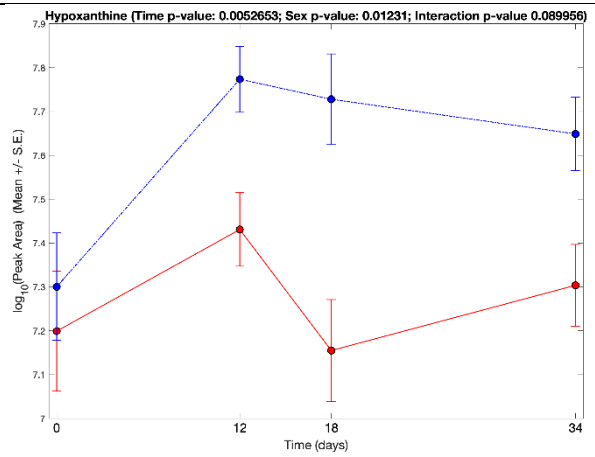
Time (n=3)



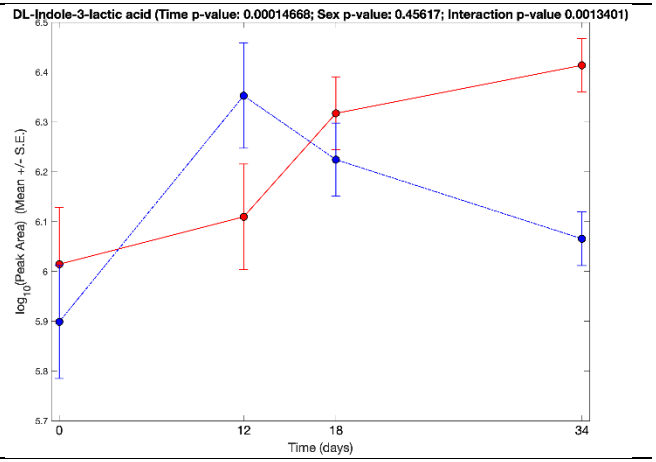


Sex (n=2)

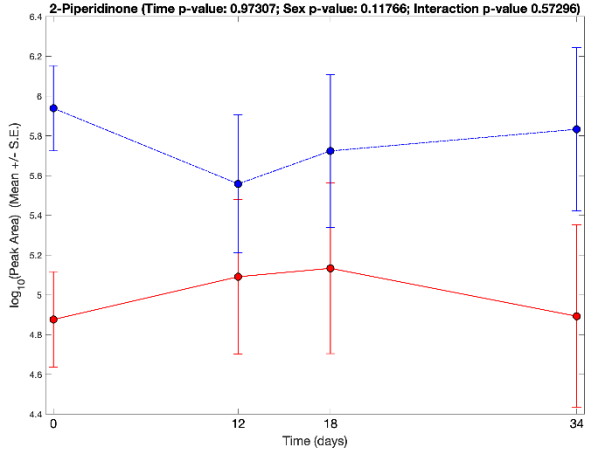


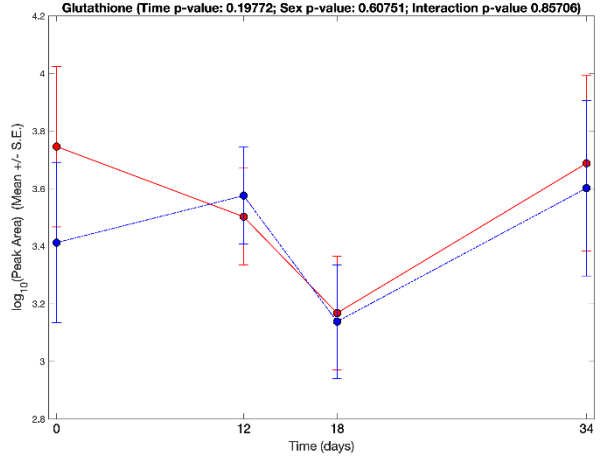


Time & Sex (n=2)



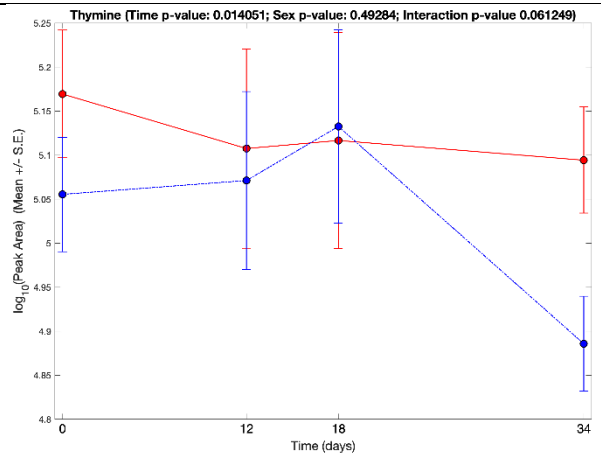
Time × Sex interaction (n=1)

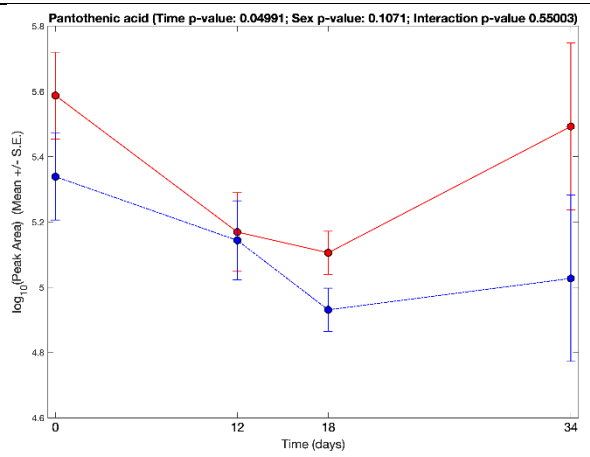


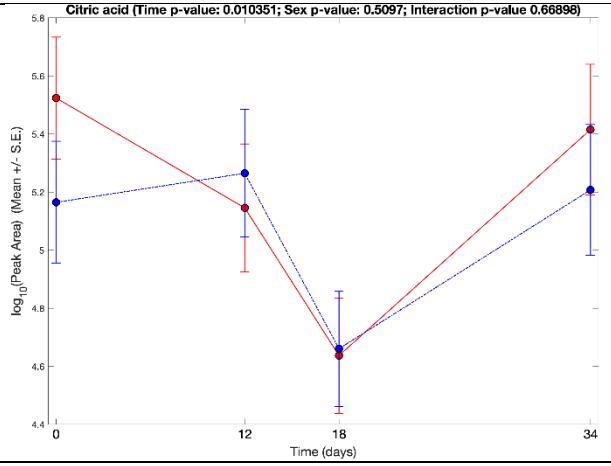


Not significant (n=2)

