Molecular and genetic approaches to the study of colour variation in *Fenneropenaeus merguiensis*

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B.Sc. (Microbiology and Biotechnology)

Submitted in fulfilment of the requirements of the degree of Honours

Faculty of Science, Health and Education

University of the Sunshine Coast

December 2010

ISBN: 978-1-925982-56-5

Abstract

Body colouration, an important survival, mate selection and communication mechanism for animals in the wild, has also significant commercial implications. In aquaculture, a darker body colour in prawns can increase farm profitability by AU$ 2-4 dollar per kilo of prawns. Therefore, there is a strong commercial interest in increasing colour intensity of prawns grown in captivity. In this study, the focus was on *F. merguiensis*, and the determination of factors that could be involved in colour formation in this species.

Molecular techniques were employed to clone and isolate crustacyanin subunits, genes known to be responsible for colouration in other crustacean species, from the muscle/cuticle tissue of *F. merguiensis* prawns and to develop gene specific primers to quantify the levels of crustacyanin gene expression in the cuticle of prawns displaying three different colour phenotypes (albino, light and dark). Additionally to the gene expression studies, astaxanthin, the primary carotenoid that is responsible for the typical red/orange colour of the cooked prawn, was extracted and its levels in the albino, light and dark prawns analysed. To gain a broader understanding of the genes expressed in the muscle/cuticle tissue of *F. merguiensis* prawns, messenger RNA was isolated from these tissues and transcriptome analysis carried out using Roche 454 next generation sequencing. Analysis of the transcripts was performed with blast2go and the transcriptome sequences used to develop customised microarrays, which were deployed to assess the cuticle tissue of albino, light and dark *F. merguiensis* prawns for novel genes potentially involved in colour formation in this prawn species.

Crustacyanin subunits were shown to be significantly ($p < 0.01$) higher expressed in light coloured prawns compared to dark coloured prawns, while the opposite was found for the total astaxanthin levels. In albino prawns, crustacyanin gene expression levels and total astaxanthin levels were significantly ($p < 0.001$) lower than in light or dark prawns. In addition to the crustacyanin subunits, a suite of genes including arginine kinase, sarcoplasmic calcium-binding protein, some form of actin, troponin I and tropomyosin were found to be significantly down regulated in the cuticle tissue of albino prawns compared to lighter or darker prawns, suggesting a potential role of these genes in the formation or regulation of colour, possibly through involvement in pigment movement.

Further research into the genes highlighted in this study will increase our knowledge of colour formation in *F. merguiensis* prawns and other crustaceans.

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Abbreviations and Acronyms

%	percent
°C	degree Celsius
ADP	adenosine diphosphate
AGRF	Australian Genome Research Facility
ATP	adenosine triphosphate
AU$	Australian Dollar
BHT	butylated hydroxyl toluene
bp	base pairs
cAMP	cyclic adenosine monphosphate
cDNA	complementary deoxyribonucleic acid
cm	centimetre
cRNA	complementary ribonucleic acid
Ct	threshold cycle
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
E	reaction efficiency
e.g.	for example
EDTA	ethylenediaminetetraacetic acid
F. merguiensis	*Fenneropenaeus merguiensis*
FA	formaldehyde agarose
FAO	Food and Agriculture Organisation of the United Nations
g	gram
h	hour(s)
H. gammarus	*Homarus gammarus*
H_2O	water
His	Histidine
HRM	high resolution melting curve analysis

IPTG	isopropyl-beta-D-thiogalactopyranoside
kg	kilogram
$kg.ha^{-1}$	kilogram/hectare
LB-Amp	Luria-Bertani Broth-ampicillin
L-PGDS	lipocalin-type prostaglandin D synthase
M	slope of the curve
M. japonicus	*Marsupenaeus japonicus*
mg	milligram
MIH	moult-inhibiting hormone
min	minute(s)
MIQE	minimum information for publication of quantitative real-time
ml	millilitre
mm	millimetre
mM	millimolar
MSH	moult-stimulating hormone
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
ng	nanogram
nm	nanometre
NTC	negative template control
P. cygnus	*Panulirus cygnus*
P. monodon	*Penaeus monodon*
PCA	principal component analysis
PCR	polymerase chain reaction
PMT	photomultiplier
ppt	parts per trillion
qPCR	quantitative polymerase chain reaction
RIN	RNA integrity number
RNA	ribonucleic acid

RNase-free	ribonuclease-free
-RT	negative reverse transcription
SCR	structurally conserved regions
SD	standard deviation
SE	standard error
sec	second(s)
SOC	super optimal broth with catabolite repression
USC	University of the Sunshine Coast
UV	ultraviolet
V	volt
X-Gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
µg	microgram
µl	microlitre
µm	micrometre
µM	micromolar
ρmol	picomole

Acknowledgments

I would like to express my gratitude to my supervisors Professor Abigail Elizur and Associate Professor Wayne Knibb for their support, guidance, encouragement and willingness to share their extensive knowledge with me during the course of this honours project. I also would like to thank the other honours and PhD students in the research laboratory, as well as the postgraduates, technicians and our laboratory manager, Daniel Powell, for their support, help with questions and companionship throughout the year. I also would like to express my appreciation to Dr Peter Brooks for his help and support with the chemical side of my colour expression analysis.

A particular thank you goes to Dr Anna Kuballa who patiently and with great knowledge guided me through my honours year in the laboratory, always willing to answer my constant stream of questions.

I'm also grateful to the University of the Sunshine Coast for their generous scholarship that allowed me to continue my studies here in Australia, to the Seafood CRC for their scholarship that made all the expensive molecular work possible and to Dr Trevor Anderson, Dr Eric Boglio and Mr Courtney Remilton from Seafarm, which graciously provided me with the much needed samples for my research.

Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

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December 2010

1. Outline

Aquaculture is an important economic sector, adding a production gross value of $861.1 million (2008-2009) to the Australian economy (Pham 2010). With Asian countries like China or Thailand as strong competitors in this sector (Food and Agriculture Organization of the United Nations (FAO) 2009), the Australian aquaculture farms need to either increase their production volume or improve their product to obtain and hold a competitive edge. Quality improvement could include an increased size of the product, better taste or stronger or more appealing colouration of the animals reared in aquaculture environments. As consumers perceive colouration as an important characteristic in food (Latscha 1989), the following project will assess factors involved in colouration in *Fenneropenaeus merguiensis*, a commercially important prawn species, grown in aquaculture farms in Australia.

This thesis will be presented in the form of a traditional-write up, with the introduction and aims included at the end of the literature review. References will be formatted according to the Harvard referencing style from the University of Queensland, Australia.

2. Literature review

Nature's play with colours and different shades and patterns can have a variety of functions. In terrestrial animals, for example, colour can have a role in thermoregulation, with paler colours often found in desert areas where reflection of light is important, and darker colours in animals living in tropical areas, where they aid in enhanced water evaporation and UV light protection (Caro 2005, 2009). Colouration also functions as defence against predators by changing colour or pattern as can be seen in octopuses (Stuart-Fox et al. 2006) or by matching the background to conceal themselves (Caro 2009; Stuart-Fox & Moussalli 2009). Some spiders, for instance, mimic ants through their colouration, appearing as if they would have three instead of two segments. As ants are considered unpalatable by many predators, this proves to be a successful way of ensuring the spiders' survival (Théry & Casas 2009). Another way of predator protection is through warning colouration as can be observed in the poison arrow frog (Osorio & Srinivasan 1991), where the body colour signals that the animal is potentially toxic or distasteful (Caro 2009; Stevens & Merilaita 2009). Protection strategies, such as background adaptation of animals, however, can also be advantageous for the predator by disguising it from its prey, thus increasing the likelihood of catching the prey (Théry & Casas 2009). Additionally, colour plays an important role in mate selection and communication. For example, a brighter colour or specific colour patterns in some animals signal the potential partner that they are healthy, dominant, or have good reproductive qualities (Caro 2005; Stuart-Fox & Moussalli 2009; Taylor & McGraw 2007). This means that more colourful males are more likely to find a partner than less colourful ones. Examples for this are jumping spiders that preferentially mate with more intensely coloured males, or lionesses that favour lions with darker mane over males with a lighter one (Caro 2005; Taylor & McGraw 2007).

Similar to terrestrial animals, colouration in aquatic animals, such as prawns, appears to play a large role in protection from predators. Prawns in particular seem to have three main defence strategies against predators. First, most are able to change their colour intensity to adapt to their surroundings, camouflaging them in the process (Stuart-Fox & Moussalli 2009; Tume et al. 2009). Second, some bottom- and open water dwelling prawn species have a transparent body combined with disruptive colouration (colour markings that disguise the outline of the animal's body) that will make it difficult for the predator to discern these prawns (Caro 2005; Carvalho et al. 2006). Third, animals living in deeper water and having a red coloured body can become virtually invisible due to the absorption of red wavelengths that happens at the

water's surface. Additional to the protection from predators, colouration can also protect these aquatic animals from UV radiation, ensuring the continued health of the animal (Tlusty et al. 2009).

Whereas colour intensity is a means of survival for the wild prawn, at a commercial scale it determines the price at which the prawn can be sold. Prawns sold at the market usually are ranked according to a colour chart, with darker coloured prawns scoring higher than less intense coloured prawns due to customer perception and preference. As a higher score quite often means an increase in profit of about AU$ 2-4 per kilo of prawn (Tume et al. 2009), there is a substantial commercial interest in improving the colour of prawns reared in aquaculture farms. Therefore, to evaluate options for colour improvement in aquaculture prawns, this thesis reviews and summarizes different factors that have an effect on colour in prawns.

2.1 Carotenoids and colour

A variety of pigments exist in crustaceans, with the fat soluble carotenoids believed to be the most important ones in colouration (Chayen et al. 2003; Latscha 1989; Sachindra et al. 2005). Natural carotenoids that are responsible for a number of colours can be found in animals, plants and microorganisms (Anderson 2000; Sachindra et al. 2005; Weesie et al. 1999). However, animals are unable to produce these lipid soluble molecules, but can use carotenoids synthesised by plants, fungi and microorganisms (Anderson 2000; Mann et al. 2000; Ponce-Palafox et al. 2006). Uptake of these molecules usually happens through the ingestion of food rich with these pigments or by a symbiotic relationship with organisms that are able to produce them (Wade et al. 2009). Of the over 600 carotenoids known today (Ponce-Palafox et al. 2006), most are derivatives of the acyclic polyene called lycopene ($C_{40}H_{56}$), formed from the original molecule by processes such as chain elongation, insertion of oxygen or by hydrogenation (Latscha 1989). Natural carotenoids are usually found in the all-*trans* form, as this structure is more stable and less light sensitive than the *cis* form (Calo et al. 1995; Ilagan et al. 2005).

According to several studies, of the various carotenoids found in nature, astaxanthin appears to be the main pigment responsible for colour in crustaceans and more importantly in the prawn species *Fenneropenaeus spp.* (Ilagan et al. 2005; Latscha 1989; Sachindra et al. 2005; Tume et al. 2009; Wade et al. 2005), accounting for approximately 65 to 98 % of all the carotenoids in this species (Latscha 1989). Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) or $C_{40}H_{52}O_4$ is produced by microorganisms like *Phaffia rhodozyma* (red yeast), *Agrobacterium aurantiacum* or

Haematococcus pluvialis (green algae) and belongs to the group of xanthophylls (composed of hydrogen, carbon and oxygen) (Calo et al. 1995; López et al. 2004; Yuan & Chen 2000). Crustaceans take up astaxanthin through their diet, either from natural sources like green algae or in aquaculture through food supplements. After uptake the pigment is transported through the digestive system to the epidermis, where it is stored in subepidermal chromatophores (cells that contain the pigment) (Latscha 1989; López et al. 2004; Tlusty et al. 2009). Other than the epidermal layer, astaxanthin is also found in the exoskeleton, with the literature indicating that colouration observed in crustaceans is mainly due to the astaxanthin detected in these two locations (Tume et al. 2009; Wade et al. 2005; Wade et al. 2009). In fact, Wade et al. (2008) argued that without carotenoids supplied in the diet of clawed lobsters, colour formation in this animal would be prevented.

In wild and cultured crustaceans and penaeids (a family of prawns, such as *Fenneropenaeus merguiensis*) in particular, three stereoisomers of the pigment astaxanthin can be found; the optical isomers (enantiomers) (3S, 3'S)- and (3R,3'R)-astaxanthin and the diasteromeric *meso* form (3S,3'R)-astaxanthin (Figure 2.1) (Cyanotech 2001; Latscha 1989; Matsuno et al. 1984).

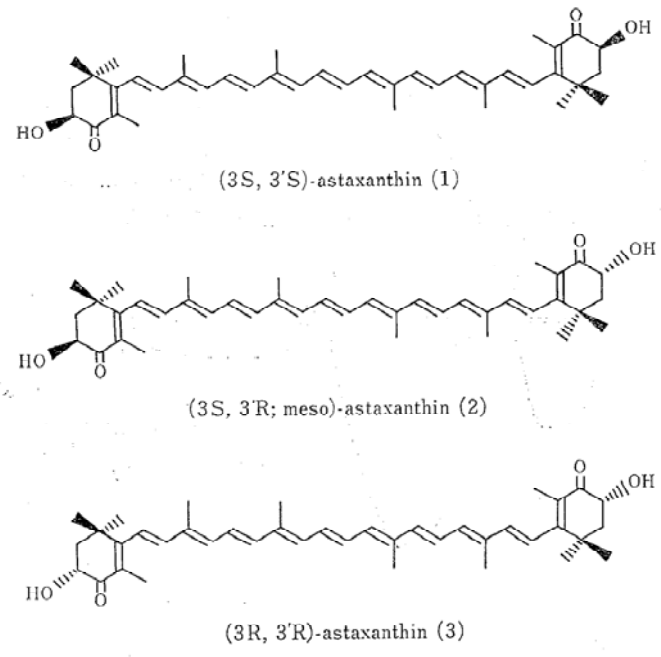

Figure 2.1 Molecular structure of the three stereoisomeric forms of astaxanthin (Matsuno et al. 1984).

This correlates with the composition of synthetic astaxanthin that is used as feed supplement and which consists of all three stereoisomers (Mann et al. 2000).

Interestingly, however, Mann et al. (2000) also point out that astaxanthin produced in nature from marine organisms is found to be in the (3S,3'S)-astaxanthin enantiomer form rather than the three stereoisomer mixture of the synthetically produced astaxanthin. The authors added that it has not been determined yet, what the significance of the astaxanthin stereoisomer form in the food source is.

According to Latscha (1989) and Tume et al. (2009), astaxanthin exists in three forms in prawns; as free astaxanthin, monoester and diester, with the astaxanthin esters generally being the main form found in the crustacean tissue. Wade et al. (2005) tested the distribution and levels of all three forms in red and white western rock lobsters and found a significantly higher level of non-esterified astaxanthin in red lobster shells compared to white lobster shells and significantly larger amounts of esterified astaxanthin in the red lobster epithelium compared to the white lobster. The authors concluded that due to its lipophilic character, the form astaxanthin takes influences where and how it will be incorporated in the lobster. Also, they suggested that a direct relationship exists between the levels of esterified astaxanthin and shell colour, thus making esterification an important factor in the control of shell colour in lobsters.

Another study by Tume et al. (2009) also observed a change from the free to the esterified astaxanthin form during light adaptation in prawns, with the pigment concentrated in the esterified form in brighter light conditions, and more dispersed in the free form during lower light conditions, causing lighter and darker colouration respectively. Additionally, it has been found that prawns have the ability to structurally modify carotenoids like canthaxanthin or β-carotene by forming, for example, hydroxy or keto groups to convert them into astaxanthin (Boonyaratpalin et al. 2001; Tlusty et al. 2009; Wade et al. 2005).

Additionally to the form astaxanthin takes (free or esterified), the quantity and distribution of astaxanthin also plays a role in the colour intensity of crustaceans (Tlusty et al. 2009). For example, Wade et al. (2008), Tume et al. (2009) and Stuart-Fox & Moussalli (2009) argue that crustaceans are able to change their colour by adjusting the amount of pigment in the chromatophores and exoskeleton (linked to diet and how much astaxanthin can be ingested), changing the amount of chromatophores present in a tissue and by dispersing or concentrating the pigment inside the chromatophores.

These findings indicate that the intensity of prawn colour depends not on one single factor, but on a variety of factors, such as the amount of astaxanthin ingested by the prawns, the form it takes, where it is stored and how spread out or concentrated the pigment is. Also, when derivatives of the pigment are fed to the prawns, binding

properties of the molecules can be different. For example, Anderson (2000) pointed out that astaxanthin can bind to two areas in the muscle, compared to canthaxanthin that has one weaker binding location, making the bond less stable during processes such as freezing.

2.2 Crustacyanin and astaxanthin

Crustacyanin, which forms a complex with the ingested astaxanthin, is another important factor involved in prawn colouration. Crustacyanin is a protein that is expressed in crustaceans and binds to astaxanthin, forming a carotenoprotein complex. This complex stabilizes the highly reactive pigment, while in turn the pigment provides stability to the proteins' tertiary and quaternary structure (Mantiri et al. 2004; Wade et al. 2009). By binding to the protein, a water-soluble complex is produced that allows the carotenoprotein complex to be transported through aqueous environments and across cell membranes (Mantiri et al. 2004; Weesie et al. 1997). Czeczuga et al. (2005) detected three types of carotenoid and crustacyanin complexes, α-, β-, and γ-crustacyanin, each of them bound to the pigment astaxanthin, with, for example, α- and β-crustacyanin found in *Palaemon adspersus* and γ-crustacyanin in *Crangon crangon*. However, the authors pointed out that α-crustacyanin was the prevalent form they found in crustaceans.

A consensus in the literature indicates that the β-crustacyanin form is comprised of two protein subunits, bound to two astaxanthins shared between the proteins (Figure 2.2). Eight of these β-crustacyanin heterodimeric molecules then bind irreversibly with each other to form α-crustacyanin, which carries 16 astaxanthin molecules in total (Chayen et al. 2003; Cianci et al. 2002; Keen et al. 1991; Wade et al. 2009; Weesie et al. 1999). In the lobster *Homarus gammarus*, two types of protein subunits were detected, type 1 and type 2, also referred to as crustacyanin C and crustacyanin A respectively in the literature. Type 1 includes protein subunits A1, C1 and C2 and type 2 is comprised of subunits A2 and A3, with each subunit being able to bind one astaxanthin molecule. Different combinations of type 1 with type 2 protein subunits then combine to form a β-crustacyanin heterodimer (Chayen et al. 2003; Cianci et al. 2002; Ilagan et al. 2005; Keen et al. 1991; Wade et al. 2009).

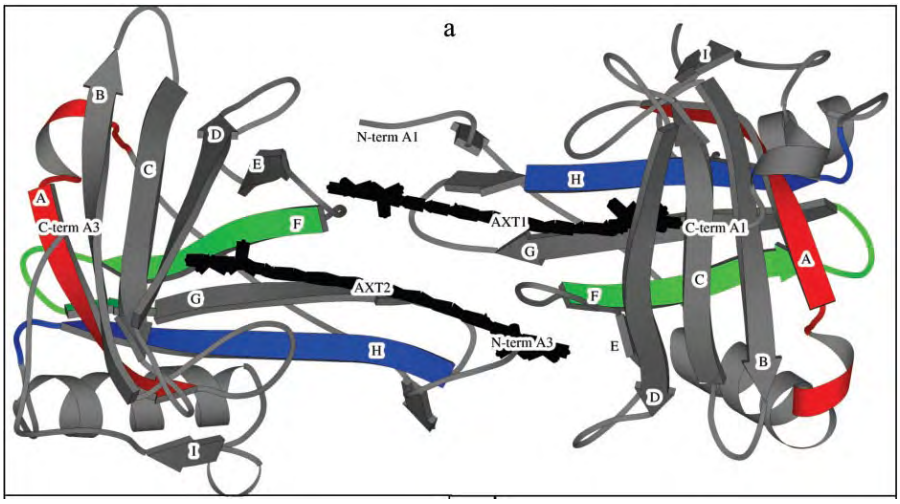

Figure 2.2 Schematic of a crustacyanin dimer, formed with one A3 and one A1 subunit, binding two astaxanthin (AXT) molecules. Matching blue, green and red colours indicate regions of consensus (Cianci et al. 2002).