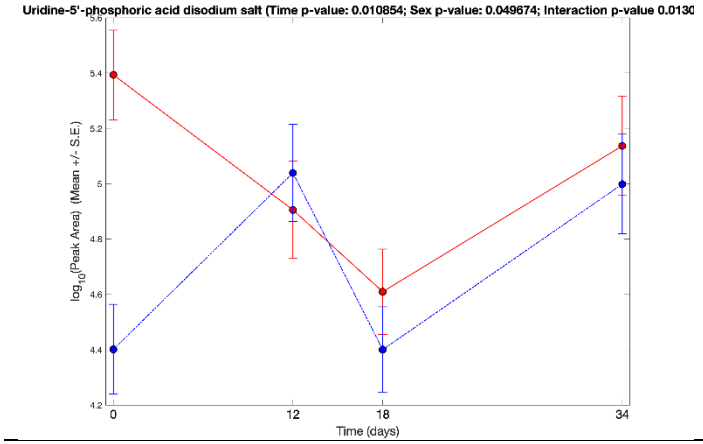
Cluster B (n=6)



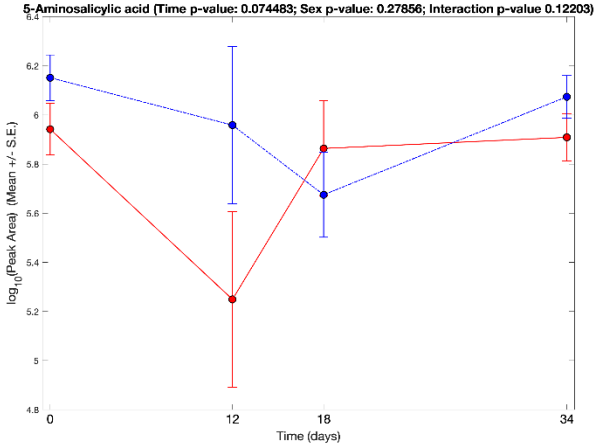


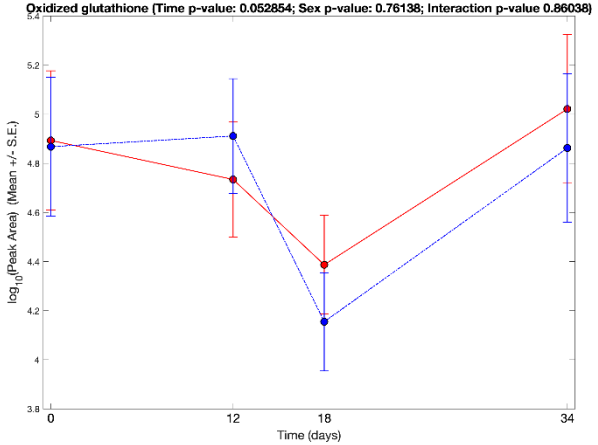


Time (n=3)



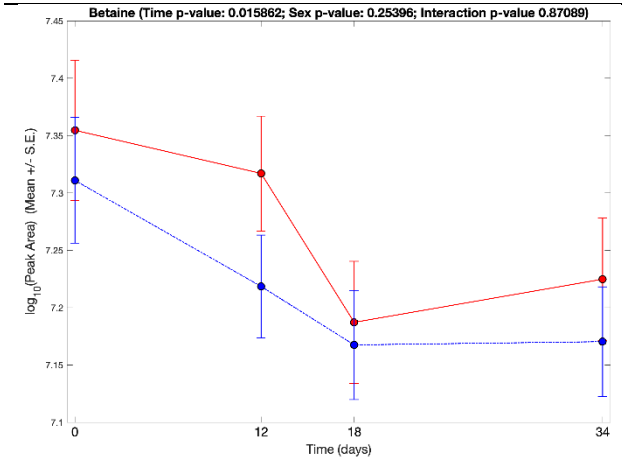
Time × Sex Interaction (n=1)

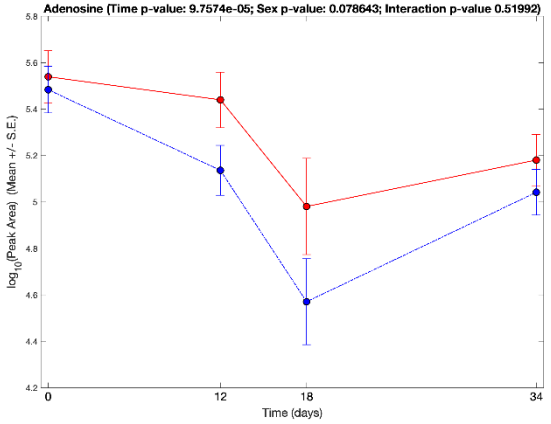


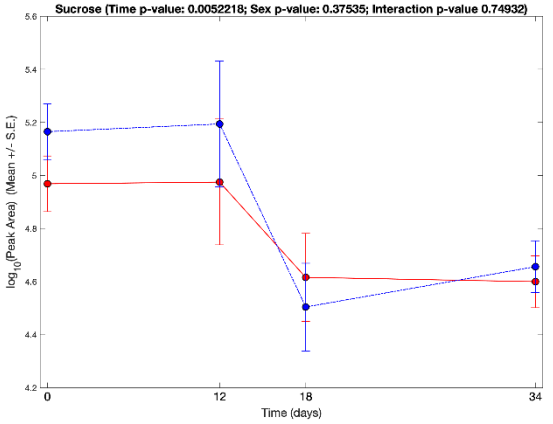


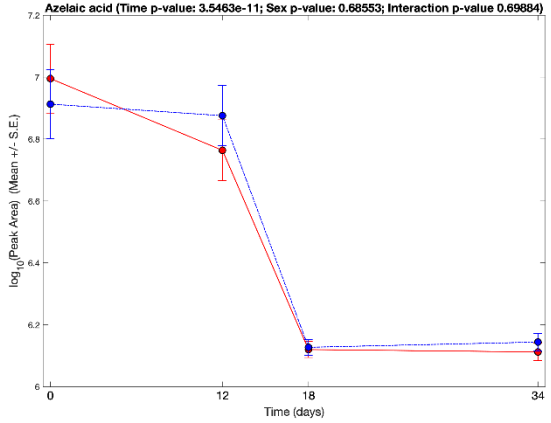
Not significant (n=2)

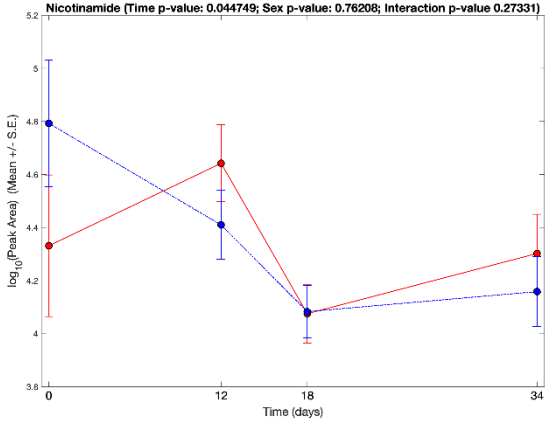
Cluster C

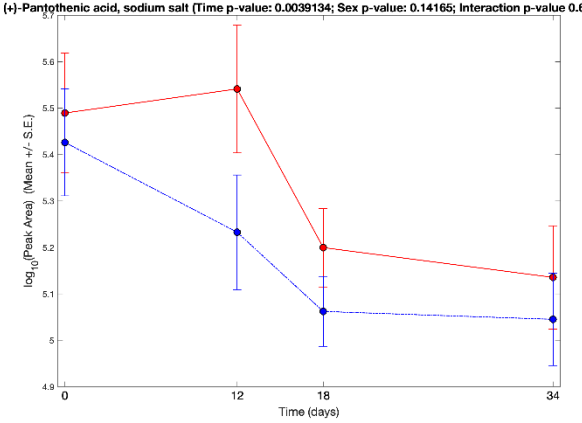




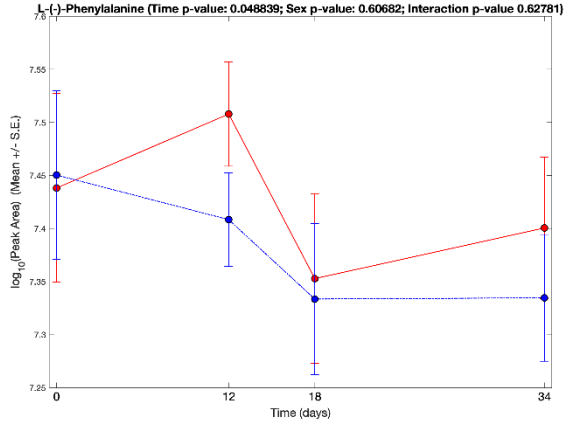




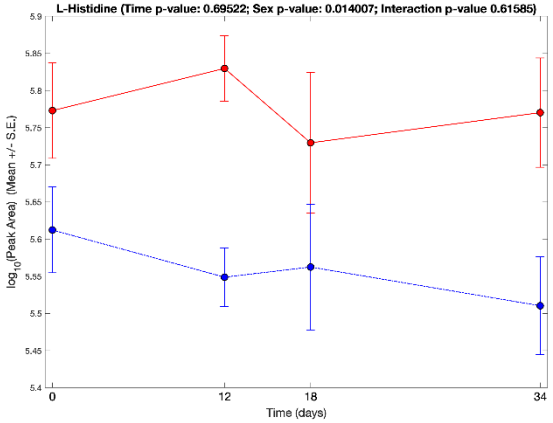




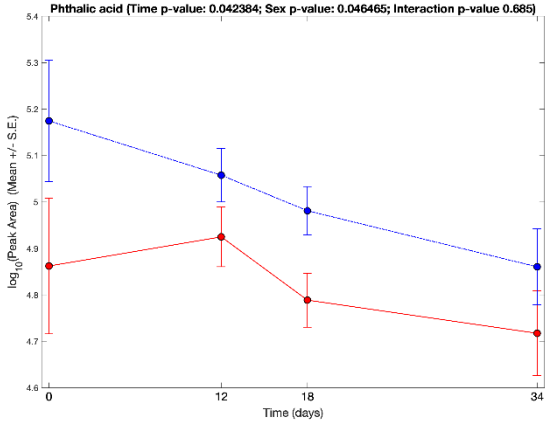
Time (n=7)



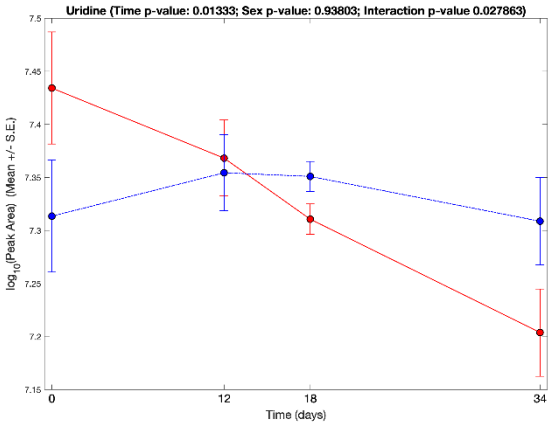
Time (n=7)

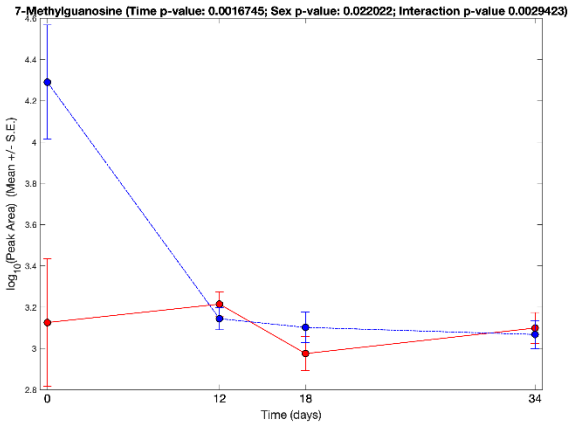


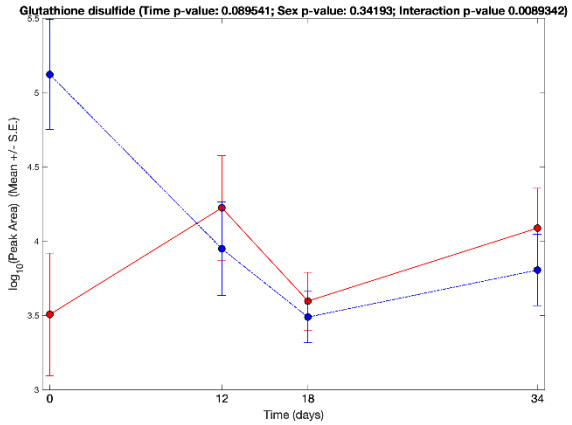
Sex (n=1)