According to research into the crustacyanin complexes in *H. gammarus*, the form of the astaxanthin molecules bound to β- and α-crustacyanin is subject to certain restrictions. For example, Chayen et al. (2003), Cianci et al. (2002) and Weesie et al. (1997) argue that for a successful non-covalent binding of the astaxanthin molecule, the size and form of the pigment cannot vary greatly. Also, they indicate that only all-*trans* astaxanthins with keto and methyl groups at correct positions can bind to the protein. Furthermore, they suggest that the keto groups found on positions 4 and 4' play an important role in forming the coloured β-crustacyanin carotenoprotein and that the methyl groups located on positions C20, C19 and C20', C19' are important in holding the chromatophore, in which it is enclosed, in its correct form and position (Figure 2.3).

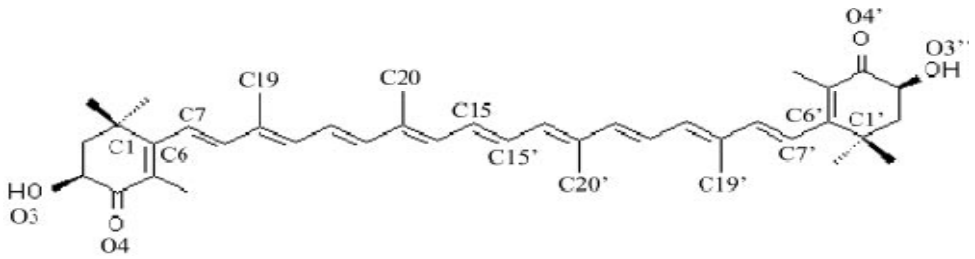

Figure 2.3 Molecular structure of the centrosymmetric astaxanthin with the locations for the keto and methyl groups labelled O4, O4' and C19, C20, C20' and C19' respectively (Cianci et al. 2002).

Chayen et al. (2003) and Cianci et al. (2002) also indicate that the two astaxanthin molecules found in a β-crustacyanin complex are shared equally between the two protein subunits, with each protein subunit being bound to the C1 – C6 ring of one astaxanthin molecule and to the C1' – C6' ring of the second astaxanthin molecule

(Figure 2.3). As Chayen et al. (2003) and Helliwell (2010) outline, both rings are bound to the protein subunits through hydrogen bonds, with the C4 keto group of the astaxanthin carotenoid binding to a water molecule in the protein, while the C4' keto group associates with a His residue of the protein subunit.

Upon binding of the astaxanthin molecules with the protein subunits, a large bathochromic shift can be observed that moves from an absorption wavelength of approximately 480 nm of the unbound astaxanthin to around 632 nm for the bound astaxanthin molecule. A great amount of research has been invested so far to define the factors that cause this roughly 150 nm large spectral shift. Furthermore, most of this research appears to have been done on the β-crustacyanin complex in *H. gammarus* that is comprised of the protein subunits A_1 and A_3 (Chayen et al. 2003; Helliwell 2010; Ilagan et al. 2005; Weesie et al. 1999).

In order to determine the factors involved in the 150 nm spectral shift, a model of crustacyanin and the bathochromic shift in the lobster *H. gammarus* has been built and discussed by several authors (Chayen et al. 2003; Keen et al. 1991; Weesie et al. 1999). It was established that the bathochromic shift observed in this crustacean was from the orange-red of the astaxanthin molecule (wavelength ~ 480 nm) to the blue colour of the crustacyanin-astaxanthin complex in the living lobster (absorption of β-crustacyanin at ~ 590 nm, of α-crustacyanin at ~ 630 nm) to a red colour of varying intensity in the cooked lobster (Cianci et al. 2002; Ilagan et al. 2005; Weesie et al. 1997).

Weesie et al. (1999) tested a proposed model for the shift in which the bound astaxanthin was twisted around its double bonds. This twisting appears to be a structural change that happens when the carotenoid binds to the protein and was believed to be one of the main reasons for the spectral shift observed (Helliwell 2010; Ilagan et al. 2005). However, while Weesie et al. (1999) were unable to prove this theory, Ilagan et al. (2005) indicate that the structural change of the pigment could play a role in the spectral shift, albeit only a small one. Furthermore, Ilagan et al. (2005) suggest that the bathochromic shift is mainly caused by dimerization of the pigment in the α-crustacyanin complex. On the other hand, Weesie et al. (1999; 1997) argued that full protonation of both keto functionalities of the astaxanthin molecule could be the main reason for the bathochromic shift in the lobster. In comparison, Cianci et al. (2002) and Helliwell (2010) indicate that a variety of factors play a role in the spectral shift observed. For example, they suggest that by bringing the two end rings of the pigment into one plane with the polyene chain of the pigment, the conjugation (two p-

orbitals overlap and allow delocalisation of pi electrons across the aligned orbitals) will be extended, allowing an increase in the delocalisation of the electrons, which will lower the energy gap between ground and excited state of the molecule and contributes to the spectral shift (Cianci et al. 2002; Helliwell 2010; Weesie et al. 1999). Another important factor according to Cianci et al. (2002) and Helliwell (2010) is polarization of the astaxanthin due to the hydrogen bonds the keto groups at the end rings of the carotenoid form with the water molecule bound to the protein subunits and the His residue of the subunits. Helliwell (2010) also adds that exciton reactions between the two bound astaxanthin molecules could have an additional effect on the bathochromic shift.

Researchers have also attempted to determine the tissues in which the crustacyanin complexes can be found. For instance, Tlusty et al. (2009) describes how astaxanthin is transported through the digestive system into the epidermis of the lobster *Homarus americanus*, from where it is transferred into the endo- and exocuticle, where two protein subunits bind to the astaxanthin molecules to form β-crustacyanin. After binding, the complex is moved into the epicuticle where α-crustacyanin is formed. In *Penaeus japonicus*, α-crustacyanin with an absorbance wavelength of 640 nm was detected in the hypodermis of the animal (Muriana et al. 1993). Furthermore, Wade et al. (2009) observed α-crustacyanin in the exocuticle and the outer epithelium of the lobster *Panulirus cygnus* (Figure 2.4).

Little knowledge exists regarding the mechanism that is used to transport the pigment to the epithelium in crustaceans. Nevertheless, studies on cats, dogs and Atlantic salmon indicate that after ingestion and digestion, astaxanthin is found in the duodenum bound to micelles together with fatty acids, bile salts and other fat-soluble molecules. Due to the hydrophobic nature of astaxanthin, it is believed that these micelles physically contact the mucosal cells in the duodenum to facilitate the passive diffusion of the pigment through the cells (Aas et al. 1999; Park et al. 2010; Parker 1996; Rajasingh et al. 2006). From the mucosal cells, astaxanthin is transported to the blood via chylomicrons (one of the four main lipoprotein classes) (Parker 1996; Rajasingh et al. 2006; Rang et al. 2007). In the blood, astaxanthin is thought to be associated with high density lipoproteins (HDLs) as well as albumin which carry the pigment to the liver or the muscle cells (Østerlie et al. 1999; Park et al. 2010; Rajasingh et al. 2006). HDL is then believed to be responsible for transporting the pigment from the muscle cells to the skin (Aas et al. 1999; Rajasingh et al. 2006). Although these pathways of absorption and transport were observed in species other than crustaceans, the literature reviewed appears to describe the same pathways across the

different species, making it likely to be relevant in crustaceans as well. Additionally, Rajasingh et al. (2006) indicate, that the transport of astaxanthin through the body of Atlantic salmon follows a similar pattern in other marine species.

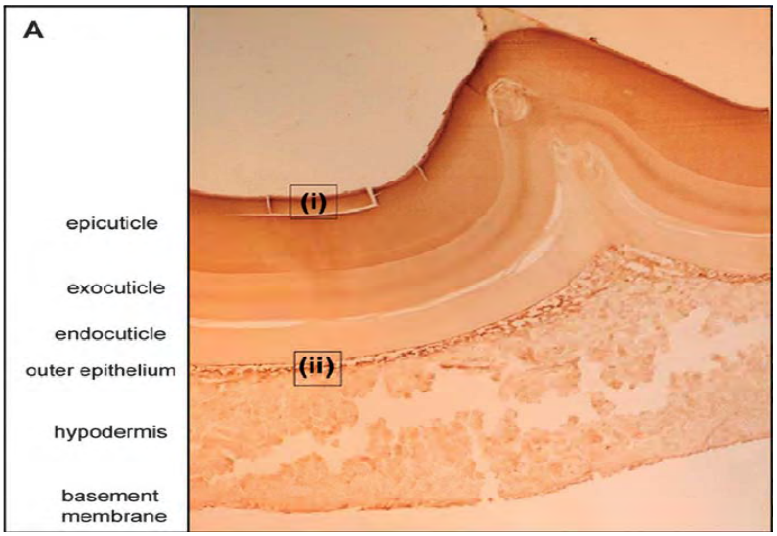

Figure 2.4 Histological image of the shell and epithelium of the lobster *Panulirus cygnus*, with (i) and (ii) indicating where α-crustacyanin was detected (Wade et al. 2009).

The non-covalent bond connecting the astaxanthin molecules with the protein subunits can be cleaved by cooking or dehydration (Cianci et al. 2002). Both methods result in a denaturation of the carotenoproteins, which relaxes the hydrogen bonds that hold the carotenoid, causing the release of the astaxanthin (Cianci et al. 2002; Helliwell 2010). According to Ponce-Palofox et al. (2006) and Anderson (2000), this released astaxanthin imparts the characteristic red colour on the cooked crustacean, with the strength in colour depending on the level of astaxanthin that had been bound to the protein. Furthermore, while denaturation of the protein is reversible in dehydrated tissue, cooking appears to irreversibly break the hydrogen bonds between the protein and its carotenoid (Cianci et al. 2002).

Additionally to the information gained about the bathochromic shift in crustaceans, modelling crustacyanin and its binding properties enabled researchers to confirm crustacyanins' affiliation with the lipocalin superfamily along with proteins such as apoliprotein D, insecticyanin or bilin-binding protein (Cianci et al. 2002; Wade et al. 2009). These proteins are all known for their high structural but low sequence similarity, with up to three short regions in their amino acid sequence generally conserved (Grzyb et al. 2006), a property which Wade et al. (2009) used, combined with other methods, to screen 58 different animal species such as *Daphnia*, *Drosophila* and *Panulirus* for the presence of crustacyanin.

Although extensive work has been done on the binding of astaxanthin to crustacyanin and the crystal structure of the carotenoprotein in lobster, little is known about crustacyanin in other crustaceans, especially in *Penaeus* (Chayen et al. 2003; Wade et al. 2009). In addition, most research appears to centre on models of crustacyanin, with hardly any information on the nucleic acid structure of the crustacyanin subunits available for any crustacean species; in particular, none appears to exist for *Fenneropenaeus merguiensis*.

2.3 Moulting and colouration

Another factor that could have an effect on colouration in crustaceans is the moult stage the individual animal is in. Moulting, which is important for the animals growth, is a cyclic event in a crustacean's life that includes the degeneration of the old cuticle, the synthesis and hardening of a new cuticle and the shedding of the old exoskeleton (Hoang et al. 2003; Kuballa & Elizur 2008; Musgrove 2000; Promwikorn et al. 2004). Throughout the moult cycle, a variety of changes can be observed in the crustaceans, such as structural and molecular modifications, as well as changes in the behaviour of the animals (Graf 1986; Longmuir 1983; Promwikorn et al. 2004). A series of factors have been identified that influence the moult cycle in crustaceans. These factors range from the gender of the animal to its developmental stage, with the most influential one being the temperature of the water in which the animals live (Hoang et al. 2003). All these factors appear to have an effect on the hormones responsible for the control of the moult cycle. Two types of hormones have been recognised, a moult-stimulating (MSH) and a moult-inhibiting hormone (MIH) (Hoang et al. 2003; Promwikorn et al. 2004). MSH, also called ecdysone, is synthesised by an endocrine gland called Y-organ and transformed into 20-OH-ecdysone in various tissues and organs. The function of this hormone is to stimulate the retraction of the epidermis from the cuticle (apolysis) as well as the shedding of the old cuticle (ecdysis or exuviations). MIH on the other hand regulates/inhibits the synthesis and secretion of ecdysone and is produced by the X-organ, found in the eye-stalks (Chang et al. 1993; Graf 1986; Hoang et al. 2003; Promwikorn et al. 2005; Yudkovski et al. 2010). However, as Hoang et al. (2003) points out, the exact way both hormones interact with each other to regulate moulting in the animal is still unclear.

The moult cycle in crustaceans can be separated into four main stages; postmoult (A and B stage), intermoult (C stage), premoult (D stage) and ecdysis or exuviations (E stage) (Graf 1986; Kuballa et al. 2007; Musgrove 2000). Furthermore, according to Musgrove (2000), every moult-stage, except the ecdysis stage, can be split into

substages, with the number of substages varying with the species observed. In general, in postmoult the new cuticle is formed and hardened, with the epidermis attached to the cuticle. Also, during postmoult, a slight colouration of the cuticle can be seen. During intermoult, the colour of the cuticle has fully developed and the maximum strength of the cuticle has been reached. Once the intermoult stage has finished, the animal enters the premoult stage in which the epidermal tissue starts to retract from the cuticle, the organs and tissues prepare for exuviations and the formation of the new cuticle starts below the old one. Additionally, parts of the old exoskeleton like some proteins, chitin and calcium will be digested, reabsorbed and stored by the animal while it is in premoult. During ecdysis, the last stage in the moult cycle, the animal sheds its old cuticle (Graf 1986; Kuballa et al. 2007; Longmuir 1983; Promwikorn et al. 2004; Yudkovski et al. 2010).

Although Longmuir (1983) disagreed with prior research that determined intermoult as a stage of no morphological change, more recent research has supported that intermoult was a relatively stable phase with the formation of the new cuticle complete and only very low levels of moult related activity observed (Promwikorn et al. 2005; Promwikorn et al. 2004; Yudkovski et al. 2010).

A variety of methods are used for moult-staging crustaceans. Graf (1986) suggested histological examination of the integument, which he believes to be a very accurate but lengthy and difficult method of moult-staging. Other methods proposed are a physical determination of the carapace hardness and the observation of the setal development and epidermal retraction (Graf 1986; Longmuir 1983; Promwikorn et al. 2004).

Although differences in colouration were seen by Graf (1986) across the moult stages, with the strongest colour found in the intermoult stage, no information has been found in the literature that outlines the exact implications of the non-intermoult stages on colouration in the respective animal. However, considering the location of the carotenoprotein and the characteristic degeneration of the old cuticle and synthesis of the new cuticle during moult, it is likely that moulting has an effect on colouration in crustaceans.

2.4 Novel genes and colour

Genes not previously associated with colouration in crustacean, might also play a role in colour formation. For instance, Lijavetzky et al. (2006) described a variety of genes that are involved in the anthocyanin (pigment) biosynthesis pathway in table grapes, as well as different alleles of VvMybA1, a Myb gene that has been strongly indicated in

table grape skin colouration. This gene appears to be an essential regulator of the expression of one of the biosynthesis pathway genes. Similar to the anthocyanin biosynthesis pathway genes found in the table grapes, Liu et al. (2003) observed genes necessary for the synthesis of the pigment lycopene in the tomato fruit. In their research, the authors determined 19 regions in the genome that influence the strength of the red colour in the tomato fruit. They also indicate that a variety of other genes that are not involved in the pigment synthesis pathway directly play a role in colouration. These genes are believed to impact positively on the amount of plastids (synthesise and store the pigment) and pigment produced, and they are thought to regulate the accumulation of lycopene. Additionally, Liu et al. (2003) indicated that mutations caused during ripening might also play a role in the formation of colour.

In flowers, Aida et al. (2000) and Mol et al. (1999) observed genes important in the anthocyanin biosynthesis pathway like chalcone synthase or dihydroflavonol-4-reductase, as well as genes that encode a variety of cytochromes that act together to hydroxylate the β-ring in anthocyanin, causing the flower colour to turn a shade of blue. In addition, Mol et al. (1999) found ten genes that are associated with a regulatory role in the pH level of the flower plants vacuoles, which the authors also linked to a colour shift into the blue range.

Billingham & Silvers (1960) and Little (1958) on the other hand based their research around the colour variation found in animals. They concentrated on melanocytes and melanin and the genes involved with skin and hair colouration. Billingham & Silvers (1960) had the focus of their research on the pigmentation in mice and at the time of publication, they had observed 25 loci with over 35 genes that they believed to be of importance in coat colouration. They also pointed out that about 20 of these genes were thought to have additional functions aside from colour formation. Some of the genes they detected appear to function in controlling the amount and type of pigment synthesised, the availability of products necessary for pigment formation, the way in which the pigment granules are deposited, as well as their shape and size.

While Billingham & Silvers (1960) only looked at mouse colouration, Little (1958) observed coat colour in a larger range of animals such as rats, rabbits, guinea pig and dogs and also concluded that a multitude of genes are responsible for coat colouration in the animals more thoroughly researched. Like Billingham & Silvers (1960) he described a variety of genes and their alleles directly and indirectly (through regulating gene expression) involved in colour formation.

No research has been invested so far into determining the existence of novel genes directly involved in colour formation along with crustacyanin in crustaceans or into genes that might have an effect on colour in crustaceans through regulating the expression of crustacyanin, by modifying the pigment astaxanthin or by affecting the storage capacity, size, amount or distribution of chromatophores in the crustacean tissue.

2.5 Other factors responsible for colouration

In addition to the importance of the crustacyanin-astaxanthin complex in colour formation, factors such as hormones, background colour or light intensity play a role in colour development in crustaceans. Fingerman and Fingerman et al. (1969; 1994) have found that the chromatophores that are dispersed mainly through the integument are controlled by two hormones, a pigment-dispersing and a pigment-concentrating hormone. The release of both hormones is believed to be triggered by the stimulation of the X-organ SG complex and the postcommissural organ with neuromodulators such as 5-Hydroxytryptamine or dopamine (Fingerman et al. 1998; Fingerman et al. 1994; Pinder et al. 1999). It was also proposed that these hormones act on the cell membrane of the chromatophores instead of entering the cell (Fingerman 1969). Additionally, the concentration and dispersion during light and dark adaptation, respectively, observed by Tume et al. (2009) is believed to be controlled by the two hormones described above (Fingerman et al. 1994; Pinder et al. 1999).

Other factors playing a role in crustacean colour formation are background colour of the pond and light conditions (Tlusty et al. 2009; Tume et al. 2009). Tlusty et al. and Wade et al. (2009; 2005) agree that lighting and dark background colour result in a more intense colour in the crustaceans affected. In particular, Tlusty et al. (2009) discovered that exposure of the American lobster to UV light had a greater effect on colour intensity than background adaptation, however, when UV was absent, the lobsters responded to the dark pond background with an increase in colouration.

2.6 Importance of colour in the context of this research project

All the factors outlined above have been shown or could be indicated to affect the colour observed in wild and farmed crustaceans and/or other animals. Research so far has concentrated on the different forms of astaxanthin and its storage and binding to crustacyanin as well as on models of crustacyanin built by association with the lipocalin superfamily and on crystal structures obtained from various lobster species. Furthermore, research into the prawn species that are commercially important to

Queensland and the various factors that might control colouration in these marine animals is still in its early stages.

The main prawn species considered commercially important in Queensland are *Penaeus monodon* (black tiger prawn), *Fenneropenaeus merguiensis* (banana prawn) and *Marsupenaeus japonicus* (kuruma prawn) (Lobegeiger & Wingfield 2010). These three prawn species can be distinguished from each other through a variety of characteristics, with one of them being their very distinctive body colouration. For instance, *P. monodon* is known for its greenish grey to brown body colour with discernible dark grey or brown and light yellow transverse banding pattern across the animals' abdomen, as well as a transverse band in a light yellow colour toward the end and often also in the middle of the carapace (the head of the prawn) (Figure 2.5a). Similar to *P. monodon*, *M. japonicus* also shows distinctive transverse bands of a dark brown or red brown colour along the abdomen and the uropods (tail of the prawn). Also, the rostrum (the spike like part above the eyes) and the carapace are transversely banded, with the non-banded part of the body in a light yellow or pinkish colour (Figure 2.5c). Compared to the other two commercial species, *F. merguiensis* is known for a completely different body colour pattern. The body colour of this prawn is of a green-greyish, pinkish or light yellow colour, with no banding but green-grayish small spots or speckles spread over the whole body of the prawn (Figure 2.5b) (Dall 1957; Fischer & Bianchi 1984).

Figure 2.5 Body colour pattern of the three commercially important prawn species in Queensland. **a)** *P. monodon*, picture courtesy Prof. Abigail Elizur, **b)** *F. merguiensis*, picture courtesy of Seafarm **c)** *M. japonicus*, picture courtesy of Dr Peter Duncan.