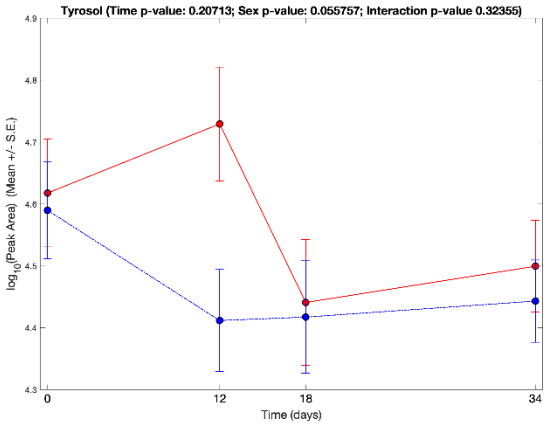
Time & Sex (n=1)

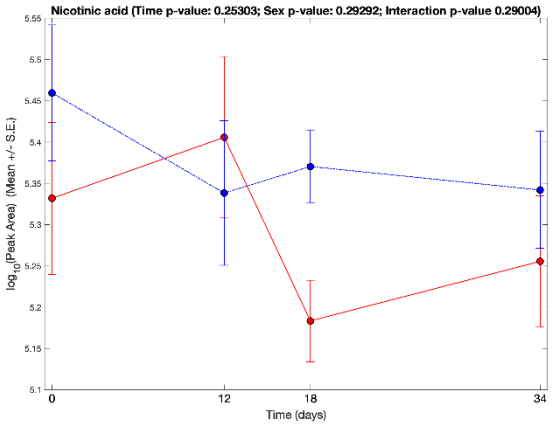


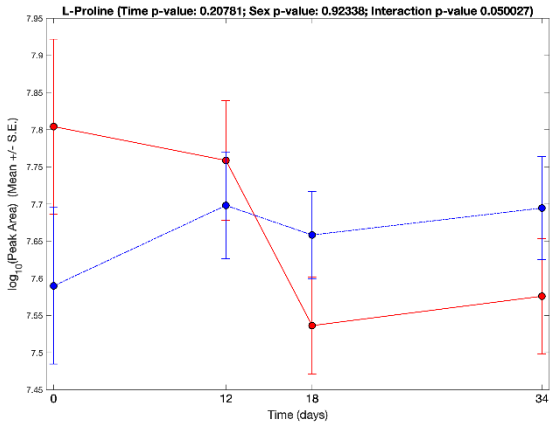


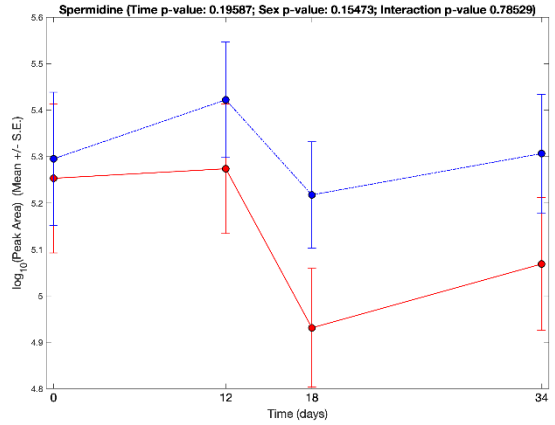


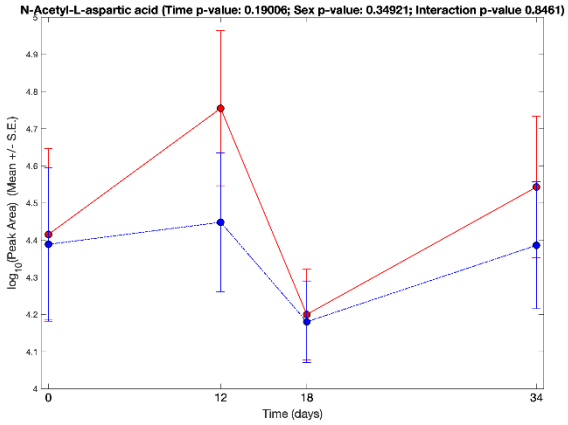
Time × Sex Interaction (n=3)

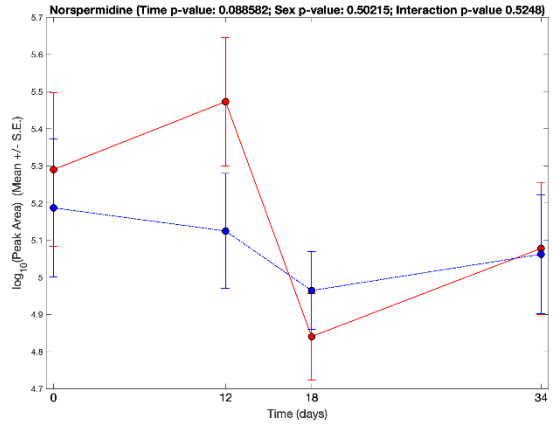




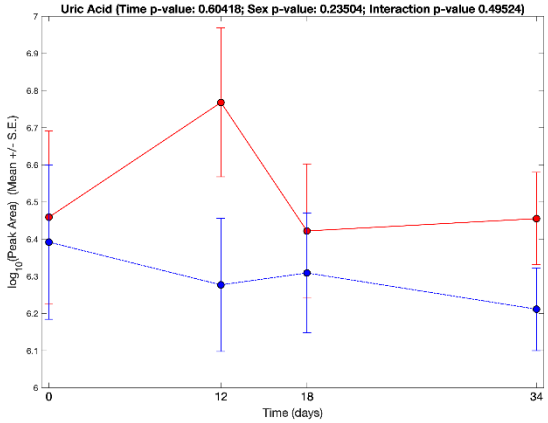






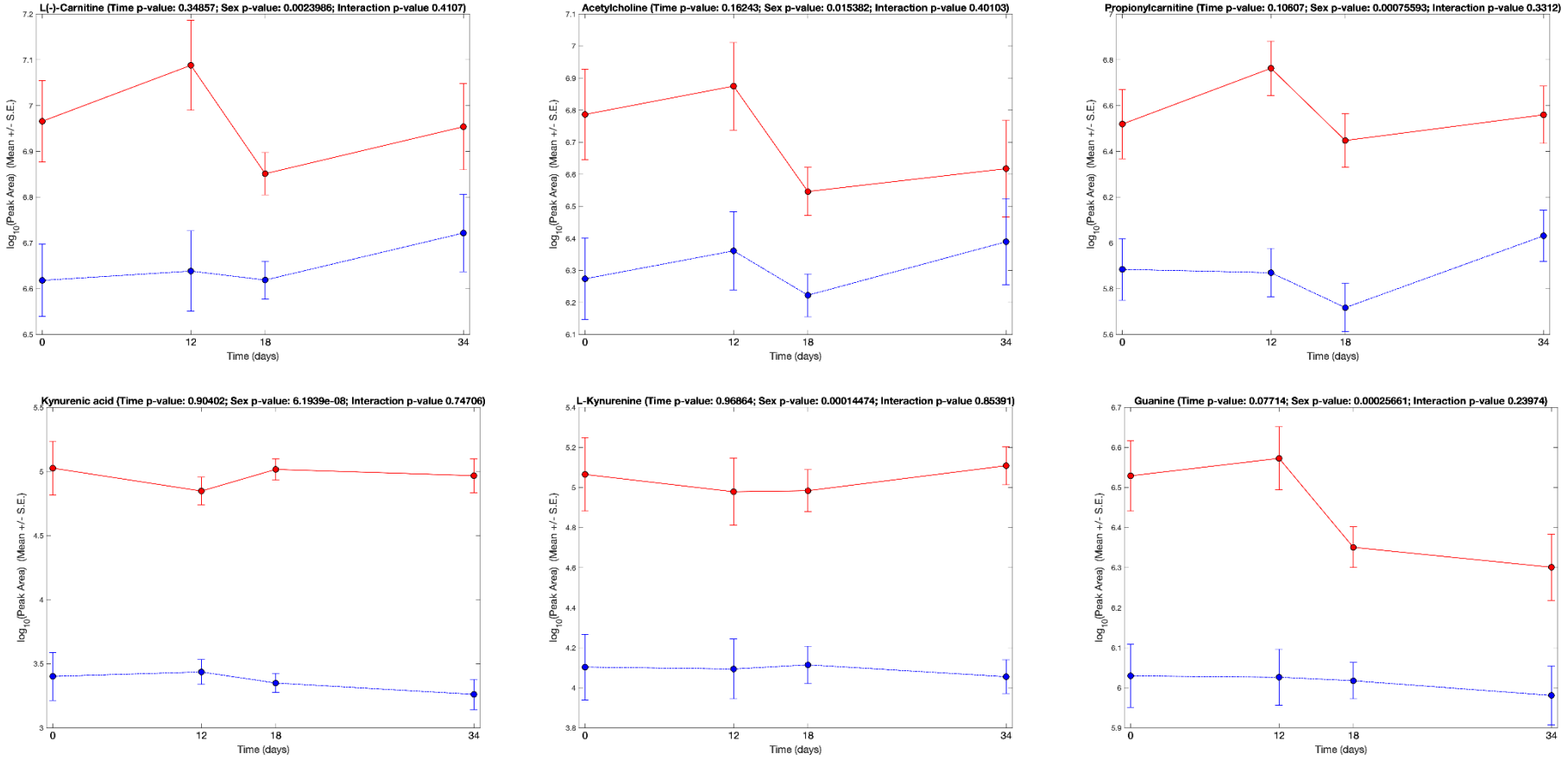


Not significant (n=7)

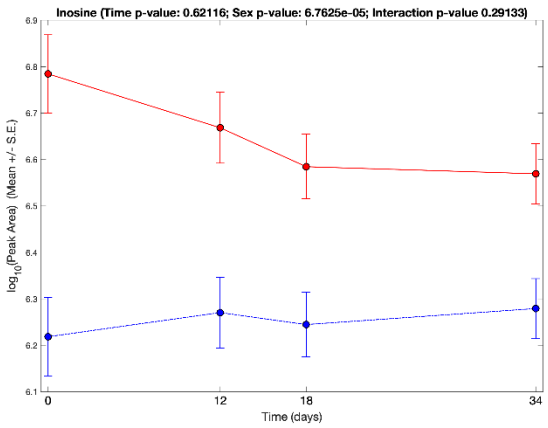


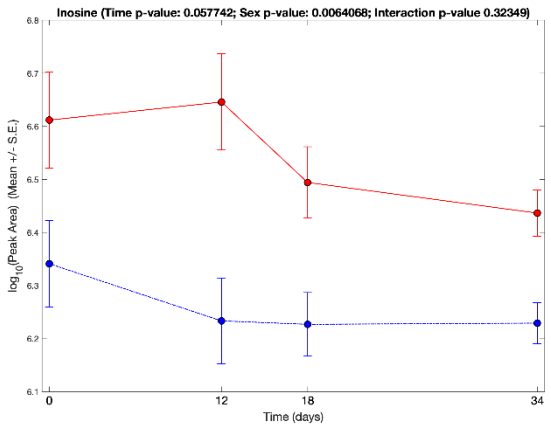
Not significant (n=7)

Cluster D

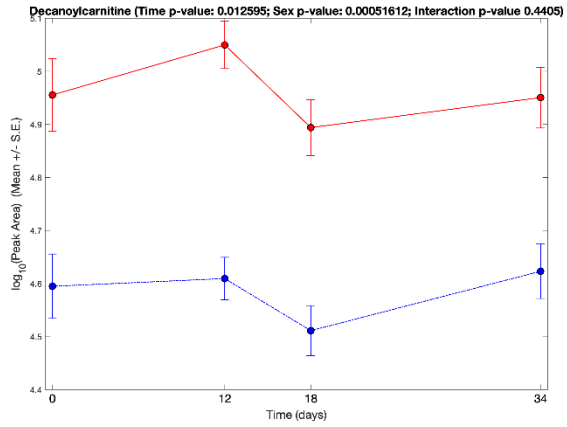


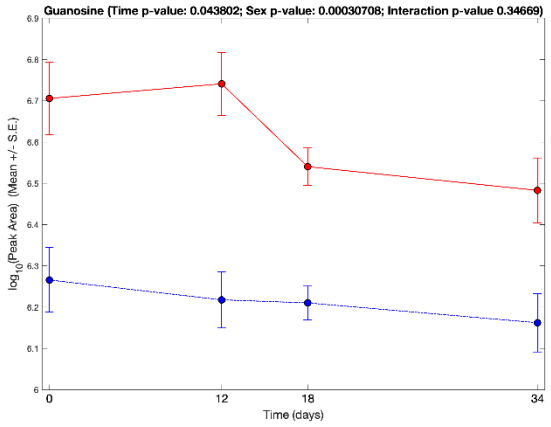
Sex (n=8)