In Queensland, *P. monodon* and *F. merguiensis* accounted for approximately 35% and 37% of the 6274 tonnes and $72.9 million respectively of the total wild prawns caught in 2008-09. Moreover, all three commercial prawn species reared in aquaculture farms in Queensland, were responsible for most of the about 4000 tonnes of prawns produced through aquaculture in Australia in 2008-09 (Pham 2010). Compared to 2007-08, the amount of the three commercial prawn species caught in the wild and produced in aquaculture farms in Queensland rose substantially in 2008-09. In detail, the catch rate of *P. monodon* and *F. merguiensis* in the wild rose by 20% (Pham 2010), and aquaculture production rate of all three prawn species increased by 30% and 32% to 3821 tonnes and $54.6 million respectively in Queensland (Lobegeiger & Wingfield 2010). This indicates that the demand for these three prawn species has also increased since 2007-08. However, while demand and production have grown, the aquaculture farms operational in Queensland declined from 25 to 22 farms in 2008-09 (Lobegeiger & Wingfield 2010). As aquaculture is similar to any other type of business, production has to be profitable and competitive with products from other farms nationally and more importantly with imported products from overseas such as large prawn producers from Asia (FAO 2009). However, the loss of three aquaculture farms from 2007-08 to 2008-09 suggests that, additional to the global economic crisis, competition has become stronger and that the affected farms were not profitable enough to keep the farms operational. So in order to protect the remaining aquaculture farms, strategies have to be developed that will increase their harvesting yield as well as the quality of the product to stay competetive in this business. As customer perception very strongly influences their purchaser behaviour, Queensland aquaculture farms might not necessarily have to try and compete with the large producers such as Thailand or China solely by raising through-put on the farms. Instead the farms could improve traits that appeal to the customer and so set their product apart from the prawns produced in other countries. For instance, Latscha (1989) points out in his study that customers generally consider the colouration of a product as one of the main traits, as purchasers often link colour to the quality of the product. Considering this, as well as the unusual body colour pattern of *F. merguiensis*, one of the main commercial prawn species in Queensland, efforts were extended in this study to understand the factors behind colouration in *F. merguiensis*, with the final aim of giving the aquaculture farms in Queensland a tool for selecting the best broodstock to produce a high quality product that will be appealing to the buyers as well as set these prawns appart from the prawns sold by other countries. Furthermore, the price for *F. merguiensis* is dependent on the intensity of the prawns' body colour (increase in colour intensity can raise the price for these individuals up to AU$ 2 to 4 per kilo) and an increase in the body colour

of the sold product can be directly translated into an increase in profit for the aquaculture farm selling these prawns (Tume et al. 2009).

So in order to gain an understanding of the genes involved in colour formation in *F. merguiensis*, genetic factors responsible for colour variation (the different body colour intensities observed in the prawns) in this species need to be determined. To achieve this, the study concentrated on the following specific aims:

- To isolate the crustacyanin subunit A and C sequences in *F. merguiensis*.
- To use quantitative PCR to determine crustacyanin gene expression levels in the cuticle tissue of *F. merguiensis* individuals of varying colour.
- To determine the levels of the pigment astaxanthin in *F. merguiensis* individuals of varying colour.
- To carry out transcriptome sequencing and analysis to gain an understanding of the genes actively expressed in the muscle/cuticle tissue of *F. merguiensis*.
- To development a customised microarray by using the data obtained from the transcriptome and to use the array to find novel genes in the cuticle tissue of *F. merguiensis* that could be involved in colouration in this prawn species.

3. Materials and Methods

This project consists of four research sections, a) isolation of the crustacyanin subunit A and C transcript sequences, b) absolute quantitative polymerase chain reaction (qPCR) to measure gene expression levels of crustacyanin and spectrophotometric colour analysis, c) next generation sequencing (transcriptome) and analysis of the cuticle/muscle tissue, and d) microarray development and analysis of prawns displaying different colour intensities. All four components link into each other and will have similar sampling and analysis methods, so the description of the procedures for all four sections will be combined in this methodology. Also, in the context of this study, the term “cuticle” refers to the endocuticle and outer epithelium (Figure 2.4).

3.1 General sample information

All samples used in this study were raw, freshly collected *Fenneropenaeus merguiensis* (*F. merguiensis*) prawns, generously provided by Seafarm, Cardwell/Mossman. Before collection, the prawns were grown in open, aerated ponds at the Seafarm Cardwell grow-out site under the conditions detailed below.

Grow-out ponds at the Cardwell and Mossman site varied in size from 0.1 ha to 2 ha (Figures 3.1 and 3.2), with water temperatures ranging between 19 ºC and 35 ºC (mainly around 22 ºC - 29 ºC) and a salinity of 15 ppt to 36 ppt (mostly between 22 ppt - 32 ppt). Water for the ponds was extracted from a creek that is connected to the Hinchinbrook channel and filtered by shadecloth socks or 500 µm filters. As necessary, up to 30 % of the pond water was exchanged every day. Turbidity of the ponds was measured with a Secchi disk and could vary from 15 cm to 50 cm Secchi depth, but efforts were exerted to keep the level at 25 cm. One box of a non-toxic aquatic blue dye (Nuturf, Australia) was added to each pond to initially darken the ponds to inhibit the growth of benthic algae until diatoms and green algae growth reached a level of coverage to protect the pond environment.

During the first 9 to 15 weeks, all *F. merguiensis* prawns were fed with a starter feed (CP or Ridleys, Australia) that did not have any astaxanthin added, until the prawns had reached a weight of approximately 7 g. When the prawns weighed about 7g, the feed was switched to a grower feed (CP or Ridleys, Australia) that contained 30 ppm/kg astaxanthin. Approximately 4 times daily, up to 300 $kg.ha^{-1}$ of the feed was blown into the ponds from all sides until the prawns reached harvest size (starting with a body weight of about 15 g). Harvesting was done by removing the monk board and collecting the prawns in a net. Once the prawns were harvested, up to 450 kg of

prawns were placed into large bins and 1 ml/l Prawnfresh (Xyrex, Ireland) added to the harvested prawns (contains the antioxidant 4-hexylresorcinol that inhibits melanosis). The bins were then kept at 13 ºC – 16 ºC for up to 2 h before freezing at the Cardwell site, and at 0 ºC for 8 – 14 h before freezing at the Mossman site. All *F. merguiensis* prawn samples sent to the research laboratory at the University of the Sunshine Coast (USC) were collected during harvesting periods to minimise the stress on the animals in the individual ponds.

Figure 3.1 Aerial picture of the prawn farm in Cardwell (courtesy of Seafarm).

Figure 3.2 Close-up of the prawn farm ponds with the aerators (courtesy of Seafarm).

3.2 Isolation of the crustacyanin complementary DNA (cDNA) sequences

3.2.1 Sample collection

Muscle and cuticle tissue from four *F. merguiensis* prawn samples, immersed in RNAlater™ (Ambion, Austin, TX) in individual tubes were sent by truck from Seafarm, Cardwell to the USC research laboratory. On arrival, the samples were placed for short-term storage at -20 °C until processed.

3.2.2 RNA isolation, quality control and quantification

Total RNA was extracted from the epithelial and muscle tissue (1-2 mm, 27-29 mg) of two randomly chosen prawns, using the RNeasy Plus Mini kit (Qiagen, Victoria, Australia) according to manufacturers guidelines (RNeasy Plus Mini Handbook 10/2005, including optional steps, http://www.qiagen.com). Homogenisation of the samples was performed by using two RNase free rotor-stator homogenisers (Qiagen, Victoria, Australia), and the extracted RNA eluted in 40 µl of RNase-free water (G Biosciences®, St. Louis, USA). The eluted RNA was then aliquoted out into two tubes of 20 µl each and stored at -80 ºC.

To test the integrity of the extracted RNA, a 1.2 % formaldehyde agarose (FA) denaturing gel was prepared by mixing 0.6 g of agarose (Sigma-Aldrich, Castle Hill, Australia) with 5 ml of 10x FA gel buffer (a total volume of 1 l was prepared by mixing 41.9 g of 3-[N-morpholino] propanesulfonic acid with 4.1 g of Na-acetate $3H_2O$ and 20 ml of 0.5 M EDTA and adjusting the pH of the buffer with NaOH to pH 7.0; all reagents were from BioLab, Victoria, Australia) and 45 ml of diethyl pyrocarbonate (DEPC) water (Sigma-Aldrich, Castle Hill, Australia; 1 ml per 1 l MilliQ water) in a conical flask. The mixture was then heated in a microwave until the agarose had completely dissolved. After cooling the liquid down to approximately 65 ºC, 0.5 µl of ethidium bromide (10 mg/ml, Sigma-Aldrich, USA) and 0.9 ml of 37 % formaldehyde (Amresco®, Ohio, USA) were added, before the solution was poured into a gel plate and left to set. After solidification, the gel was placed into a gel electrophoresis tank filled with 1x FA gel running buffer (produced by combining 50 ml of 10x FA gel buffer with 10 ml of 37 % formaldehyde and 440 ml of DEPC water) and the gel equilibrated for 30 min at 60 V.

1 µl of 5x loading buffer (Promega, USA) was added to 4 µl of each eluted RNA sample, as well as to 1.5 µl of RNA ladder (Promega, USA). The sample-dye and ladder-dye mixtures were then incubated for 5 min at 65 ºC, chilled on ice and loaded into separate wells of the equilibrated gel. After running the gel for 90 min at 60 V,

visualisation and photographing of the gel bands was done in a transilluminator (Syngene GeneGenius, England).

For quantification of the RNA samples, one quartz capillary (0.5 mm pathlength, 3 µl working volume, Amersham Bio Sciences, Sweden) was filled about two-third with RNase-free water as a blank sample, and two more capillaries with the respective samples and measured with the GeneQuant-pro spectrophotometer (Amersham Bio Sciences, Sweden) with a path length setting of 0.5 mm, µg/µl, dilution factor of 1 and a wavelength of 260/280 nm.

3.2.3 cDNA synthesis

Four 0.2 ml eppendorf tubes were set up for the cDNA synthesis, two for each of the two eluted RNA samples, with one tube set up for cDNA synthesis with random hexamer primers (50 ng/µl, Invitrogen Life Technologies, Victoria, Australia) and one with Oligo$(dT)_{20}$ primers (50 µM, Invitrogen Life Technologies, Victoria, Australia). cDNA was synthesised from 1 µg of total RNA, using the SuperScript™ III First-Strand Synthesis SuperMix Kit (Invitrogen Life Technologies, Victoria, Australia) according to the manufacturer's guidelines (http://www.invitrogen.com). The synthesised cDNA was then stored at -20 °C.

3.2.4 Degenerate primer development

As crustacyanin subunits A and C had not been previously isolated from *F. merguiensis*, degenerate primers had to be designed for both subunits. Suitable primer regions were determined by comparing the amino acid sequences of the crustacyanin gene subunits A and C in different crustacean species with each other to define conserved areas in the two subunits. Protein sequences for primer development were downloaded from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) and aligned, using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Development of the crustacyanin subunit A primer pairs was achieved by aligning *Marsupenaeus japonicus* and *Penaeus monodon* (Accession numbers ACL37116 and ACL37117 respectively for primer pairs A1, A2 and A3) and *Litopenaeus vannamei* and *M. japonicus* (Accession numbers DQ858916 and ACL37116 for primer pair A4) protein sequences with each other to determine possible primer regions (Table 3.1). Crustacyanin subunit C primer pairs were designed by aligning amino acid sequences of *M. japonicus* with *Cherax quadricarinatus* (Accession numbers ACL37122 and

ACL37121 for primer pair C1) and *M. japonicus* with *P. monodon* (Accession numbers ACL37122 and ACL37123 for primer pair C2) (Table 3.1).

Primers were synthesised by GeneWorks (Hindmarsh, Australia). A stock solution of 100 µM was prepared for each primer by re-dissolving the primers with RNase-free water, as well as a 10 µM working solution of the primers, which was needed for polymerase chain reactions (PCRs). Primer stock and working solutions were stored at -20 ºC.

Table 3.1 Degenerate primers for crustacyanin subunits A and C.

Primer Name	Primer sequence*	Tm (ºC)	Crustacyanin subunit
C1F	5' CCNAAYCCNTTYGGNGARCCNCA 3'	60	C subunit
C1R	5' TGNGGYTCNCCRAANGGRTTNGG 3'	60	C subunit
C2F	5' GAYACNGAYTAYGARAA 3'	41	C subunit
C2R	5' TTYTCRTARTCNGTRTC 3'	41	C subunit
A1F	5' TAYCARCCNTAYAC 3'	40	A subunit
A1R	5' GTRTANGGYTGRTA 3'	40	A subunit
A2F	5' GGNAARATHTAYCCNACNAANGAYTTYCC 3'	59	A subunit
A2R	5' GGRAARTCNTTNGTNGGRTADATYTTNCC 3'	59	A subunit
A3F	5' GARTTYGGNTTYGTNTT 3'	41	A subunit
A3R	5' AANACRAANCCRAAYTC 3'	41	A subunit
A4F	5' GANACNGAYTAYGAYAC 3'	43	A subunit
A4R	5' GTRTCRTARTCNGTNTC 3'	43	A subunit

* **N** = A, C, T or G; **Y** = C or T; **R** = A or G; **H** = A, C or T; **D** = A, G or T

3.2.5 PCR on random hexamer and oligo(dT_{20}) cDNA

The approximate length of the expected amplicons from different primer combinations was determined by aligning crustacyanin cDNA gene sequences from *M. japonicus* and *P. monodon* (Accession numbers FJ498903 and FJ498904 for subunit C, Accession numbers FJ498897 and FJ498898 for subunit A) with each other and counting the bases from the start of the forward primer to the end of the reverse primer. Primer pairs predicted to amplify the longest segments were then used for the PCR.

A PCR mastermix, containing 2.5 µl of 10x PCR reaction buffer, 2 mM of $MgCl_2$, 200 µM of dNTPs, 1 U of Taq (all reagents from Fisher Scientific, Australia) and 17.9 µl of RNase-free water was prepared. 22 µl of the mastermix was added to 1 µl of template cDNA and 200 nM each of the respective forward and reverse primer (Table 3.2). Additionally, two negative controls were included in the PCR reaction, by using RNase-free water as template.

Table 3.2 Primer and cDNA combinations used in the PCR amplification reaction, including the two negative control reactions.

Template cDNA	Forward primer	Reverse primer
Sample 1, oligo(dT_{20})	A1F	A3R
Sample 1, oligo(dT_{20})	A1F	A4R
Sample 1, oligo(dT_{20})	C1F	C2R
Sample 1, random hexamer	A1F	A3R
Sample 1, random hexamer	A1F	A4R
Sample 1, random hexamer	C1F	C2R
Sample 2, oligo(dT_{20})	A1F	A3R
Sample 2, oligo(dT_{20})	A1F	A4R
Sample 2, oligo(dT_{20})	C1F	C2R
Sample 2, random hexamer	A1F	A3R
Sample 2, random hexamer	A1F	A4R
Sample 2, random hexamer	C1F	C2R
RNase-free water	A1F	A3R
Sample 1, oligo(dT_{20})	C1F	oligo(dT) adaptor
Sample 1, oligo(dT_{20})	A1F	oligo(dT) adaptor
Sample 1, oligo(dT_{20})	A2F	oligo(dT) adaptor
RNase-free water	A2F	oligo(dT) adaptor

PCR amplifications were carried out in a thermocycler (Maxygene Thermocycler, Axygen, USA), starting with an initial 1 min at 95 ºC, followed by 35 cycles of a 30 sec denaturation step at 94 ºC, a 30 sec annealing step at 39 ºC and an extension step at 72 ºC for 30 sec. Final extension was done at 72 ºC for 10 min, after which the amplified products were held at 4 ºC.

To examine the amplification products, a 1.5 % DNA gel was prepared by mixing 0.75 g of agarose with 50 ml of 0.6x TBE buffer (Amresco®, Ohio, USA) and heating the mixture in the microwave until all of the agarose had dissolved. Once the solution had cooled to about 65ºC, 0.5 µl of ethidium bromide was added, the solution mixed and then poured into a gel plate. When the gel had solidified, it was placed into a gel electrophoresis tank filled with 0.6x TBE buffer.

A DNA dye was prepared by mixing 600 µl of RNase-free water with 400 µl of glycerol (Amresco®, Ohio, USA) and adding approximately 0.01 g of bromophenol blue (Amresco®, Ohio, USA). The dye was vortexed, 1.5 µl mixed with 4 µl of each of the PCR products and the mixture loaded into the gel wells. Additionally, 1.5 µl of a 100 base pairs (bp) DNA ladder (Axygen, Union City, USA, 0.1 µg/µl) was loaded into the gel and the gel run for 30 min at 95 V. The rest of the PCR products were then

stored at -20 ºC and once the gel run had finished, the bands visualised and photographed (Syngene GeneGenius, England).

To determine if the correct sequences had been amplified with the primer pairs used, a second PCR with nested primers (primers that bind to a small base sequence within the segment the original primer pair amplified) was done. The PCR mastermix was prepared as described above (see 3.2.5), using 22 µl of mastermix, 1 µl of template and 200 nM each of the forward and reverse primers as outlined in Table 3.3. Furthermore, negative controls in which template was exchanged with RNase-free water, were added to the PCR reaction and the amplification reaction carried out according to the parameters listed above (see section 3.2.5).

Table 3.3 Combination of primers and template used in the nested PCR.

Template cDNA	Forward primer	Reverse primer
A1F and A3R PCR product	A1F	A4R
A1F and A3R PCR product	A2F	A3R
A1F and A4R PCR product	A1F	A2R
RNase-free water	A1F	A2R
C1F and oligo(dT) adaptor	C1F	C2R
A1F and oligo(dT) adaptor	A2F	oligo(dT) adaptor
A1F and oligo(dT) adaptor	A1F	A3R
A2F and oligo(dT) adaptor	A2F	A3R
RNase-free water	A2F	A3R

Amplification was tested by running the nested PCR products on a 3 % DNA gel. The gel was prepared and visualised as described above (see 3.2.5).

3.2.6 Cloning of the 3' end of crustacyanin subunits A and C

3.2.6.1 Gel purification

A 1 % DNA gel was loaded with 4-16 µl of the PCR products listed in Table 3.4, always leaving two wells empty between each PCR product to facilitate a clean extraction of each individual band later, and the gel run and visualised as described in section 3.2.5.

Table 3.4 Specification of the PCR products chosen to be gel purified and cloned.

PCR product
A1F and oligo (dT) adaptor with A2F and oligo(dT) adaptor
A2F and oligo(dT) adaptor with A2F and A3R
Sample 1, oligo(dT_{20}) with C1F and oligo(dT) adaptor
Sample 1, oligo(dT_{20}) with A1F and oligo(dT) adaptor
Sample 1, oligo(dT_{20}) with A2F and oligo(dT) adaptor

Strong, relatively clean gel bands were cut out of the gel under UV light and the DNA extracted from the gel pieces with the QIAquick Gel Extraction Kit (Qiagen, Victoria, Australia) according to the manufacturer's instructions (Handbook 03/2008, including optional steps, http://www.qiagen.com). To increase the quantity of the extracted DNA, the same 30 µl of elution buffer were re-used during the DNA elution process. Recovery of the purified DNA was visualised with a 1 % DNA gel (section 3.2.5).

3.2.6.2 Ligation and transformation

Ligation reactions were set up for the purified PCR products described in Table 3.4. In these reactions, the purified products were ligated to a pGEM®-T Easy Vector (Promega, USA) by mixing 5 µl of 2x Rapid Ligation Buffer, 1 µl of pGEM®-T Easy Vector (50 ng/µl) and 1 µl of T4 DNA Ligase (Promega, USA) per single reaction and adding up to 3 µl of the purified product. Ligation tubes were incubated at 25 ºC for 1 h before they were placed at 4 ºC overnight.

For the transformation step, LB-Amp 50 agar plates, 0.1 M IPTG (isopropyl-beta-D-thiogalactopyranoside) stock solution and 50 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside) were prepared. For the agar plates, 500 ml of MilliQ water were filled into a 1 ml shott-bottle, 12.5 g of LB mix (Amresco®, Ohio, USA, catalogue # J106-500g) and 7.5 g of agar bacteriological / agar no. 1 (Amresco®, Ohio, USA, catalogue # J637-500g) added, mixed and the solution heated in the microwave for approximately 4 min until the liquid turned a clear yellow colour. The agar was then autoclaved for 15 min at 121 ºC and cooled down in a water bath to 55 ºC. While the solution cooled, 50 mg of ampicillin (Amresco®, Ohio, USA) were mixed with 1 ml of MilliQ water, vortexed and filter sterilised to make up a 50 µg/ml ampicillin solution. 500 µl of the solution was then mixed into the 55 ºC warm agar and the LB-Amp 50 agar poured into sterile Petri dishes and left to solidify.