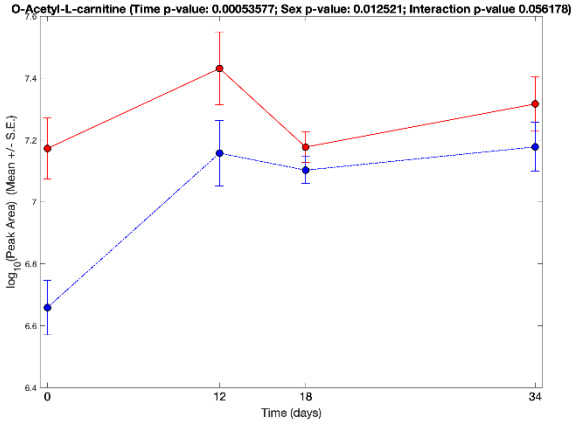
Sex (n=8)



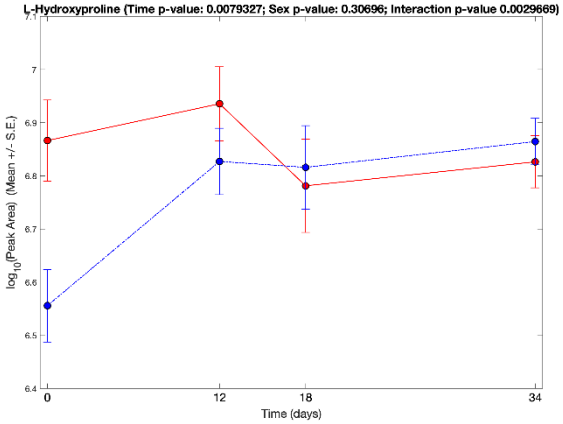


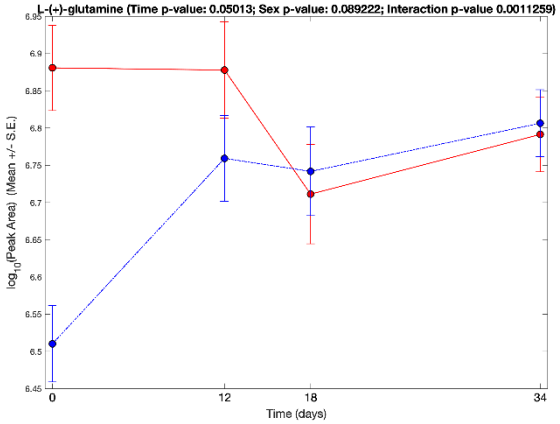
Time & Sex (n=2)

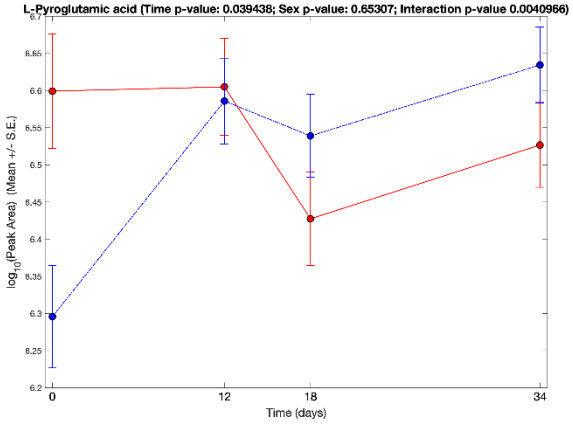
Cluster E



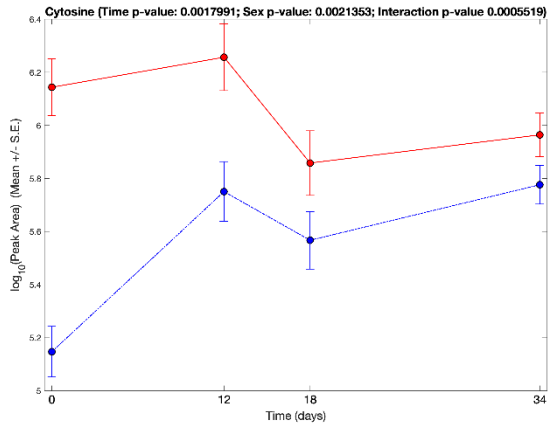
Time & Sex (n=1)