The 0.1 M IPTG stock solution was prepared by placing 0.238 g of IPTG (Amresco®, Ohio, USA) into a 15 ml falcon tube and adding RNase-free water to reach a final volume of 10 ml. After vortexing the mixture, it was filter sterilised, split into aliquots of 1 ml each and stored at 4 ºC.

As X-Gal is light sensitive, the 50 mg/ml X-Gal solution was prepared in a 15 ml falcon tube covered with aluminium foil. For this solution, 100 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Amresco®, Ohio, USA) were dissolved in 2 ml of N,N-dimethyl-formamide (Amresco®, Ohio, USA) and the mixture then aliquoted into two

1.7 ml eppendorf tubes protected with aluminium foil, each containing 1 ml of X-Gal. Storage of the aliquots was at -20 ºC.

Immediately before transformation, 20 µl of the X-Gal and 100 µl of the IPTG solution were spread onto the dry LB-Amp 50 agar plates. Also, the SOC medium (super optimal broth with catabolite repression) (Fermentas Life Sciences, Canada) was preheated in a 42 ºC water bath before it was used in the transformation.

After the overnight incubation of the ligation reaction, *Escherichia coli* JM109 competent cells (Promega, USA) were defrosted on ice and very gently mixed once the cells had thawed. 50 µl per ligation reaction were then pipetted into chilled 15 ml falcon tubes and 2 µl of the centrifuged ligation reaction added to the cells. To test the transformation efficiency, 1 µl of the pUC18 control plasmid (Fermentas Life Sciences, Canada) was added to 50 µl of competent cells. The tubes with the cell/ligation and cell/control plasmid mixture were gently swirled and incubated on ice for 20 min. After incubation, the cells were heat shocked for 45 sec in a 42 ºC water bath and then immediately placed back on ice for 2 min. 900 µl of the pre-warmed SOC medium were then aliquoted out into each of the cell tubes and the tubes incubated in a 37 ºC temperature controlled shaker (Edwards Instrument Company, Australia) for 1 h at 225 rpm.

As soon as the incubation step was finalised, 100 µl and 200 µl of each transformation reaction were spread onto the pre-prepared LB-Amp 50 IPTG-X-Gal agar plates and the plates incubated at 37 ºC overnight.

3.2.6.3 Colony and nested PCR

Plates were removed from the incubator after the 37 ºC overnight step and observed for blue and white colonies. Eight white colonies were randomly chosen for each transformation reaction, along with two blue colonies as a negative control. A sterile 200 µl pipette tip was used to transfer a very small amount of cells from each chosen colony to a LB-Amp 50 (without X-Gal and IPTG) agar plate, labelled with a numbered grid, and the pipette tip with the rest of the cells placed in pre-prepared 0.2 PCR tubes filled with 25 µl of PCR mastermix. The mastermix used contained 18.9 µl of RNase-free water, 2.5 µl of 10x PCR reaction buffer, 2 mM of $MgCl_2$, 200 µM of dNTPs, 1 U of Taq (all reagents from Fisher Scientific, Australia), as well as 200 nM each of a M13 forward and a M13 reverse primer (GeneWorks, Hindmarsh, Australia) per single reaction.

Following the transfer of the cells to the LB-Amp 50 agar plate and the PCR tubes, the agar plate (spot plate) was incubated at 37 ºC overnight and the prepared PCR tubes placed into the thermocycler. The amplification conditions for the colony PCR consisted of an initial heat step at 95 ºC for 5 min to disrupt the cells and allow access to the DNA and 25 cycles of denaturation at 94 ºC for 30 sec, an annealing step at 48 ºC for 30 sec and an extension step at 72 ºC for 2 min. The final extension was carried out at 72 ºC for 10 min, before the PCR products were held at 4 ºC.

To determine whether the colonies used for the amplification carried the correct DNA insert, a nested PCR was run. As colonies grown from the ligation reaction were carrying either crustacyanin subunit A or crustacyanin subunit C DNA inserts, two PCR mastermixes were prepared (see section 3.2.5), with the primer pair C1F and C2R used for crustacyanin subunit C inserts and primer pair A4F and A3R for crustacyanin subunit A inserts. 24 µl of the respective PCR mastermix were then added to 1 µl of template from the amplified colony PCR and the amplification reaction carried out as described in section 3.2.5.

1 % DNA gels were prepared for the colony PCR and nested PCR, run at 120V for 35 min and the bands visualised and photographed.

3.2.6.4 Sequencing and analysis

27 of the colony PCR products were selected, 10 µl of each pipetted into individual 0.2 ml eppendorf tubes and the tubes placed on a 56 ºC warm heat block for 1 h with their lids open to evaporate the liquid from the samples. The prepared samples were then submitted to Macrogen, Korea for sequence analysis.

Once the sequence analysis results were received, the sequences were cleaned and analysed with Sequencher v4.1 (Gene Codes, USA).

3.2.7 Cloning of the 5' end of crustacyanin subunits A and C

3.2.7.1 cDNA synthesis

The SMART^{TM} Race cDNA kit (Clontech, US) was used to synthesise first-strand cDNA from 460 ng of total prawn muscle/cuticle RNA (total RNA extracted in section 3.2.2) and from human placental total RNA as control (included in the SMART^{TM} Race kit) according to the manufacturer's guidelines (Protocol No. PT3269-1, http://www.clontech.com). 100 µl of Tricine-EDTA buffer were added to the cDNA synthesis reactions to dilute the cDNA product and the product then stored at -20 ºC.

3.2.7.2 PCR and nested PCR on 5' end cDNA

To gain only cDNA that code for crustacyanin subunit A and subunit C, first a PCR reaction was performed with the first-strand cDNA as a template, combined with each of the 6 degenerate reverse primers outlined in Table 3.1. PCR mastermix for this reaction was prepared as described in section 3.2.5, and 22 µl each added to 1 µl of first-strand cDNA template and 200 nM of one of the six reverse primers (A1R, A2R, A3R, A4R, C1R and C2R). Furthermore, to test the success of the cDNA synthesis, 22 µl were also added to 1 µl of control cDNA and 200 nM of control primer (included in the clontech kit). As the target was to obtain the 5' end of both crustacyanin subunit sequences, no forward primers were added to the PCR reactions. PCR amplification conditions followed the PCR amplification protocol described in 3.2.5.

After amplification, PCR products were examined on a 1.5 % DNA gel as illustrated in section 3.2.5.

Next, a nested PCR was carried out to determine whether the previous PCR reaction successfully amplified the desired crustacyanin subunit A and subunit C transcripts and to produce PCR products that could be used for cloning. A PCR mastermix was prepared as described in section 3.2.5, and 22 µl of it added to 1 µl of template and 200 nM of forward and reverse primer each (see Table 3.5). Amplification and storage parameters were as previously described in 3.2.7.2.

Table 3.5 PCR template and primer combinations for the nested PCR.

PCR template	**Forward primer**	**Reverse primer**
PCR product from 5' race cDNA and A3R	A1F	A2R
PCR product from 5' race cDNA and A4R	A1F	A2R
PCR product from 5' race cDNA and A2R	A1F	A2R
PCR product from 5' race cDNA and C2R	C1F	C2R
PCR product from 5' race cDNA and A3R	Universal primer mix*	A4R
PCR product from 5' race cDNA and A4R	Universal primer mix*	A2R
PCR product from 5' race cDNA and A2R	Universal primer mix*	A1R
PCR product from 5' race cDNA and C2R	Universal primer mix*	C1R
RNase-free water (control)	Universal primer mix*	A4R

* from SMART^{TM} Race cDNA kit, Clontech, US

3.2.7.3 Cloning

From the nested PCR reaction in 3.2.7.2, the cleanest and brightest PCR products were chosen, 16 µl from each of these products gel purified, and ligation,

transformation and colony as well as nested PCRs carried out on these products as previously described in sections 3.2.6.1 – 3.2.6.3.

3.2.7.4 PCR purification and sending for sequencing

PCR products from ten white colonies were sent to the Australian Genome Research Facility (AGRF), Queensland for sequence analysis. The PCR products were purified with the QIAquick PCR purification kit (Qiagen, Victoria, Australia) as outlined in the manufacturer's guidelines (QIAquick Spin Handbook, 03/2008, Qiagen, http://www.qiagen.com) and the quantity of the purified products determined with the NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, USA). All samples were prepared for sending according to AGRFs requirements (total volume of 12 µl, comprised of the M13F primer at a concentration of 9.8 ρmol and the purified product at a concentration of 12 to 18 ng).

3.2.8 GenBank submission and further analysis of both subunits

Sequences obtained through the 3' and 5' cloning for the crustacyanin subunit A and subunit C were aligned with each other in Sequencher v4.1 (Gene Codes, USA) to form a consensus sequence for each subunit. Ambiguous bases in the consensus sequence were solved by choosing the most abundant base form. The reading frame as well as the start and stop codons were determined by translating the consensus sequence in ExPASy (http://au.expasy.org/tools/dna.html) into its protein sequence.

The full length cDNA consensus sequences for crustacyanin subunits A and C, starting from the start codon and ending in the stop codon, were submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and are accessible under Accession numbers HM370278 and HM370279 for subunit A and subunit C, respectively. Deduced protein sequences were aligned with published crustacyanin subunit A and C protein sequences using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Affiliations of both consensus sequences with the large lipocalin family were assessed using InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/).

3.3 Analysis of colour variants with absolute qPCR, spectrophotometer and visual observation

3.3.1 Sample collection

85 and 87 *F. merguiensis* samples, classified as having light and dark colouration respectively were collected from Seafarms Cardwell grow-out site during the harvesting period. Both sample groups were collected from ponds 205, 203 and 218. For the

sample collection, prawns were randomly picked and then physically moult-staged by testing the strength of the prawns' carapace to ensure that the prawns were all in intermoult, before they were placed into -10 ºC cold brines (salt concentration approximately 250 g/l) until they had cooled to 4 ºC. To reach temperatures below -10 ºC, the prawns were frozen in a blast freezer for about 2 – 3 h.

Seafarm also sent ten albino prawns that had been collected over one week from different ponds at the Mossman and Cardwell grow-out sites. All albino prawns were moult-staged with the microscope, placed into individual bags and frozen in the blast freezer for about 1 h. All prawns were kept below -10 ºC until they were sent to the USC research laboratory on dry ice, where they were immediately placed at -80 ºC.

At USC, six prawns were randomly chosen from the light and dark groups (one prawn for each colour and pond group) and used to determine whether tissue degradation had happened during collection and transport.

Furthermore, five extra *F. merguiensis* samples were chosen for testing two different methods of RNA extraction to determine the extraction method with the best possible yield and quality and to produce a high concentration of RNA that could be used for the production of a standard curve for the absolute qPCR experiment.

3.3.2 Determination of gene expression with absolute qPCR

3.3.2.1 Primer development

The consensus sequences for crustacyanin subunit A and C, obtained through cloning in the first part of the study were used to develop gene specific primers for absolute qPCR. To obtain primers with melting temperatures (Tm) above 60 ºC, a primer length of approximately 20 bases, low self annealing and a product size of 200 bp or below, each sequence was imported into Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and the optimal parameters set for a Tm of 63 °C, 20 bases primer length and 200 bp product size. Primer3Plus then highlighted forward and reverse primer sequences in each of the two crustacyanin subunits that matched all requirements. Two and three of these primer pairs were chosen for the crustacyanin subunit A and C respectively and the primers ordered from GeneWorks, South Australia (Table 3.6).

Table 3.6 Gene specific qPCR primer pairs developed with Primer3Plus, their individual Tm's and sequences, as well as the size of the amplicons.

Primers	Primer sequence	Tm (°C)	Amplicon size (bp)	Crustacyanin subunit
SubA1F	5' CGCTGCATCCACTCCAACTA 3'	62.3	225	subunit A
SubA1R	5' GCAGGAATACACGCAGGCATA 3'	63.3		
SubA2F	5' CAGGGCAAGATCTACCCCACA 3'	64.5	175	subunit A
SubA2R	5' GGGAGAACACGAAGCCGAAC 3'	64.3		
SubC1F	5' TGCTGGCACATGGTACGAAA 3'	63.6	205	subunit C
SubC1R	5' TGCCTCGTAGTCCACGGAAA 3'	64.0		
SubC2F	5' CGAGAACTTCGCGTGCATCT 3'	63.9	178	subunit C
SubC2R	5' TCCACCCTGAGCTGTCTTCG 3'	63.9		
SubC3F	5' TGCCGACAAGATCCCTGACT 3'	63.1	167	subunit C
SubC3R	5' CGTTGCGGACACACTTCTTG 3'	62.8		

3.3.2.2 Determination of optimal RNA extraction method

Two prawns were chosen, placed on dry ice and the cuticle tissue of the first abdominal segment, marked with a red arrow in Figure 3.3, dissected off the prawn and stored in RNAlater™.

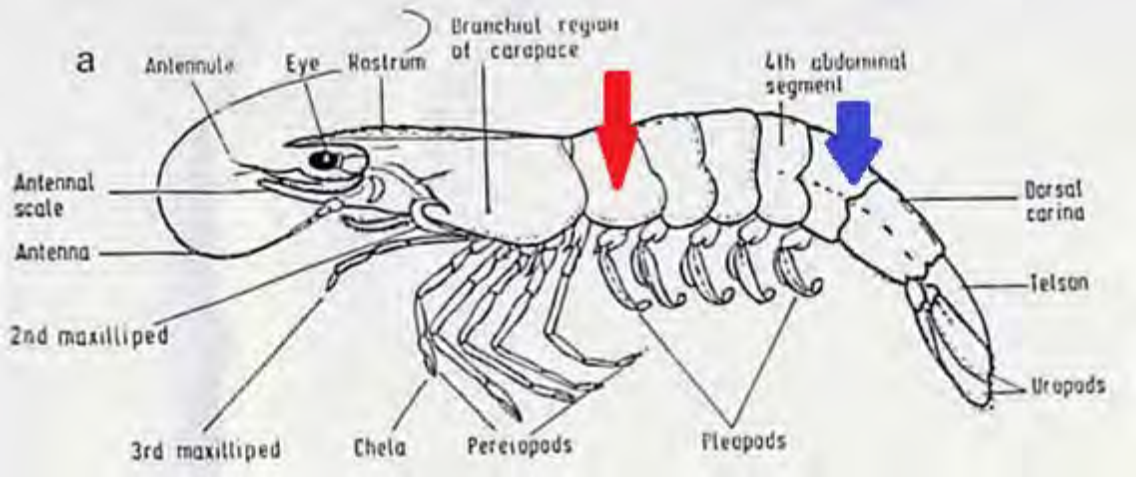

Figure 3.3 Location of the segments used for gene expression and colour analysis. The red arrow points to the segment from which the cuticle tissue has been dissected off to test for gene expression levels. The blue arrow indicates the whole segment taken for the colour extraction in 3.3.3. Diagram adapted from Wickins & Lee (2002).

16 - 19 mg of tissue from the test samples were taken and the RNA extracted with the RNeasy Plus Mini kit as described in section 3.2.2. After extraction, the quality and quantity of the RNA was tested with the NanoDrop2000 spectrophotometer.

The same amount of tissue for both test samples was then used to extract RNA according to the TRIzol® extraction protocol (including optional steps; Invitrogen Life Sciences, Victoria, Australia, http://www.invitrogen.com). To allow for optimal RNA extraction, homogenisation of the samples was performed with RNase-free rotor stator homogenisers, after which a 7 min incubation step at room temperature (25 ºC) was added. Once total RNA of both samples was extracted, the quality and quantity of the RNA was tested with the NanoDrop2000 spectrophotometer and by gel electrophoresis as outlined in section 3.2.2.

The quantity and quality of the RNA extracted with both methods was then compared with each other and the TRIzol® method chosen.

3.3.2.3 Determination of optimal primer pairs for absolute qPCR

500 ng of total RNA extracted with TRIzol® (Invitrogen Life Sciencies, Victoria, Australia) from the test samples (section 3.3.2.2) was used to synthesise cDNA with the QuantiTect Reverse Transcription kit (Qiagen, Victoria, Australia) according to the manufacturer's guidelines (QuantiTect Reverse Transcription Handbook 04/2005, http://www.qiagen.com). To determine if genomic contamination was present, negative reverse transcription (-RT) reactions for both extracted RNA's were also carried out by following the manufacturer's guidelines in everything, except that 1 µl of RNase-free water was used in exchange for 1 µl of the Quantiscript Reverse Transcriptase (point 4 of the handbook).

For a preliminary test of the gene specific primers developed for absolute qPCR, a PCR was run with each primer pair listed in Table 3.6 to evaluate the specificity and amplification strength of each pair. A PCR mastermix of 22 µl per single reaction was prepared as previously described in 3.2.5, and added to 1 µl of the synthesised cDNA from the test samples, together with 200 nM each of the forward and reverse primers.

PCR amplification and product visualisation were carried out as outlined in section 3.2.5, with the exception of the PCR annealing step, which was done at 60 ºC for 30 sec.

A qPCR test run was carried out with the cDNA and -RT reaction to determine optimal cycling and melt curve conditions and to select the primer pairs with the lowest threshold cycle (Ct) value, using a threshold value of 0.1.

Per single reaction, 5 µl of 2x SensiMix, 0.4 µl of EvaGreen dye (both from the kit SensiMix™ HRM, Quantace, USA) and 3.2 µl of RNase-free water were added to

200 nM of each primer pair (Table 3.6) and 1 µl of cDNA template. All amplifications were done in duplicate, with a negative template control (NTC) for each primer pair used, added in duplicate to the qPCR reaction set-up.

The qPCR was run with the following extension conditions on the Rotor-Gene 6000 (Corbett Research, Australia): initial hold step at 95 ºC for 15 min, then 40 cycles of 1) 95 ºC for 15 sec, 2) 60 ºC for 15 sec and 3) 72 ºC for 30 sec, with the last step set to acquire to Green. HRM (high resolution melting curve analysis) conditions for the qPCR were set to rise by 0.1 ºC each cycle from 70 ºC to 95 ºC, with acquiring on the HRM channel. HRM gain was set to 0.67 and green gain to 10. After the amplification, a melt curve analysis was carried out, using the Rotor-Gene 6000 software, version 1.7 (Corbett Research, Australia) to ensure that a single product had been amplified.

3.3.2.4 Validation of the qPCR reaction

Three prawn samples were placed on dry ice and the cuticle tissue from the segment indicated by the red arrow in Figure 3.3 excised and placed into RNAlater™. Total RNA was extracted from approximately 25 mg of tissue per sample according to the TRIzol® protocol (as described in 3.3.2.2). RNA from the three samples was then combined together and mixed with the RNA previously extracted (3.3.2.2). The quantity and quality of the combined RNA was tested twice with the NanoDrop2000 spectrophotometer and by running a 1.2 % RNA gel (as outlined in section 3.2.2).

500 ng of this combined total RNA were used to synthesise cDNA with the QuantiTect Reverse Transcription kit as outlined in section 3.3.2.3. To produce a standard curve of known concentration from the synthesised cDNA, a PCR was carried out to obtain crustacyanin subunit A and C cDNA segments of a known length. PCR reactions were set up as outlined in 3.2.5 (annealing step at 60 °C for 30 sec), with the combined cDNA as template (1 µl) and primer pairs SubA1F and SubA2R (200 nM each) for subunit A and primer pairs SubC3F and SubC2R (200 nM each) for subunit C.

After the PCR products were confirmed to be the correct size by gel electrophoresis (see section 3.2.5) the products were cleaned with the QIAquick PCR purification kit and the eluted products tested three times with the NanoDrop2000 spectrophotometer for its DNA quantity.

The mean concentration of both purified samples and the length of the cDNA segments amplified were used to calculate the copy number of the two purified products with the aid of the copy number calculator of the URI Genomics & Sequencing Center

(http://www.uri.edu/research/gsc/resources/cndna.html). A 20 point 1 : 5 dilution was then set up for both purified products and their respective copy numbers calculated.

Once the dilutions were prepared, a qPCR was set up as described above (see 3.3.2.3) and the dilutions run in triplicates along with triplicate –RT controls and NTCs for both primer pairs. Amplification parameters were as previously outlined in 3.3.2.3, with a melt curve analysis at the end of each run. Primer pairs used in this qPCR reaction were the pairs with the lowest Ct value for crustacyanin subunit A and subunit C. From the dilution curves produced for both subunits, the reaction efficiency (E) was calculated by the Rotor-Gene 6000 software, version 1.7 with the equation $E = [10^{(-1/M)}] - 1$, where M stand for the slope of the curve.

3.3.2.5 Determination of sample tissue quality

The six samples randomly chosen from the light and dark group of each pond to test the tissue quality were placed on dry ice and the cuticle tissue dissected off the first abdominal segment (Figure 3.3, red arrow) and placed into RNAlater™. Total RNA was then extracted from 20 mg of tissue per sample with TRIzol® as described in 3.3.2.2 and the quality and quantity of the RNA tested with the NanoDrop2000 spectrophotometer and by running the samples on a 1.2 % RNA gel (see section 3.2.2) for 60 min at 60 V.

500 ng total RNA was then used to synthesise cDNA and –RT from each sample with the QuantiTect Reverse Transcription kit (see section 3.3.2.3). Once the cDNA was prepared, a test PCR was set up and carried out as described in 3.2.5 (annealing temperature was changed to 60 °C for 30 sec), using the primer pairs SubA2F and SubA2R, and SubC1F and SubC1R. The amplified products were then run on a 1.5 % DNA gel (see 3.2.5) for 35 min at 60 V.

A qPCR reaction was set up and run under the conditions outlined in 3.3.2.3. While the samples, –RT controls of each sample and the NTCs (for primer pairs SubA2F and SubA2R, and SubC1F and SubC1R) were analysed in duplicates, dilution points 10 and 11 from the 20 point dilution curves for both subunits were run in triplicate. To test for successful amplification of the correct product, a 1 % DNA gel (see 3.2.5) was run for all samples used in the qPCR.

3.3.2.6 Sample preparation for absolute qPCR of the 50 prawns displaying a distinct body colour

20 prawns were each chosen randomly from the light and dark group of pond 205 and kept together with the 10 albino samples on dry ice at temperatures between -40 ºC and -60 ºC as much as possible during the sample preparation steps. Pond 205 was chosen for the light and dark prawns, as all prawns of this pond came from the same breeding group and were exposed to the same conditions during growth.

Each prawn was weighed and photographed individually in a dark room while frozen. For the photos, the prawns were placed on a clean, white Styrofoam box with its respective sample number, the light turned off and two consecutive photos taken with a Panasonic digital camera (5.0 mega pixel, 6x optical zoom, model DMC-LZ3) set on flash that was held immobile approximately 25 cm above the prawn. After the prawns were photographed and their weight recorded, they were placed back on ice.

The cuticle tissue of each of the 50 prawns was carefully dissected off the first abdominal segment (Figure 3.3) and the tissue placed into RNAlater™ and stored at 4 ºC overnight prior to freezing them at -80 ºC. Before the prawns were placed back on dry ice, the gender of each prawn was determined and recorded.

3.3.2.7 RNA extraction, cDNA synthesis and absolute qPCR of the 50 prawns

Total RNA was extracted from 20.0 mg of tissue of each of the 50 prawns at the same day with TRIzol® (section 3.3.2.2) and RNA quantity and quality analysed with a 1.2 % RNA gel and the NanoDrop2000 spectrophotometer as previously outlined in 3.2.2. As the MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines suggest to test the RNA for qPCR experiments with a Bioanalyzer (Bustin et al. 2009), a representation of the 50 RNA samples was also tested with a 2100 Bioanalyzer (Agilent Technologies, USA). 7 of the albino samples, 8 of the light and 7 of the dark samples were tested with the Agilent RNA 6000 Nano Chip kit (Agilent Technologies, USA) according to the manufacturer's guidelines (Agilent RNA 6000 Nano kit guide, http://www.agilent.com).

For the cDNA production, 500 ng of total RNA was used from each individual sample and processed with the QuantiTect Reverse Transcription kit. Additionally, 10 % of the samples were chosen for –RT production and the cDNA synthesis and –RT production carried out as previously described (see 3.3.2.3).

In order to determine the copy number of crustacyanin subunit A and C expressed in the samples tested, a qPCR was set up as reported in 3.3.2.3. Each sample was tested in duplicate for primer pair SubA2F and SubA2R and primer pair SubC1F and SubC1R. To be able to normalise between runs and to determine the expression level of both subunits, two points of the respective dilution curve (dilution 7 and 8, details of dilution in 3.3.2.4) were included into each run in triplicates. To rule out genomic contamination, 10 % of the samples were incorporated in duplicates as –RT controls. Additionally, every run had NTCs for the corresponding primer pair used in the individual qPCR runs and a melt curve analysis was performed at the end of each run to ensure the amplification of a single product. To minimize variation between the samples, RNA extraction, cDNA production and qPCR runs were performed for all 50 samples together at the same time.

Following the qPCR runs for all samples, a 2 % DNA check gel was carried out (see section 3.2.5).

3.3.3 Spectrophotometric colour analysis

3.3.3.1 Cooking parameters

All 50 prawn samples from 3.3.2.6 were defrosted on ice until they had reached a temperature of approximately 4 ºC. Prior to removing the prawns from their individual numbered bags for cooking, labels were prepared and attached to the small ridge between the sixth abdominal segment and the telsons (Figure 3.3). As the exoskeleton of the first abdominal segment of each prawn had been removed for the gene expression experiment, the prawns were steamed instead of boiled at approximately 100 ºC to inhibit leaching out of the pigment. Groups of 6 to 9 prawns were steamed together for 4 min, then placed on ice, photographed, repacked into their individual bags and frozen at -20 ºC for about 15 to 20 h until the extraction could be set up. For the photographs, prawns were placed in groups of 2 to 3 on a clean, white Styrofoam box with the label clearly visible, the light turned off in the dark room and two consecutive pictures taken. The camera used was a Panasonic digital camera (5.0 mega pixel, 6x optical zoom, model DMC-LZ3), set on flash and held immobile about 25 cm above the prawns.

3.3.3.2 Astaxanthin extraction and absorbance measurement

Two different colour determination methods (colorimeter or spectrophotometric analysis of pigment extract) were tested and the method listed below was chosen as the preferred way to analyse the colouration in the 50 cooked prawn samples.

For the extraction of the pigment astaxanthin from the prawn samples, a protocol described in Tume et al. (2009) was adapted. Although the authors determined that about 95 % of the pigment found in their test species (*P. monodon*) was astaxanthin and astaxanthin esters, a small amount of other carotenoids such as lutein will be included in the extracted pigment. Therefore, the extracted astaxanthin will be termed "total astaxanthin". To keep the temperature at which the extraction was carried out stable at all times, all preparations and extraction steps were executed at 4 ºC, with all the equipment used for the extraction also kept at 4 ºC.

To keep the process uniform across all samples, the fifth abdominal segment of each prawn was chosen to be used for the total astaxanthin extraction (Figure 3.3, blue arrow). The segment and the attached pleopods were cut, weighed and the weight recorded. Next, the exoskeleton of the segment was carefully separated from the cuticle tissue and both, tissue and shell placed into a clean 25 ml beaker that was covered with aluminium foil to inhibit evaporation of the solution used for the extraction and to provide protection for the photosensitive pigment. 10 ml of 0.05 % butylated hydroxy toluene (BHT), dissolved in 100 % acetone (both reagents from BioLab, Victoria, Australia) were filled into the 25 ml beakers to completely cover the excised segment and shell. The beakers were then placed in a closed Styrofoam box and stored overnight in a dark place at 4 ºC. After leaving the extraction for approximately 20 h, the acetone solution with the extracted pigment was decanted into a clean 20 ml volumetric flask, covered partially with aluminium foil and closed with a plug. The procedure was then repeated on the same segments with 10 ml of fresh 0.05 % BHT in acetone. About 22 h later, the acetone with the extracted total astaxanthin was decanted into the respective 20 ml volumetric flasks and the solution adjusted to a total volume of 20 ml.

The UV spectrophotometer (UV-1800, Shimadzu) was blanked with the 0.05 % BHT in acetone, before a full range wavelength scan (350 nm to 750 nm) was carried out to confirm the wavelength of 477 nm for the total astaxanthin absorbance measurements as indicated in Tume et al. (2009). Each sample was then measured in duplicate at a wavelength of 477 nm and mean absorbance and amount of total astaxanthin in µg/g of wet weight calculated.

3.3.4 Visual colour determination

Colour intensity of the 50 cooked prawns was ranked by six USC academic and research staff by observing pictures of the cooked prawns. The photographs taken from the cooked prawns were uploaded onto one single computer to eliminate

differences in the screen set-up and allow for consistent conditions during the ranking process. Staff were asked to score the colour of each prawn according to a grading colour score card for *Penaeus monodon* (courtesy of Seafarm, commercially available from Aqua-Marine Marketing, Australia) from 1 to 12. To be in line with industry proceedings, this score card was used, as the industry assesses colour intensity in *F. merguiensis* according to the *P. monodon* colour card. Also, for consistency, all staff assessing the colour intensity of the cooked samples ranked the samples according to the colour strength in the segments indicated in Figure 3.4.

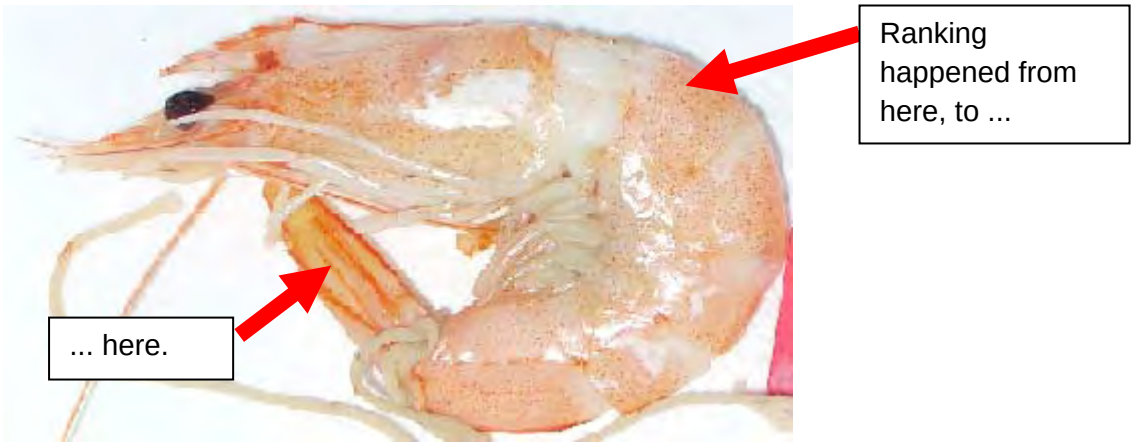

Figure 3.4 Ranking of prawn colour. Red arrows and text boxes outline the range of the area the rankers were asked to score.

3.3.5 Analysis of the results obtained from the three colour experiments

To determine, whether significant differences in pigment quantity and crustacyanin subunit A and subunit C gene expression levels existed between the three different colour groups (albino, light and dark), the non-parametric Kruskal-Wallis test with Bonferroni's correction was used to lower the risk of type 1 error (as suggested in Field (2005), p. 550). This test was chosen as the data violated the assumptions of normality (tested using Levene's test and Kolmogorov-Smirnov test). Furthermore, non-parametric Kendall's tau correlation analysis was carried out to determine whether a relationship existed between the level of gene expression and the amount of pigment extracted from the tissue. All statistical analysis was performed using PASW Statistics 18 (SPSS Inc., Illinois) and the results reported as mean ± 1 standard error, unless otherwise noted.

3.4 Next generation sequencing and analysis

Dr Anna Kuballa went to Seafarm, Cardwell to collect 75 *F. merguiensis* prawn samples during the harvesting period. To ensure genetic variability for the

transcriptome analysis, the samples chosen came from eight different breeding groups, with a mixture of male and female prawns. The cuticle and part of the muscle tissue were dissected of the prawn samples, placed into RNAlater™ and stored at 4 ºC for 3 days before the samples were frozen at -80 ºC.

At the USC Genecology research laboratory, the samples were paired into groups of 3 – 4 prawns and a total of 24 mg of tissue per group excised from the *F. merguiensis* sample tissue. RNA was then extracted from each group with the RNeasy Plus Mini kit (see 3.2.2) and its quality and quantity assessed with the NanoDrop2000 spectrophotometer and by gel electrophoresis (see 3.2.2).

Total RNA from all sampling groups was then combined into one sample and messenger RNA (mRNA) isolated with the Oligotex mRNA kit (Qiagen, Victoria, Australia) as outlined in the manufacturers guidelines (Oligotex® Handbook, 05/2002, http://www.qiagen.com). To increase the amount of polyA mRNA isolated, the supernatant obtained in step 5 of the protocol was reused for another mRNA isolation, beginning with the addition of 15 µl of oligotex suspension and incubating the sample at 70ºC for 3 minutes. After this step, the protocol was followed again, starting from step 4 of the oligotex handbook. Elution of the mRNA was done three times with 25 µl of 70º warm OEB buffer (included in the Oligotex mRNA kit) to gain an additional increase in mRNA isolated. The triple elutions were then tested for their total RNA quantity and quality with the NanoDrop2000 spectrophotometer and every elution that gave a value above 0 ng/µl was added together into one combined sample. However, as a low volume, high concentration mRNA was required, the combined mRNA sample was concentrated with the RNeasy Mini Elute™ Cleanup kit according to the manufacturer's guidelines (RNeasy® Mini Elute® Cleanup Handbook, 12/2007, Qiagen, http://www.qiagen.com) and re-eluted in 40 µl of RNase-free water (included in the Mini Elute kit, Qiagen).

The prepared sample was then sent to AGRF in Brisbane, where Roche 454 next generation sequencing was conducted on the non-normalised mRNA. Raw sequence reads were processed using the CLC Genomics workbench software (CLCBio, Denmark). After removing the adaptor sequences, the transcriptome was *de novo* assembled into contigs (continuous sequences, produced by aligning overlapping sequences from the next generation sequencing project), using the standard parameters set by the CLC software. Analysis and functional annotation of the transcriptome reads was performed, using the blastn function of the blast2go software

(http://www.blast2go.org/start_blast2go) with the parameters set automatically by the software and the NCBI database.

In addition to the muscle/cuticle samples sent to AGRF for next generation sequencing, *F. merguiensis* tissues samples from the androgenic gland, hepatopancreas, stomach, nervous system, eyestalk and male and female gonads were prepared by the Genecology group and the isolated mRNAs sent for next generation sequencing. Messenger RNA isolation from these tissues was performed as described for the muscle/cuticle tissue above in section 3.4. Sequencing results from these samples were also analysed with the CLC Genomics workbench software to determine the presence or absence of the crustacyanin subunit A and C genes in these tissues.

3.5 Microarray development and analysis

3.5.1 Microarray design

Contigs and singletons (sequences not used in the contigs and representing one single unique sequence) produced with the CLC Genomics Workbench software and analysed with blast2go and the NCBI database were cleaned up by deleting sequences that were below 100 bp long and by removing (when it was towards the end) or exchanging an “N” base (N = base A, C, T or G) with either of the bases that appeared to fit best into the sequence pattern (e.g. a T for an N in a sequence like CCTCTCCTCCTCTCCTCCNC), before the sequences were used in the microarray design. PolyA tails were removed and ribosome sequences (e.g. 18S or 28S ribosomal RNA) as well as sequences that had high matches (above 80 %) across the full length of the sequence with ribosomal RNA were deleted from the data used for the microarray. Since the sequencing direction of the 454 Roche next generation system was unknown, the CLC Genomics Workbench software (CLCBio, Denmark) was used to produce the reverse complement of all sequences. Crustacyanin subunit A and C sequences obtained through the cloning processes in 3.2 were also added to the microarray design. All sequences were then incorporated into one single FASTA file and uploaded onto the eArray site of Agilent (http://www.agilent.com). Once uploaded, four probes with a length of 60 bases each were produced per sequence. The probe file was then downloaded and control probes/replicate probes chosen from it, copied and pasted into a control.txt file. This file was then uploaded into the eArray system (http://www.agilent.com) and used along with the original probe file to design a 4x44K customised gene expression microarray.

3.5.2 Samples used for the microarrays

The ranking results from section 3.3.4 were statistically analysed with PASW Statistics 18 by determining the mean and standard deviation (SD) of each of the 50 samples. The mean values were then sorted from highest to lowest number and disregarding the 10 albino samples, the 12 individuals with the highest value (classified as “dark” colour in the microarray experiment) and the 12 individuals with the lowest mean value (classified as “light” colour) were chosen. Also, concentrating only on the 10 albino samples, the 9 individuals with the lowest mean value were considered “albino” samples for this experiment. Individual prawns of the three main categories (dark, light and albino) were then randomly combined into four light, four dark and four albino groups, by using random numbers generated with Microsoft Office Excel 2007 (Microsoft Corporation, Australia). For the light and dark categories, each of the 4 groups was comprised of 3 individuals. For the 4 albino groups, three groups consisted of 2 albino individuals each, and one group contained 3 albino individuals.

3.5.3 Labelling, hybridisation and scanning of the microarrays

For the labelling reaction, 540 ng of total RNA (for the groups composed of three individuals, each prawn contributed 180 ng of total RNA; for the groups composed of two samples, each prawn contributed 270 ng of total RNA) were used per group. The total RNA of each group was first transcribed into cDNA, from which labelled cRNA (complementary RNA) was synthesised, using the Quick Amp Labeling kit (one-colour) according to the One-Colour Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling) protocol (Agilent technologies, Australia, http://www.agilent.com). The labelled cRNA was then purified with the Qiagen RNeasy kit (Qiagen, Victoria, Australia) as recommended by the manufacturer (http://www.qiagen.com). After purification, the quantity and quality of the labelled cRNA, as well as the dye incorporation were determined with the NanoDrop2000 spectrophotometer.

Three 4x44K customised gene expression microarrays were set up for hybridisation as shown in Figure 3.5. For each of the four light and dark groups, as well as the “Albino 1” group, 1650 ng of purified, labelled cRNA were used for the hybridisation reaction. However, the concentration of cRNA for the other three albino groups were too low after the labelling and purification reaction, so only 1050 ng of cRNA were used for the “Albino 2” group and 825 ng for the “Albino 3” and “Albino 4” groups.

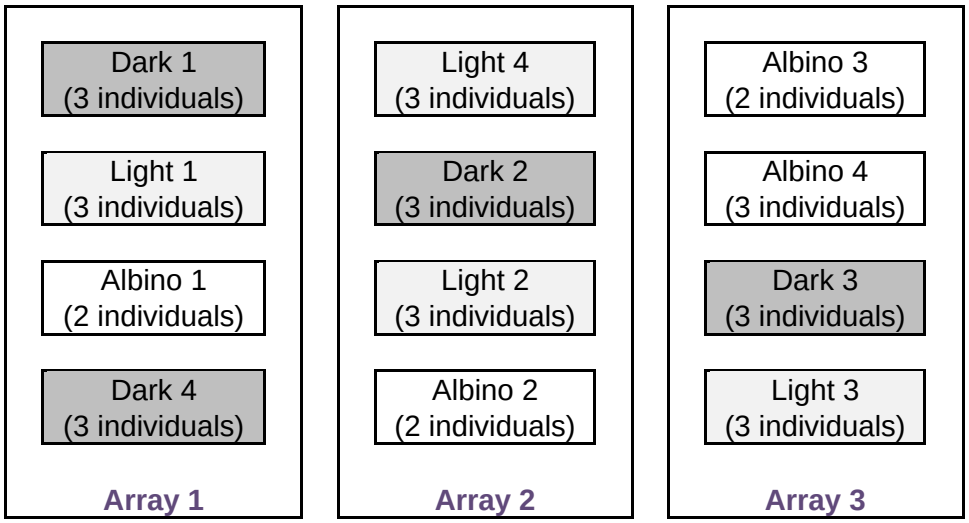

Figure 3.5 Set up of the four albino, light and dark groups on the three 4x44K customised gene expression microarrays.

Although labelling and purification was done on all 12 subgroups in one day, only one array could be hybridised per day as just one hybridisation chamber was available. The samples for the other arrays were kept in a black light-impenetrable storage box and stored at -80 °C until used. During the hybridisation reactions, which were carried out according to the One-Colour Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling) protocol, the arrays were tightly covered with aluminium foil to protect the light sensitive dye, and incubated at 65 °C for 17 h in a Shake 'n' Stack Hybridisation oven (Thermo Fisher Scientific, USA).

After hybridisation, the arrays were washed, using the Gene Expression Wash Buffer kit as recommended in the One-Colour Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling) protocol and then scanned in the GenePix 4000B Microarray scanner (Molecular Devices, California, USA). Scan parameters were 100 % power, pixel size of 5 µm, lines to average: 1, focus position of 0 µm and channel green at 532 nm. The photomultiplier (PMT) gain of the scanner was set at 500 and then raised by 10 every consecutive scan until 700 was reached. Each microarray scan was visualised with the GenePix®Pro 6.0 (Molecular Devices, California, USA).