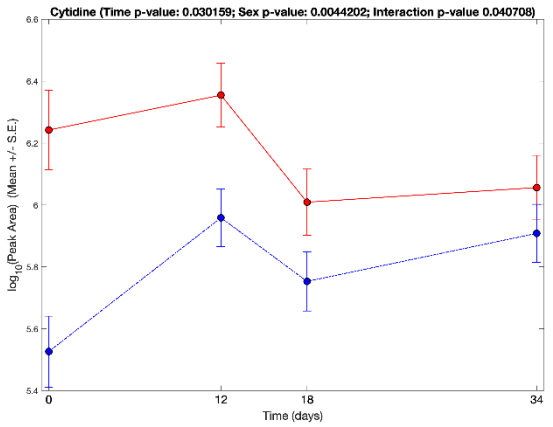


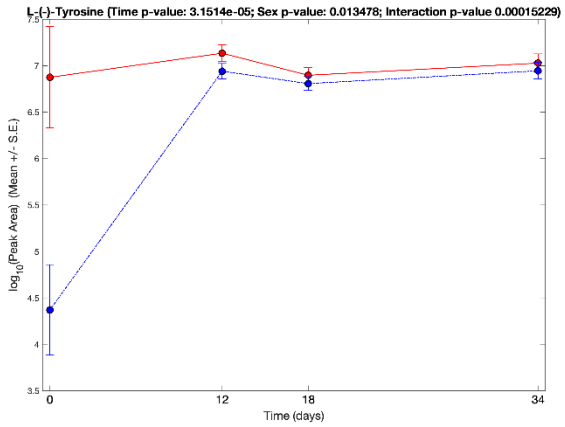


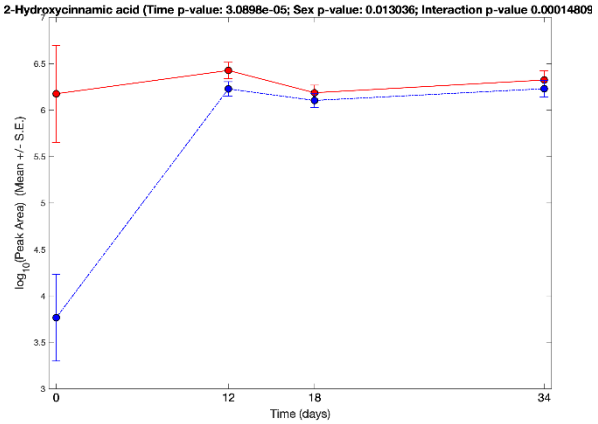


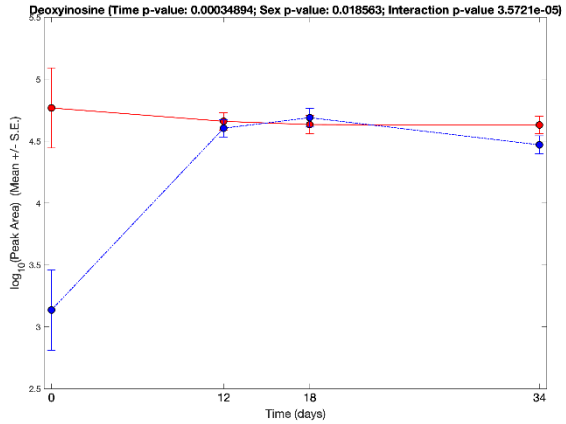
Time × Sex Interaction (n=9)

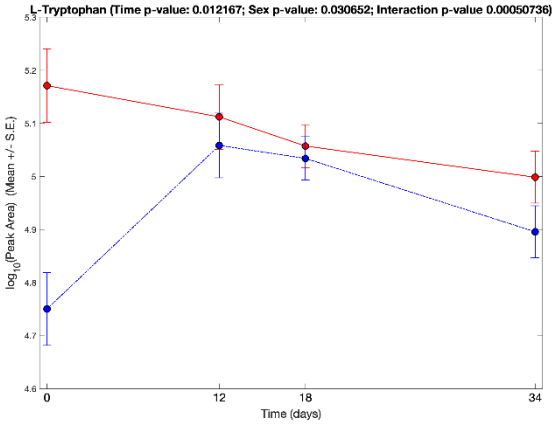












Time × Sex Interaction (n=9)

B.3. Values calculated for Nernst Equation for oxidative stress

		metabolite peak area			E_{hc} (mV)	
Sex	ID_TP	GSH	GSSG	E_{hc} (mV)	Mean	±SE
Female	FH1_1	6159.99	15620.98	-340.04	-331.1	15.7
Female	FH1_2	11145.33	43121.80	-342.23		
Female	FH1_3	4156.09	6852.08	-340.52		
Female	FH1_4	366.49	588.93	-309.68		
Female	FH2_1	1997.34	1807.76	-338.81	-350.8	19.3
Female	FH2_2	12916.20	6064.02	-371.19		
Female	FH2_3	2313.77	4650.20	-330.46		
Female	FH2_4	13325.05	12324.21	-362.89		
Female	FH3_1	3885.31	2604.47	-351.20	-356.9	28.4
Female	FH3_2	23001.73	2627.44	-396.73		
Female	FH3_3	3742.96	2685.30	-349.85		
Female	FH3_4	8148.12	61424.65	-329.65		
Female	FH4_1	2345.58	1607.28	-344.44	-359.2	39.1
Female	FH4_2	9834.59	50724.46	-336.93		
Female	FH4_3	3037.54	4548.65	-337.73		
Female	FH4_4	65356.88	4187.19	-417.56		
Female	FH5_1	8061.48	13988.11	-348.36	-369.6	52.7
Female	FH5_2	11786.81	97362.06	-333.21		
Female	FH5_3	4583.91	4267.03	-349.11		
Female	FH5_4	276399.57	7059.47	-447.86		
Male	MH1_1	3624.44	998002.91	-273.08	-327.1	36.5
Male	MH1_2	11904.93	61611.08	-339.34		
Male	MH1_3	3677.25	4307.15	-343.33		
Male	MH1_4	9198.54	13005.39	-352.68		
Male	MH2_1	4147.82	123040.49	-303.40	-376.3	56.5
Male	MH2_2	6847.35	801.84	-380.86		
Male	MH2_3	5978.19	669.20	-379.70		
Male	MH2_4	112732.31	1947.63	-441.37		
Male	MH3_1	6565.67	1183504.98	-286.14	-352.7	46.5
Male	MH3_2	9411.63	10729.69	-355.74		
Male	MH3_3	8866.07	1401.89	-380.33		
Male	MH3_4	15340.78	2212.93	-388.54		
Male	MH4_1	14798.76	3502.84	-381.72	-363.1	13.6
Male	MH4_2	7741.96	11413.04	-349.93		
Male	MH4_3	13886.58	12570.93	-363.69		
Male	MH4_4	6532.57	4687.53	-357.00		
Male	MH5_1	2599.74	80005.20	-296.94	-348.9	38.6
Male	MH5_2	26811.71	9154.34	-384.65		
Male	MH5_3	12029.47	5493.51	-370.63		
Male	MH5_4	11410.62	40727.08	-343.56		

Appendix C - Permits for conducting research with *C. teniumanus* and *C. cainii* at ECU.

C.1. Regulation 16: Licence to keep fauna for educational or public purpose.

Permit No. WW001595 - Department of Biodiversity, Conservation and Attractions (DBCA) (formerly Department of Parks and Wildlife - DPaW) valid 07-07-2017 to 06-07-2018.

DEPARTMENT OF PARKS AND WILDLIFE

Department of Parks and Wildlife

Enquiries: 17 DICK PERRY AVE, KENSINGTON, WESTERN AUSTRALIA
Telephone: 08 9219 9000
Facsimile: 08 9219 8242

Correspondence: **Locked Bag 30 Bentley Delivery Centre WA 6983**

PAGE NO. 1 WW001595

RECEIPT NO. **AMOUNT** $0.00

WILDLIFE CONSERVATION ACT 1950
REGULATION 16

LICENCE TO KEEP FAUNA FOR EDUCATIONAL OR PUBLIC PURPOSE

WAIVED FEE

THE UNDERMENTIONED PERSON MAY KEEP THE SPECIES OF FAUNA LISTED BELOW, SUBJECT TO THE CONDITIONS ENDORSED ON AND ATTACHED TO THIS LICENCE, AT THE PREMISES SPECIFIED.

DIRECTOR GENERAL

CONDITIONS

1 The licensee shall comply with the provisions of the Wildlife Conservation Act 1950, Wildlife Conservation Regulations 1970 and any notices in force under this legislation.
2 The licensee must comply with all relevant local authority by-laws.
3 This licence shall be displayed in a prominent position in the premises at the property specified on this licence.
4 The maximum number of each species specified below may be varied subject to lawful acquisition and breeding pursuant to the provisions of Wildlife Conservation Regulations 1970 regulation 16.
5 Marron to be obtained from captive stock held at the Department of Fisheries facility in Pemberton.
6 Further conditions (numbered 1 to 6) are attached.