3.5.4 Microarray analysis

For the analysis of the three microarrays, each block (a 4x44K microarray has 4 blocks per microarray) was assessed for the scan with the best possible PMT gain (determined by an approximately equally low amount of oversaturated or white points) and the files of the blocks at the chosen setting saved separately. These blocks were

then normalised and analysed with the GeneSpring GX 11.0 (Agilent Technologies, Australia) software to determine genes that were differentially expressed across the three colour groups. Normalisation was carried out according to the suggestions of the GeneSpring GX 11.0 program (threshold raw signal to 1.0 and percentile shift algorithm to 75th percentile, no base line transformation). Unpaired t-tests with Benjamini and Hochberg FDR (Benjamini & Hochberg 1995) multiple testing corrections were used for the statistical analysis of the data to correct for the occurrence of Type I errors. As different amounts of total labelled RNA were used for 4 blocks hybridised with the albino samples, a K-means clustering analysis (samples with similar expression profiles are grouped together, using a variety of clustering algorithms) was performed on these samples to determine, whether the albino data was successfully normalised as well.

4. Results

In this study, RNA extracted from *F. merguiensis* prawns was used to isolate the crustacyanin subunit A and subunit C gene sequences, genes that have been strongly indicated in colouration in other crustaceans. Furthermore, *F. merguiensis* individuals of different colour intensities were analysed for their levels of gene expression (crustacyanin and other genes) in their cuticle tissue, utilising three important molecular methods, qPCR, microarrays and next generation sequencing. Additionally, to obtain a broader picture of the factors involved in body colouration of *F. merguiensis*, astaxanthin was also extracted from the above differently coloured prawns and assessed in relation to the gene expression level of crustacyanin.

4.1 Isolation of the crustacyanin cDNA sequences

4.1.1 Quality and quantity assessment of the extracted RNA

Total RNA was extracted from the muscle and cuticle tissue of two *F. merguiensis* prawn samples for the synthesis of cDNA. Following RNA extraction, the integrity of the total RNA was examined on an RNA gel. RNA bands observed on the gel were faint but relatively clean, indicating that the RNA was only slightly degraded. RNA quantification gave total RNA amounts of 0.057 µg/µl to 0.230 µg/µl for the samples, with these low RNA values correlating with the faint appearance of the gel bands. Values obtained for the 260/280 wavelength spectra (between 2.174 and 2.219) indicated that the extracted RNA had a fairly high level of purity.

4.1.2 Degenerate primer design and use in PCR reactions

12 degenerate primers were developed, based on crustacyanin amino acid sequences from other crustaceans (Table 3.1). Four of these primers were designed for the crustacyacyanin subunit C and eight for subunit A. By aligning crustacyanin subunit A and C gene sequences from *M. japonicus* and *P. monodon*, the expected segment length that each chosen primer pair encompassed was determined. The longest segments for each subunit were built with primer pairs A1F and A3R and pair A1F and A4R for subunit A and primer pair C1F and C2R for subunit C. Shorter segments were built with primer pairs A1F and A2R, A2F and A4R, A2F and A3R and A4F and A3F (Figures 4.1 and 4.2). As long segments were desired in general, most of the PCRs and cloning performed involved the primer pairs enfolding larger segments, with the primer pairs spanning shorter regions mainly used for nested PCRs.

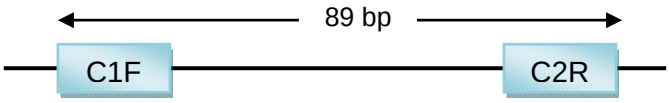

Figure 4.1 Schematic of the expected primer pair length for the degenerate crustacyanin subunit C primers.

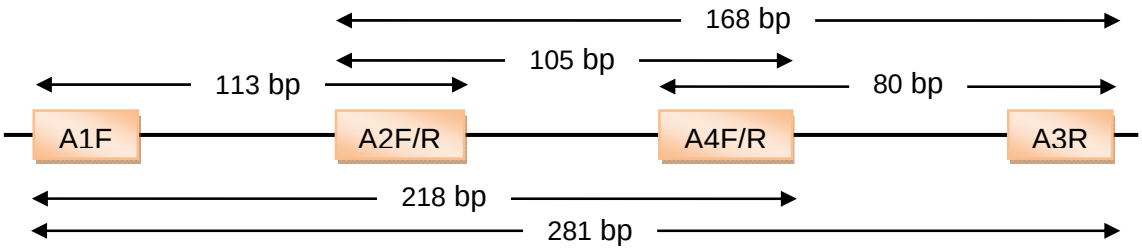

Figure 4.2 Schematic of the expected primer pair length for the degenerate crustacyanin subunit A primers.

Check gels run on the cDNA PCR amplification reactions with the primer pairs spanning the larger regions resulted in gel band sizes of ~ 220 bp to 280 bp for subunit A gene segments and ~ 90 bp for subunit C gene segments as was expected. Nested PCR reactions carried out to determine that the correct segments had been amplified in the PCR reactions with primer pairs like A1F and A3R were observed as ~ 160 bp to 220 bp large gel bands. Gel electrophoresis for all gels showed clean, strong bands, suggesting that the desired bands had been amplified in the PCR and nested PCR reactions. Furthermore, negative controls included in the PCR reactions showed that amplification reactions, reagents and techniques used were clean and did not introduce any error in the form of primer dimers.

4.1.3 Cloning of crustacyanin subunits A and C

Gel purification of the PCR amplified 3' and 5' crustacyanin segments was controlled by running a check gel on a small amount of purified product. Gel electrophoresis results showed clear, single DNA bands of between 400 bp and 500 bp length for all segments. These sizes were expected as amplification was performed with oligo(dT) adaptor (for 3' race) or universal primers (for 5' race), resulting in segments of more than 100 bp to 200 bp long segments, depending on the forward (for 3' race) or reverse (for 5' race) degenerate primer used for product amplification.

Successful ligation and transformation reactions of the purified PCR products were observed through the growth of distinctive blue and white colonies on the LB-Amp 50 spread plates and pure blue colonies on the plate incubated with cells carrying the pUC18 control plasmid. For the colony PCR (3' and 5' colony PCR), 90 colonies in total

were chosen from the spread plates. 86 of these colonies were white colonies that contained either the crustacyanin subunit A or subunit C insert, depending on the primer pair used in the PCR amplification prior to the gel purification. DNA gels performed on the colony PCRs showed strong, clean bands of slightly variable size for nearly all white colonies chosen, as well as for the four blue colonies included as control (Figure 4.3).

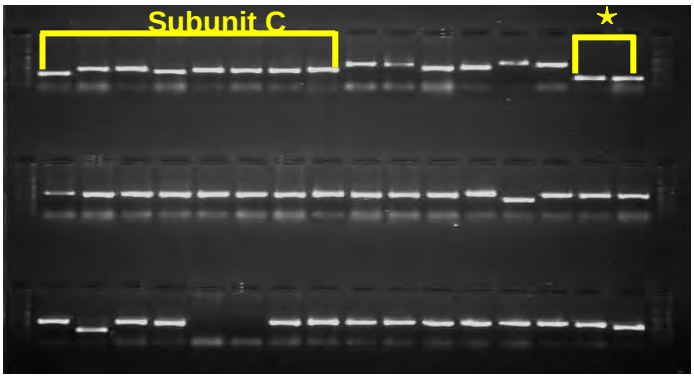

Figure 4.3 Example of a colony PCR check gel. Amplification of the chosen colonies was performed with the universal M13 primer pair. ⋆ Size of the vector alone (from blue control colonies); gel lines not marked are all vector plus DNA inserts from colonies carrying crustacyanin subunit A segments. Wells 1 and 18 at the beginning and end of each row were loaded with a 100 bp DNA marker.

The observed variation in band size was expected, as different primer combinations were used in the cloning process, with the two control samples (⋆) being approximately 200 bp long, while the DNA plus vector inserts from the white colonies appeared to be around 300 to 500 bp long.

Nested PCRs on the 90 samples from the colony PCR and subsequent check gels, confirmed that the colonies chosen carried the correct insert (band sizes of 80 bp to 90 bp), however no bands were detected for the blue colonies, indicating that the cloning process was successful. Of the 90 samples, a total of 37 were chosen for sequencing. Determination of these 37 samples was according to their product size, with inserts chosen that showed slight variation in the insert size, to increase the chance of finding potential isoforms of the subunits.

4.1.5 Sequence analysis and submission to GenBank

Sequence analysis of the 37 samples revealed eleven crustacyanin subunit A sequences and five sequences for crustacyanin subunit C. Consensus sequences for crustacyanin subunit A and subunit C were produced from 15 of these sequences and the two consensus sequences then published on GenBank (Figures 4.4 and 4.5).

Sequence reads of the remaining 21 sequences were of poor quality and were excluded from the analysis.

```
   1 atgttgaagg cactcgtagc tgctgccctc gtggctcttg tcgcagccga cagcattcca
  61 gatttcgtag ctcctggaaa ttgtgccaga gtggcaaatc aagaaaactt cgaccttcgt
 121 agatatgctg gtcgctggta tcaggtaaag atcatcgaca acccctacca gccatacact
 181 cgctgcatcc actccaacta cgactactct gactctgact acggctttag ggtgaccaca
 241 gctggattaa cccccagcaa cgagtaccgc agactgcagg gcaagatcta ccccacaacg
 301 gacttcccca aagcccacat gctcattgat ttccctactg ctttcgccgc tccctacgaa
 361 gtgatcgaga ctgactacga cagctatgcc tgcgtgtatt cctgcattga cacagacaag
 421 tacaagtctg agttcggctt cgtgttctcc cgtactccac agaatgccgg ttcagcgatc
 481 gacaggtgtg cctccgtctt ccgcaggaac ggcgtcgact tctcgtcttt caatgtagtt
 541 cctcattcat ccgaatgttc ttacagggga tag
```

Figure 4.4 Nucleotide sequence of crustacyanin subunit A. Start (ATG) and stop (TAG) codon are highlighted in blue; Accession number of the sequence is HM370278.

```
   1 atgaagactt cgttgctgtg tctcgttctc gttgcagcgg tcgctgccga caagatccct
  61 gactttgtcg tgccaggaaa atgccctgcc gtagacgagc gaatgctcta caatcagcag
 121 aggcctaacc atcagaagta tgctggcaca tggtacgaaa ttgcactcac aaacaaccca
 181 taccaactga tcaagaagtg tgtccgcaac gaatatacct atgatggaag caagttcaac
 241 gtcagatcta ctggttctga tgcttatgga aacgcagtta cacgtaatgg tcaggttctg
 301 cccaatccat tcggtgaacc tcatctttcc gtggactacg aggcatcctg gatggctccc
 361 tacgtgattc tggacactga ctacgagaac ttcgcgtgca tctacagctg cgccggccac
 421 aacttcggct attatgccga cttcgccttc atcttctccc gctcgccaag tctcgctgac
 481 agatattaca ggcgttgtga agccgccttc atgagcatcg gtgtcgaccc ctctcgcttc
 541 acgaagacag ctcagggtgg atcctgctcc tacaacacta actacaggtc ttggtag
```

Figure 4.5 Nucleotide sequence of crustacyanin subunit C. Start (ATG) and stop (TAG) codon are highlighted in blue; Accession number of the sequence is HM370279.

Translation of the nucleotide sequences of both crustacyanin subunits into a deduced protein sequence and alignment of these sequences with their shorter counterparts for

M. japonicus and *P. monodon* indicated areas of consensus between the different prawn species (Figures 4.6 and 4.7). Although both subunits appear to have larger areas of consensus between the three prawn species, crustacyanin subunit A seems to be more conserved between the three species than crustacyanin subunit C. However, as only partial crustacyanin subunit A and C cDNA sequences were available for the prawn species *M. japonicus* and *P. monodon*, while the crustacyanin sequences obtained for *F. merguiensis* through cloning were complete cDNAs, it is difficult to make full comparisons between the three different prawn species.

Analysis of the protein sequence with InterProScan determined that both consensus sequences belonged to the large lipocalin family. All three structurally conserved regions (SCRs) unique to the lipocalin family were found in the putative protein sequences for crustacyanin subunit A and C, as well as the three motifs with their conserved amino acid sequence (GxW in motif 1, TDY in motif 2 and R in motif 3) (Charron et al. 2002; Flower et al. 1993) and six cysteine residues. To determine the locations of these SCRs and motifs, sequences from the study by Flower et al. (1993) were used as guideline. Comparison of the three SCRs and motifs across the three different prawn species shows that these regions were highly conserved (Figures 4.6 and 4.7).

```
                                                          <--- SCR 1 ---
Sub_A_F. merguiensis    MLKALVAAALVALVAADSIPDFVAPGNCARVANQENFDLRRYAGRWYQVK 50
Sub_A_P. monodon        ------------------------------------LRRYAGRWYQVK 12
Sub_A_M. japonicus      ------------------------------------LRRYSGRWYQTQ 12
                                                           ****:*****.:

                     -->
Sub_A_F. merguiensis    IIDNPYQPYTRCIHSNYDYSDSDYGFRVTTAGLTPSNEYRRLQGKIYPTT 100
Sub_A_P. monodon        IIDIPYQPYTRCIHSNYDYSDSDHGFRVTTAGLTPNNEYRRLVGKIYPTN 62
Sub_A_M. japonicus      IIDNAYQPYTRCIHSNYDYSDSDYGFKVTTAGFSPNNEYLRLQGKIYPTK 62
                        *** .******************:**:*****::*.*** ** ******.

                                        <----- SCR 2 ---->       <-- SCR 3
Sub_A_F. merguiensis    DFPKAHMLIDFPTAFAAPYEVIETDYDSYACVYSCIDTDKYKSEFGFVFS 150
Sub_A_P. monodon        DFPKAHMLIDFPSAFAAPYEVIETDYDSYSCVYSCIDTDRYKSEFGFVFS 112
Sub_A_M. japonicus      DFPAAHMLIDFPTVFAAPYEVIETDYDTYSCVYSCIDWNGYKSEFGFVFS 112
                        *** ********:.*************:*:******* : **********

                        -->
Sub_A_F. merguiensis    RTPQNAGSAIDRCASVFRRNGVDFSSFNVVPHSSECSYRG 190
Sub_A_P. monodon        RTPQNAGSAIDRCASV----------------------- 128
Sub_A_M. japonicus      RTPQTTGPANDKCASV----------------------- 128
                        ****.:*.* *:****
```

Figure 4.6 Alignment of protein sequences for crustacyanin subunit A. * indicates areas of consensus, space, . and : indicate areas of variability. Accession numbers were HM370278, ACL37116 and ACL37117 for *F. merguiensis*, *M. japonicus* and *P. monodon*, respectively. Blue highlighted segments correspond to motifs with the yellow inserts marking the conserved amino acids in these motifs. SCR stands for structurally conserved regions, and grey highlights the six cysteine residues.

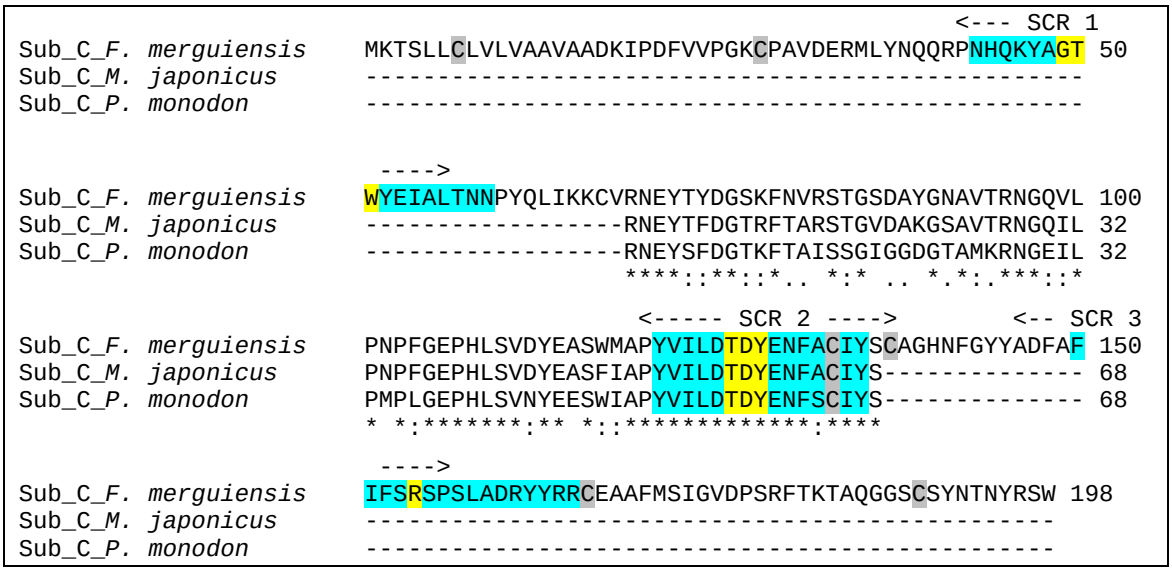

Figure 4.7 Alignment of protein sequences for crustacyanin subunit C. * indicates areas of consensus, space, . and : indicate areas of variability. Accession numbers were HM370278, ACL37122 and ACL37123 for *F. merguiensis, M. japonicus* and *P. monodon*, respectively. Blue highlighted segments correspond to motifs with the yellow inserts marking the conserved amino acids in these motifs. SCR stands for structurally conserved regions, and grey highlights the six cysteine residues.

Additionally to the two full length crustacyanin subunit sequences, one of the eleven sequences for crustacyanin subunit A appeared to be an isoform of this subunit, as 108 bp towards the end of the segment did not match with any of the other ten sequences (Figure 4.8). As this sequence was so clearly different to the other ten sequences found for subunit A, it was not taken into consideration when the consensus sequence was formed for subunit A (Figure 4.4). The cDNA sequence not shared between the putative isoform and the subunit A consensus sequence has the characteristics of an intron, due to the GT-AG sequence found at the beginning and end of this segment which is typical of an intron-exon boundary. No deduced isoforms were detected in the sequences for subunit C (Figure 4.5).

Figure 4.8 Alignment of the *F. merguiensis* consensus sequence for crustacyanin subunit A (HM370278_SubA) with the subunit A isoform. The first 80 bp of the putative isoform and the last 181 bp of the HM370278_SubA were not shown, as they were not relevant for this comparison. * indicates areas of consensus, a space indicates areas of variability; blue highlighted bases indicate the putative intron-exon boundary.

4.2 Colour analysis with absolute qPCR, spectrophotometer and visual observation

4.2.1 Determination of gene expression with absolute qPCR

In the second part of this project, absolute qPCR, a sensitive molecular method, was used to assess the absolute level of gene expression of the crustacyanin subunit A and subunit C genes in the cuticle tissue of *F. merguiensis* prawns of different colour intensities. For this, gene specific primers were developed from the newly cloned crustacyanin subunit A and subunit C consensus sequence (see section 4.1). While the light and dark samples sent from Seafarm had been tested on site to be in intermoult, microscopic analysis of the albino samples by Seafarm determined that three samples were in the late postmoult stage, two in intermoult and five samples were in early to

late premoult. As albinos are rare events in the aquaculture farm environment and a sample size of two was too small for statistical testing, results obtained from albino samples that were not in the intermoult stage have to be viewed with awareness that the moult stage might have an effect on the gene expression levels.

4.2.1.1 Determination of the optimal RNA extraction method and primer pairs for qPCR

Before the qPCR primer pairs could be tested, RNA of high quality had to be extracted from test samples. From the two RNA extraction methods tested, TRIzol® proved to be the best method as the amount of total RNA extracted from the cuticle tissue gave approximately over five times more total RNA than the RNeasy Plus Mini kit. Moreover, quality analysis of the extracted RNA with gel electrophoresis and the NanoDrop2000 spectrophotometer indicated clean, very strong bands of high quality RNA with 260/280 values of just below 2.0 for the TRIzol® samples, compared to faint bands and lower quality readings for the samples produced with the RNeasy Plus Mini kit. Therefore, all RNA extractions for the absolute qPCR experiment were carried out with trizol.

After cDNA was synthesised from the TRIzol® samples and the cDNAs PCR amplified, gel electrophoresis showed strong and clear bands of expected segment length for each PCR product (Figure 4.9). Once the primer pairs were also assessed with qPCR, primer pairs SubA2F and SubA2R (Ct of 16.31) were chosen for crustacyanin subunit A and SubC1F and SubC1R (Ct of 17.34) for subunit C as these primer pairs were most likely to have a high primer/reaction efficiency. Additionally, cycling and melt curve conditions used for the absolute qPCR run were verified to be well suited for the two chosen primer pairs.

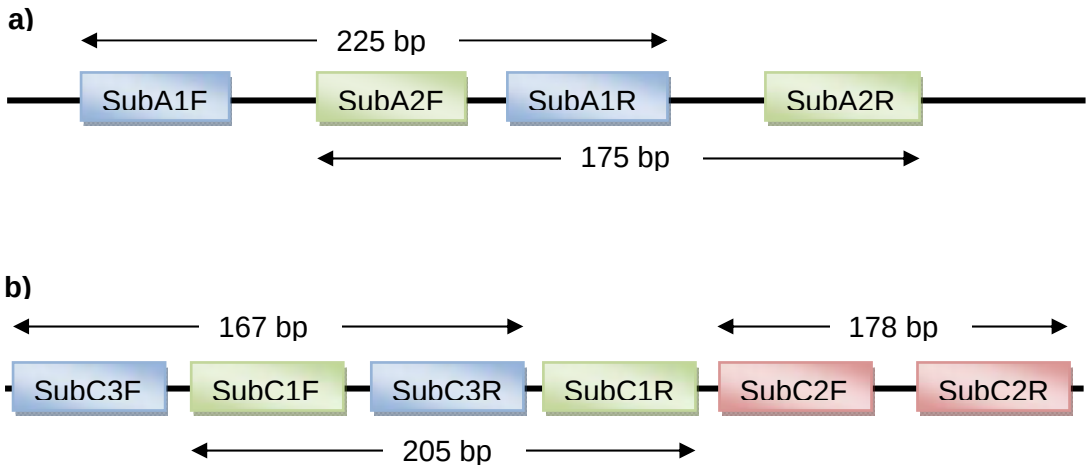

Figure 4.9 Schematic of the expected amplicon sizes of the **a)** crustacyanin subunit A and **b)** subunit C qPCR primer pairs.

Melt curves showed a single prominent peak for each sample run in triplicates, confirming that only one product was amplified (Figure 4.10a and b). Furthermore, NTCs and –RT controls added to the reaction stayed close to the base level, and well below the 0.1 threshold, indicating that the reagents used in the reaction did not introduce any error into the reaction and no genomic DNA was included in the templates used.

Results for the melt curve analysis and addition of controls (NTCs and –RT controls) observed in this first qPCR were consistently reproduced for every qPCR amplification reaction used in this project.

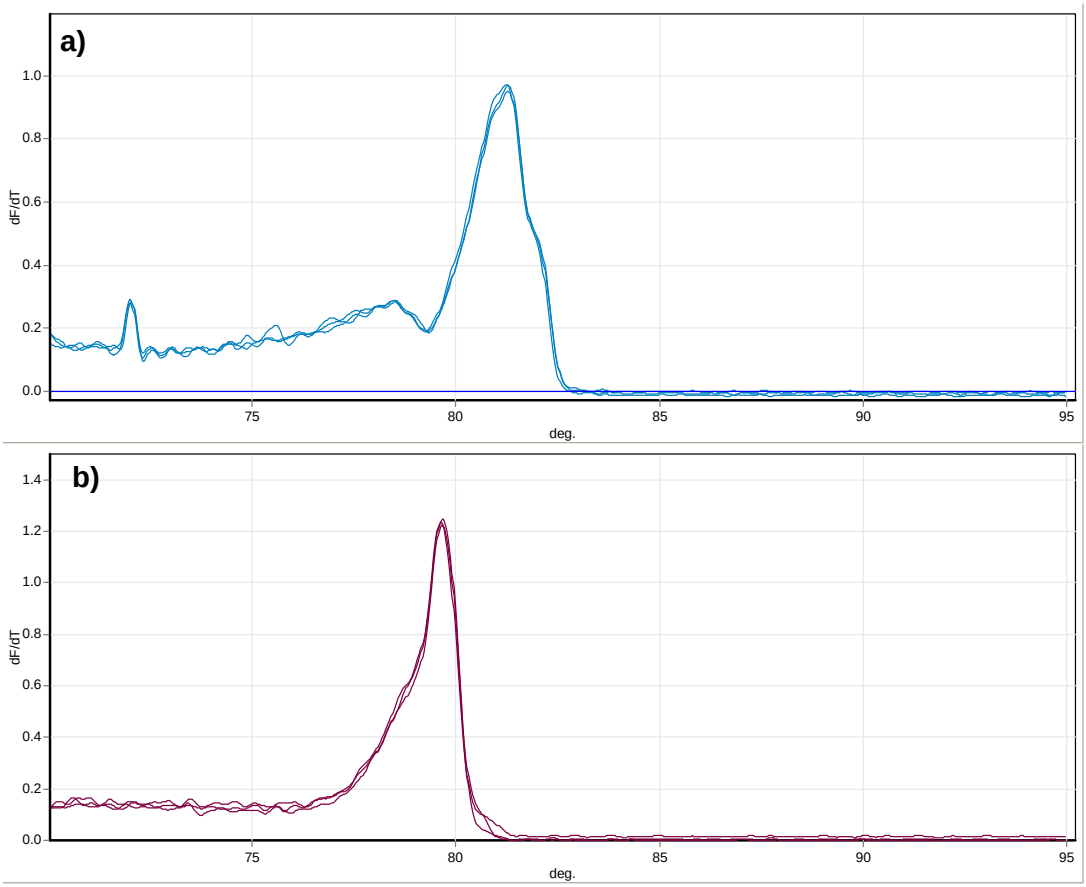

Figure 4.10 Example of a melt curve for samples analysed with primer pairs chosen for **a)** crustacyanin subunit A and **b)** subunit C. Melt curve conditions were as described in 3.3.2.3.

4.2.1.2 Validation of the absolute qPCR reaction

Total RNA used to prepare the standard dilution curves were observed as strong, clear bands on the RNA gel, with a high quality reading (260/280 of 2.04). Gel electrophoresis on the PCR products amplified with gene specific primer pairs for subunit A or subunit C confirmed that the correct segments were amplified (band sizes of ~ 170 bp to 200 bp), before the PCR products were cleaned and the gene copy

number of the two amplified cDNAs was calculated. Mean total cDNA concentration of the sample amplified with the subunit A primer pair was 69.07 ng/µl (segment length of 175 bp), and for the subunit C primer pair it was 93.67 ng/µl (segment length of 205 bp), resulting in a calculated gene copy number of 2.36 x 10^{11} and 1.68 x 10^{11} gene copies respectively.

Although a 20 point dilution curve was run on the qPCR machine for both subunits, the final standard curves for both subunits were only comprised of 13 dilutions. The first dilution step counting for the standard curve was the second dilution, as this dilution started with a fluorescence reading of 0 % in the very first cycle, steadily increasing to 100 % over the first 10 amplification cycles. Additionally, the last dilution step included in the standard curve was the 14th 1:5 dilution step, as results became unreliable after this dilution (Figure 4.11a and b). This meant that any result in the gene expression analysis that was above 9.44 x 10^9 and 6.72 x 10^9 copies for crustacyanin subunits A and C, respectively were considered to be "above detectable limit" and results below 38.66 and 27.52 gene copies for subunits A and C, respectively were considered to be "below detectable limit". Reaction efficiency, R^2 value and slope for both standard curves were determined to be 0.962, 0.999 and -3.415 for the subunit A standard curve and 0.901, 0.998 and -3.583 for the subunit C standard curve, respectively.

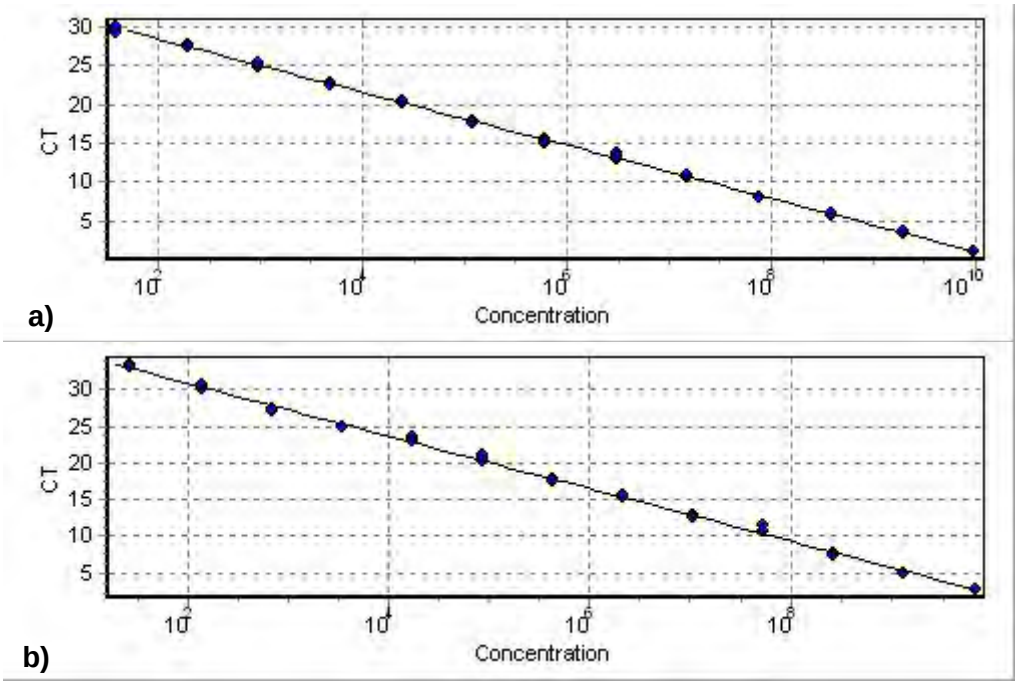

Figure 4.11 Standard curves for the adjusted **a)** crustacyanin subunit A and **b)** subunit C standard curves. The curves do not include the original sample, dilution 1 and dilutions below the 14th.

4.2.1.3 Tissue quality assessment of the prawn samples

Gel electrophoresis of the total RNA from the six test samples showed bright and relatively clean 18S bands and slightly fainter 28S bands as was expected from crustacean RNA. Although this suggests that the RNA was intact, the start of RNA degradation could also be observed by the faint smears between the bands (Figure 4.12a). Amplification of the cDNA produced from each of the six test samples, using crustacyanin subunit A (SubA2F and SubA2R) and subunit C (SubC1F and SubC1R) primer pairs and checking the product size on a DNA gel also showed clean, strong bands of expected length (~ 170 bp to 200 bp) (Figure 4.12b). Amplifying the synthesised cDNA with absolute qPCR showed that the standard curves produced for both crustacyanin subunits from the dilution curve were spread out sufficiently for the samples to fall within and be able to calculate the crustacyanin gene expression with the Rotor-Gene 6000 software.

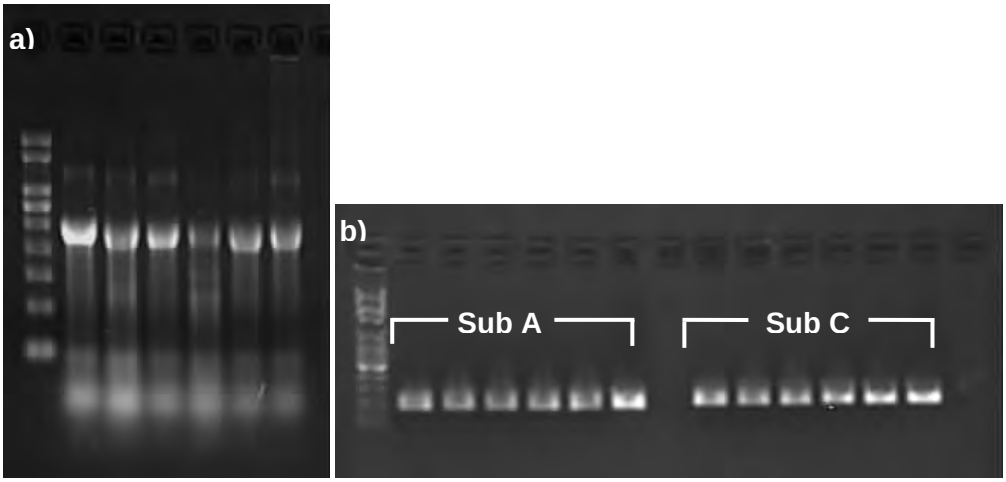

Figure 4.12 Gel visualisation of **a)** total RNA extracted from prawn cuticle samples and **b)** PCR amplified products from the six test prawn samples. Both gels were run with a 100 bp marker (left lane). “Sub A” and “Sub C” refer to samples amplified with SubA2F and SubA2R, and SubC1F and SubC1R primer pairs, respectively.

4.2.1.4 Absolute qPCR crustacyanin gene expression level analysis of light, dark and albino prawns

Total RNA extracted from the 50 prawns (20 light, 20 dark and 10 albino) chosen for the crustacyanin subunit A and subunit C gene expression level analysis, showed strong bands for 18S, lighter 28S bands with faint smears between the bands (Figure 4.13). Bioanalyzer analysis on a representation of the 50 prawns confirmed the results observed through gel electrophoresis, with a strong 18S line and a faint 28S band. RNA extracted from some albinos appears to be slightly more degraded than total RNA

extracted from the other samples (Figure 4.14). As the Bioanalyzer was designed for higher vertebrates, no RNA integrity numbers (RIN) could be determined for either of the samples.

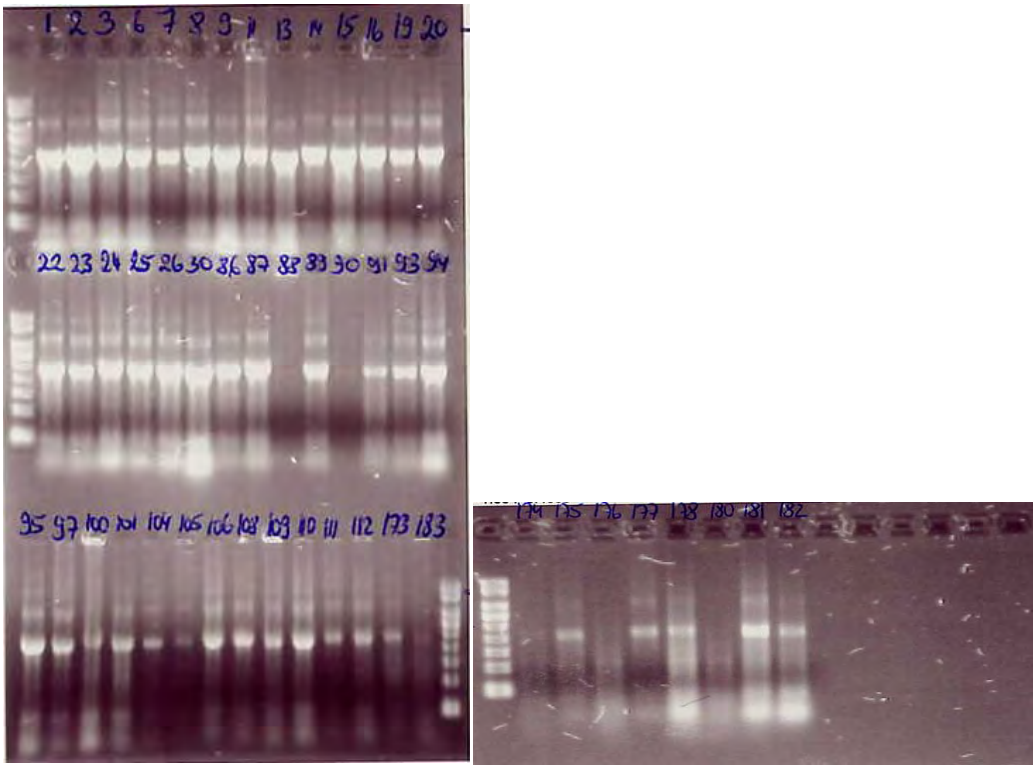

Figure 4.13 RNA gel electrophoresis visualisation of total extracted RNA from the 50 prawn samples, run with a 100 bp marker.

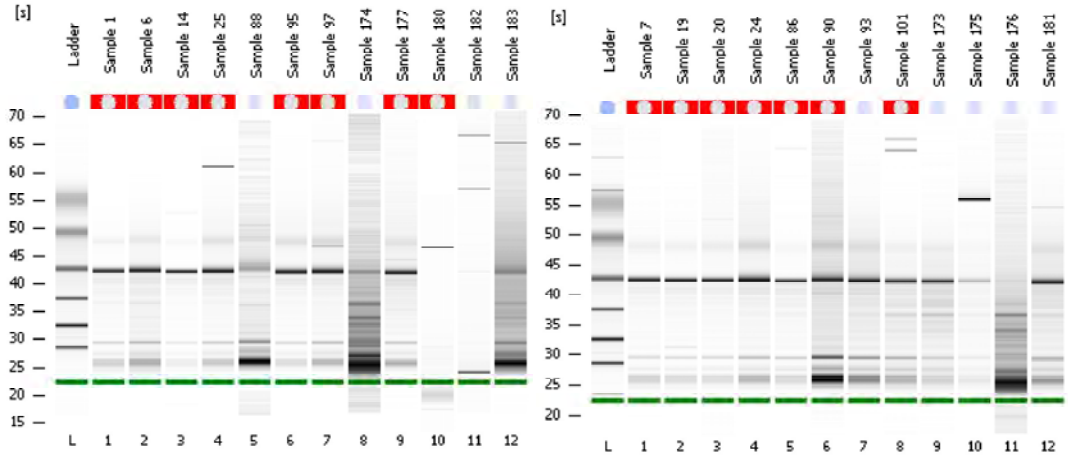

Figure 4.14 Bioanalyzer RNA pictures of total extracted RNA from a sample of the 50 prawn samples, run with the Agilent RNA 6000 Nano ladder. Gel lines for samples 180 and 182 should be disregarded.

A DNA check gel (Figure 4.15) was run on the qPCR products and confirmed the results of the melt curve analysis (Figure 4.10) done for the amplification reaction of

both primer pairs, with both, gel and melt curve, showing the amplification of one single product. NTCs and –RT controls were both observed as baseline, well below the threshold of 0.1 in the qPCR reaction and no band was observed for these samples in the DNA check gel. Gel bands were observed to be around 170 bp and 200 bp for samples amplified with the subunit A (Figure 4.15) and subunit C primer pairs, respectively.

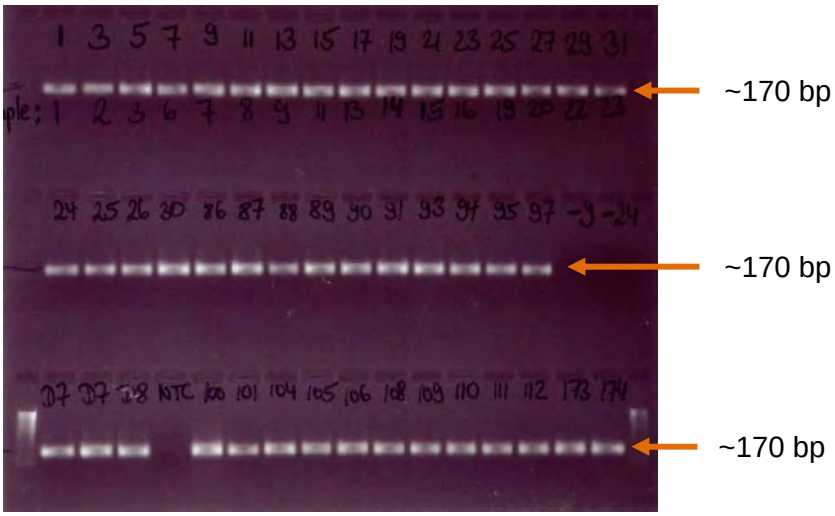

Figure 4.15 Example of a DNA check gel, depicting crustacyanin subunit A qPCR amplicons. Amplification of these samples was performed with the SubA2F and SubA2R primer pair. In the picture, -9 and -24 stand for –RT controls, D7 and D8 were from the dilution curve for subunit A and NTC stands for no template control.

Analysis of the crustacyanin subunit A and C gene expression levels in the 50 *F. merguiensis* prawns, using absolute qPCR with the appropriate standard curves allowed the determination of mean copy numbers for crustacyanin subunit A and C actively expressed in each individual prawn. Gene expression levels for crustacyanin subunit A were all below the maximal detection limit of 9.44×10^9 copy numbers and for subunit C below 6.72×10^9 copy numbers, however, four of the albino prawns expressed crustacyanin subunit A and C below the minimal detectable limit. Additionally, one of the dark prawn samples showed a crustacyanin subunit C expression level below the detectable limit. RNA gel bands for these five samples all showed a relatively faint band, except for one albino that had a stronger gel band; however, for the cDNA synthesis the same amount of RNA was always used, so results should not have been compromised. To be able to statistically analyse the data, these 5 samples, labelled “below detectable limit” were set to half of the lowest detectable limit (19.33 gene copies for subunit A and 13.76 gene copies for subunit C).