PROPERTY ECU JOONDALUP CAMPUS BUILDING 19 (RESEARCH AQUARIUM ROOM)

SPECIES 45 MARGARET RIVER HAIRY MARRON (Cherax tenuimanus)
70 SMOOTH MARRON (Cherax cainii)

PURPOSE HOLDING OF MARGARET-RIVER HAIRY MARRON (CHERAX TENUIMANUS) FOR THE DEVELOPMENT OF NON-LETHAL METHODS TO ASSESS STRESS AND REPRODUCTIVE STATUS OF A CRITICALLY ENDANGERED FRESHWATER CRAYFISH.

DATE OF ISSUE 07/07/2017
VALID FROM 07/07/2017
DATE OF EXPIRY 06/07/2018

LICENSING OFFICER

RESIDENTIAL ADDRESS: [redacted]

LICENSEE: MRS ED LETTE (EMILY DIANE)
ADDRESS C/- EDITH COWAN UNIVERSITY
100 JOONDALUP DVE
JOONDALUP WA 6027

CONDITIONS ATTACHED TO REGULATION 16 LICENCE NO . WW001595

1. Protected fauna shall not be obtained or accepted or disposed of by the licensee or any person operating under his authority unless it is first authorised in writing by the Director General.

2. A book of record shall be kept and made available for inspection at all times and the licensee shall cause to have entered therein (in accordance with Regulation 16) -

   2.1 the name and address and licence number of the person from whom the fauna was received, and the common name of the species and its condition, sex and age;

   2.2 similar entries shall be made in respect of fauna for which approval has been given for him to dispose;

   2.3 details of deaths or increase through breeding.

3. The licensee shall furnish a copy of all records to be sent to the Director General within fifteen (15) days from the expiration of this licence.

4. The fauna must be provided with adequate security, shelter, food, water and clean surroundings in accordance with the Wildlife Conservation Regulations.

5. Day to day type of enquiries for Regulation 16 license holders in the Metropolitan area are to be made through the Department's Licensing Section on (9219 9831 FAX 9219 8242).

6. Day to day type enquiries for Regulation 16 license holders in country areas are to be made through the District Wildlife Officer.

C.2. Department of Fisheries Exemption No. 2958

Facsimile Message

Government of Western Australia
Department of Fisheries

Fisheries Research Division
WA Fisheries &Marine Research
Laboratories
PO Box 20, North Beach
Western Australia 6920
Telephone +61 (08) 9203 0111
Facsimile +61 (08) 9203 0199
Website http://www.wa.gov.au/westfish

Fish for the future

To: Emily Lette
Of: Edith Cowan University
Fax: 63045070
From: Karen Santoro on behalf of Rick Fletcher
Date: 6/6/17
No. of pages including this page: 3

Dear Emily

EXEMPTION no. 2958

Thank you for forwarding the details of your research project authorised under exemption from the *Fish Resources Management Act 1994*. I am forwarding herewith your numbered and approved exemption.

You must carry the exemption on collecting trips. Please familiarise yourself with the conditions of the exemption and ensure you comply with them. **Failure to do so may result in cancellation of your research exemption.**

In particular, you **must** provide notice of your field plans to Fisheries, quoting your exemption number, by calling 1800 815 507 (condition 1). Correct notification avoids unnecessary callouts of our compliance staff, responding to reports of 'illegal activity' from concerned members of the public, and prevents unwanted complications for yourself.

Please add my name and address now to the mailing list for your final report and publications, so that your research results can be placed in the Department's marine science library for the benefit of all the state's fisheries research and managers.

Best wishes for a successful research project!

Rick Fletcher
EXECUTIVE DIRECTOR – SCIENCE AND RESOURCE ASSESSMENT DIVISION

Office use only
LATMS Number_17-47____
EXEMPTION NUMBER__2958____

Fish Resources Management Act 1994

Section 7(2) (a)

INSTRUMENT OF EXEMPTION

I, Warrick Fletcher, Executive Director, Science and Resource Assessment Division of the Department of Fisheries, HEREBY EXEMPT the persons named in **Schedule 1** from all those provisions of the *Fish Resources Management Act 1994* and subsidiary legislation thereunder which would otherwise prevent those persons from lawfully pursuing the activity described in **Schedule 2**, for the period and purpose set out in **Schedule 3**, subject to the conditions listed in **Schedule 4**.

Schedule 1
Persons

1. Emily Lette and Dr. Quinton Burnham of Edith Cowan University.

Schedule 2
Activity

1. To collect captive marron and transport them to ECU Joondalup Campus from Pemberton Fisheries Research Centre.

Schedule 3
Period and Purpose

1. From the date of signing of this instrument until 30 June 2018.
2. For the purposes of research.

Schedule 4
Conditions

1. The exemption holder must advise the Department of Fisheries by calling **1800 815 507** at least 60 minutes prior to commencing every fishing trip under the exemption, of:
   a) the Exemption number;
   b) the holders name;
   c) contact numbers;
   d) the nearest Department of Fisheries Office; and
   e) the species and number of fish intended to be transported under the authority of this

Exemption; and
g) the registration of any vehicle/s used to transport fish under the authority of this Exemption.

2. The person in charge of the transportation must carry a copy of this exemption when transporting any fish under the authority of this exemption.

3. Only the species and numbers of each as shown in Table 1 may be transported under the authority of this exemption.

Table 1

Common Name	Scientific Name	Total Numbers
Hairy Marron	Cherax tenuimanus	44 - 22 male and 22 female
Smooth Marron	Cherax cainii	68 - 34 male and34 female

4. Collection only from the Pemberton Fisheries Research Centre.

5. Transportation to be carried out by vehicle in styrofoam eskies with coolbricks and layers of foam & or hessian as recommended by the staff at Pemberton Fisheries Research Centre.

6. Hairy marron remain the property of the Department of Fisheries, and should be made available for collection if at anytime they are required by the Department of Fisheries.

7. No other fish, other than those under the authority of this exemption, are to be transported during the same trip.

8. The Executive Director, Science and Resource Assessment Division of the Department of Fisheries can at his discretion revoke this exemption at any time.

9. Copies of reports resulting from this research must be forwarded to the Director of Science and Resource Assessment Division, Department of Fisheries, PO Box 20, North Beach WA 6920.

Dr WJ Fletcher
EXECUTIVE DIRECTOR
SCIENCE AND RESOURCE ASSESSMENT DIVISION

Dated this ***second*** day of ***June*** 2017