Colour groups (albino, light and dark), into which each individual prawn had been placed in by experienced staff at Seafarm, the generous provider of the *F. merguiensis* prawn samples, were maintained throughout the colour analysis. Splitting the samples according to these colour groups (termed “colour group assigned by Seafarm”) showed that hardly any crustacyanin subunit A or C was expressed in the albino prawns (copy numbers of 1000.6 ± 463.9 and 525.4 ± 305.8, respectively). Dark prawns, which had been expected to have the highest crustacyanin subunit A and C gene expression level, expressed substantially less crustacyanin subunit A and C (copy number of 40,661.6 ± 15,020.2 and 30,185 ± 12,860.7, respectively) then the light prawns (copy number of 206,998.9 ± 63,971.3 for subunit A and 265,687.7 ± 105,295.7 for subunit C). The mean copy numbers for crustacyanin subunit A were slightly higher than the mean copy numbers for subunit C in the albino and dark prawn groups, while in the light prawns, the trend was reversed, with subunit C being higher expressed than subunit A. However, it has to be mentioned that while the raw, individual prawn data (Appendix 1) overall followed the trend observed for the mean copy numbers of each colour group, the individual copy numbers for both crustacyanin subunits were quite spread and some overlap between individuals from the light and dark groups was observed.

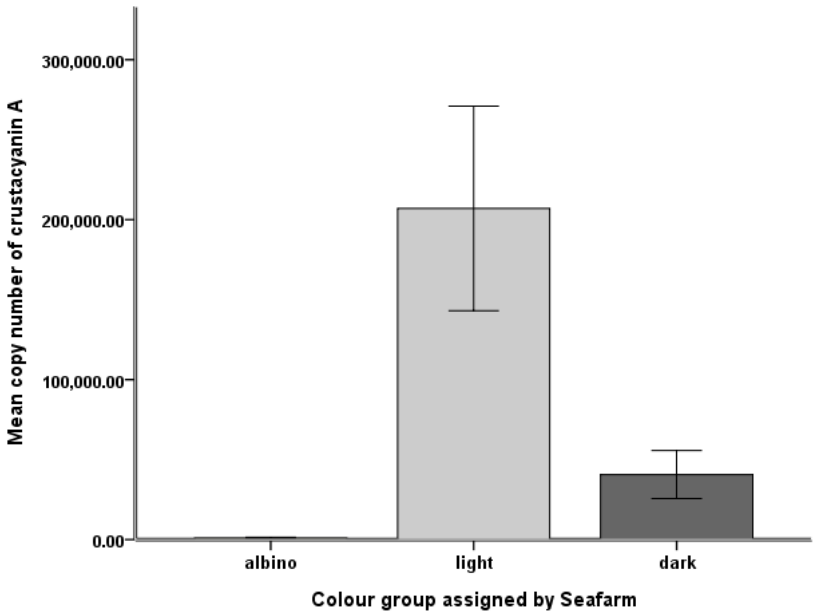

Figure 4.16 Mean crustacyanin subunit A gene copy numbers, separated into their respective colour group (albino, light and dark) as initially classified by Seafarm staff. Standard error (SE) bars for each colour group stand for +/- 1SE.

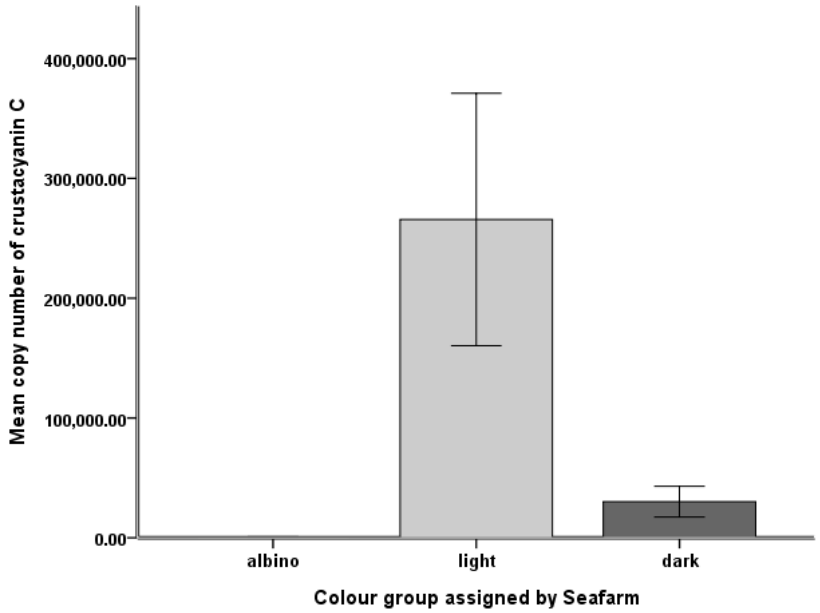

Figure 4.17 Mean crustacyanin subunit C gene copy numbers, separated into their respective colour group (albino, light and dark) as initially classified by Seafarm staff. Standard error (SE) bars for each colour group stand for +/- 1SE.

Statistical analysis of the mean crustacyanin subunit A and C copy numbers for the 20 light, 20 dark and 10 albino samples, using PASW Statistics 18, showed that the data violated the assumptions of normality and homogenous variance. Mean copy numbers of crustacyanin subunit A and C for the albino, light and dark groups were significantly non-normal distributed ($p < 0.05$) with a heterogeneous variance ($p < 0.001$) (determined using the Kolmogorov-Smirnov and Levene's test).

Analysis of the crustacyanin subunit A and subunit C gene expression levels of the 50 prawn samples, using the non-parametric Kruskal-Wallis test resulted in the rejection of the null hypothesis for both subunits ($p < 0.001$), therefore, the distribution of the crustacyanin subunit A and C mean copy numbers was different between the three prawn colour groups (albino, light and dark). Moreover, three Mann-Whitney U tests with Bonferroni's correction showed that the differences in the crustacyanin subunit A and C mean copy number distribution observed between the three colour groups (Figures 4.16 and 4.17) were significant ($p < 0.001$ for the comparisons between albino and light, and albino and dark, and $p < 0.01$ for the comparison between the light and dark colour group).

Correlation analysis of all three colour groups was performed to assess whether a relationship existed between the mean gene expression level of crustacyanin subunit A

and subunit C, using the 2-tailed, non-parametric Kendall's tau correlation test. Analysis of the albino, light and dark prawn colour groups all showed that the mean copy number of crustacyanin subunit A was significantly positively correlated ($p < 0.01$) with the mean copy number of crustacyanin subunit C.

4.2.2 Spectrophotometric colour analysis

The 50 prawn samples were steamed as a mean of cooking instead of boiled, as preliminary cooking trials showed that steaming of prawns, which had the exoskeleton removed from the first abdominal segment, did not appear to cause the pigment to leach out of the other, still protected abdominal segments, while boiling the prawns resulted in large amounts of pigment leaching out into the water.

Two different colour analysis methods (colorimeter or spectrophotometric analysis of pigment extract) were evaluated for the determination of colour in prawns (3.3.3.2). The preliminary tests showed that the pigment extraction method outlined in Tume et al. (2009) could be easily repeated consistently for all 50 prawn samples. Measurements carried out with the colorimeter, however, were difficult to duplicate consistently for all samples, due to the small scan area of this device (~ d = 2 cm), as well as the measurement differences observed over one segment of one individual prawn. Furthermore, as the prawn samples varied slightly in size, it was difficult to pinpoint the exact same spot for colorimetric measurement across all samples, further increasing the potential error in the resulting measurement. Additionally, the colour intensity was measured on the intact prawn (exoskeleton included) to include the astaxanthin embedded in the exoskeleton, which made it difficult to find a relatively flat and even surface due to the more rigid shell. These restrictions would quite possibly have lead to a very inaccurate result. Therefore, the chemical pigment extraction and spectrophotometric measurement method discussed by Tume et al. (2009) was used for the pigment analysis.

After the complete extraction of the total astaxanthin from the fifth abdominal segment of each of the 50 prawns, an initial full range wavelength measurement was performed with the spectrophotometer, confirming the wavelength of 477 nm for total astaxanthin measurement. All peaks of subsequent full range wavelength measurements, carried out for each sample, were detected around 476 nm to 477 nm.

Results obtained from the spectrophotometric colour analysis, indicate that prawns classified "dark" by Seafarm staff contained the largest amount of total astaxanthin, with a mean total astaxanthin value of 14.6 µg/g of wet weight ± 0.5 (Figure 4.18).

Slightly less total astaxanthin was detected in the light prawns (12.8 µg/g of wet weight ± 0.6), with the lowest amount of total astaxanthin found in the albino prawns (4.5 µg/g of wet weight ± 1.4) (Figure 4.18). Individual results of the total astaxanthin extraction showed that while most light and dark prawns contained total astaxanthin levels close to their respective group mean, a slight overlap in total astaxanthin results was observed for a few of the light and dark coloured prawns (Appendix 2). Furthermore, while the majority of the individual albino prawns contained low levels of total astaxanthin, one albino prawn had an elevated total astaxanthin level of 15.4 µg/g of wet weight.

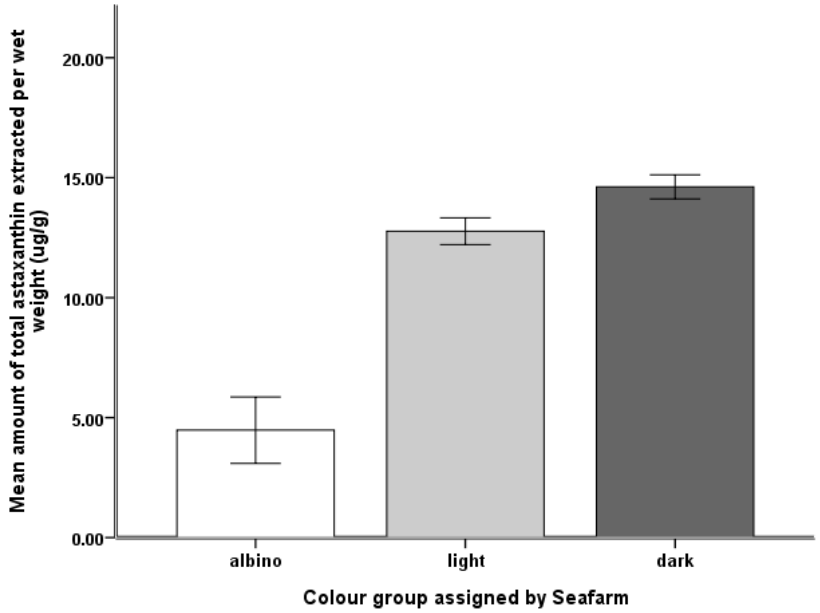

Figure 4.18 Mean amounts of total astaxanthin (in µg/g of wet weight), extracted from the 5th abdominal segment of each of the 50 prawns, and separated into the respective colour group (albino, light and dark) as initially classified by Seafarm staff. Standard error (SE) bars for each colour group stand for +/- 1SE.

Statistical analysis of the total astaxanthin results for each individual prawn, determined that the data was normally distributed, with homogenous variances ($p > 0.05$, using Kolmogorov-Smirnov and Levene's test). As a correlation analysis was also performed on the data and transformation of the raw total astaxanthin data (e.g. through Log or square root transformation) would have resulted in significance values of $p < 0.05$ for Levene's and Komolgorov-Smirnov test, the non-parametric tests were used for the statistical analysis of the raw data. The non-parametric Kruskal-Wallis test determined that there was a difference in the distribution of the mean total astaxanthin values across the three different colour groups ($p < 0.001$), with significant differences

($p < 0.001$) in the mean total astaxanthin values observed between the albino and light, and albino and dark groups, using three Mann-Whitney U tests with Bonferroni's correction. Statistical analysis of the distribution of the mean total astaxanthin values between the light and dark group also showed significant differences, however, the statistical results were just slightly below $p < 0.0167$.

Correlation analysis of the mean total astaxanthin values of each of the three colour groups, using the non-parametric Kendall's tau test, showed that there was no significant relationship ($p > 0.05$) between the mean total astaxanthin values and the mean copy numbers of crustacyanin subunit A and C of each colour group.

4.2.3 Visual colour determination

Following the steaming and photographing of the 20 light, 20 dark and 10 albino *F. merguiensis* prawns, photographs of the uncooked and cooked prawns were visually compared with each other. Results showed that colour classifications (albino, light and dark), given to the uncooked 50 prawn samples by Seafarm staff, coincided with the perceived colour intensity of the same prawns when cooked (Figure 4.19).

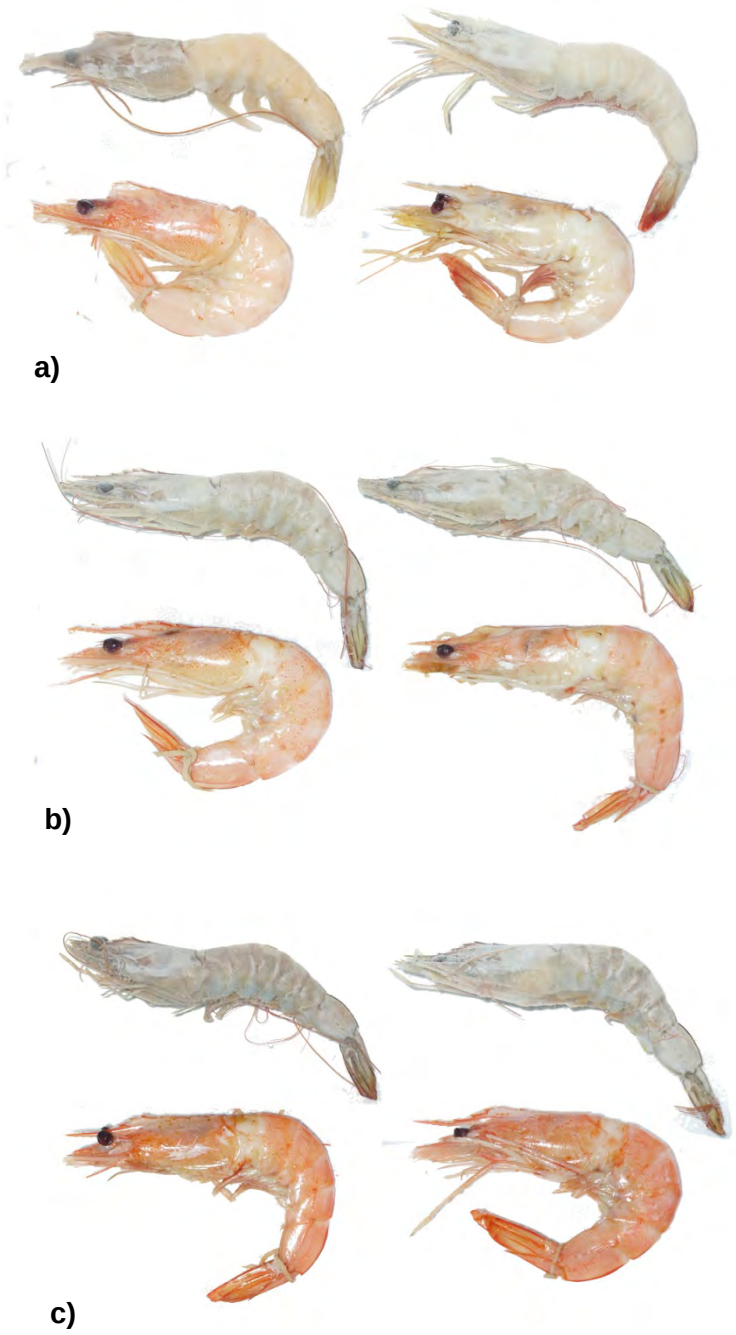

Figure 4.19 Photographs of **a)** two albino, **b)** two light, and **c)** two dark prawns before and after the cooking process. Frozen, uncooked prawns are shown in the first row, with the respective cooked prawns in the second row.

Colour intensity of the 50 prawns was also visually assessed by four male and two female USC staff, using the *P. monodon* colour chart from Aqua-Marine Marketing as ranking guide. Ranking values for each cooked prawn, given according to the perceived strength of the colour intensity, were observed to be between one and seven. Mean rank values for each individual cooked prawn were grouped into albino,

light and dark according to their respective pre-cooked colour classification, to determine whether uncooked colour classifications could be confirmed with the mean ranking values of the cooked prawns. Results of this grouping into albino, light and dark, showed that the colour classifications given by Seafarm staff overall represented the observed colour intensities of the cooked prawns fairly accurately (Figure 4.20). As depicted in Figure 4.20, the cooked albino prawns received the lowest mean ranking values, while mean rankings for the prawns classified light were approximately double, and for the prawns classified dark were about triple the mean ranking values of the albino prawns. While the mean ranking values of the prawns classified light and dark appear to be distinctly different, the difference between them was slightly less than that observed between albino and light (Figure 4.20). This smaller difference was the consequence of a minimal overlap that was observed in individual mean ranking values of light and dark prawns, with the ranking values for four light prawns falling within the range of the dark prawns, and the rankings for four dark prawns falling within the range of the light prawns (Appendix 3). Due to the nature of the ranking data (ordinal), significance of the observed differences between the three colour groups could not be determined.

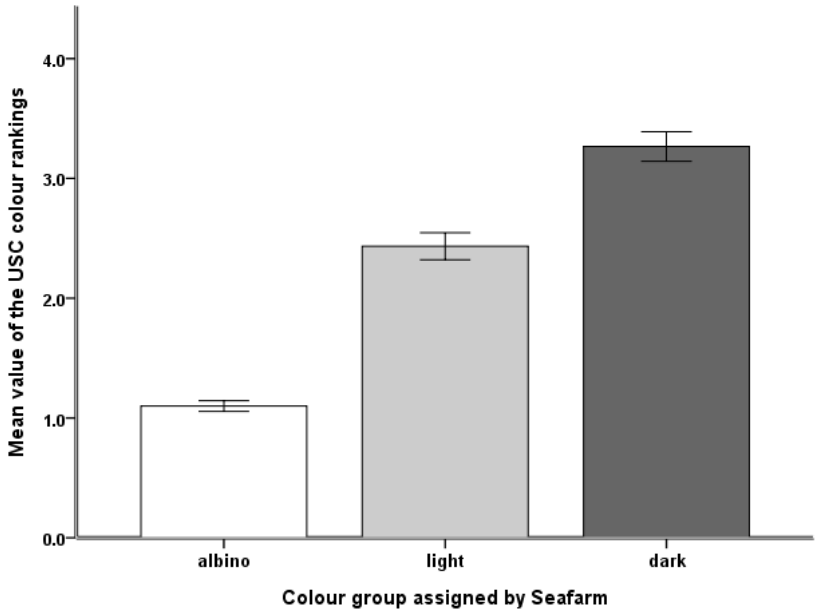

Figure 4.20 Mean values of the rankings, given by seven University of the Sunshine Coast staff, plotted according to the colour classifications assigned to each prawn by Seafarm staff. Standard error (SE) bars for each colour group stand for +/- 1SE.

4.3 Next generation sequencing and analysis

4.3.1 Sequencing results for mRNAs expressed in the muscle/cuticle tissue

Total RNA extracted from *F. merguiensis* muscle/cuticle tissue for the Roche 454 next generation sequencing project was shown to be not degraded, with RNA concentration levels ranging from 9.6 ng/µl up to 481.9 ng/µl with the 260/280 value for each sample being around 2 as was expected.

Next generation sequencing of the muscle/cuticle transcriptome data by AGRF resulted in 54928 sequence reads of varying base pair length (from ~ 30 bp to 540 bp). From these, 1455 contigs (sequence length between 45 bp and 4568 bp) were produced with the CLC Workbench, leaving 4535 singletons. Analysis of the 5990 contig and singleton sequences with the blast2go software, using blastn, resulted in sequence matches being found in the NCBI database for 3586 of these contig and singleton sequences. For the 2404 sequences that did not have any matches on the NCBI database, functional annotation was not possible and the sequences were considered to be unannotated. Functional annotations given to the 3586 sequences by the blast2go program grouped the annotated gene sequences into three divisions: molecular function, cellular component and biological process.

The results for the molecular function division indicate that almost half of the sequences code for molecules that have a binding function, followed by molecules that have some sort of catalytic or structural activity at a molecular level, with only a small component of sequences having regulatory activity (Figure 4.21).

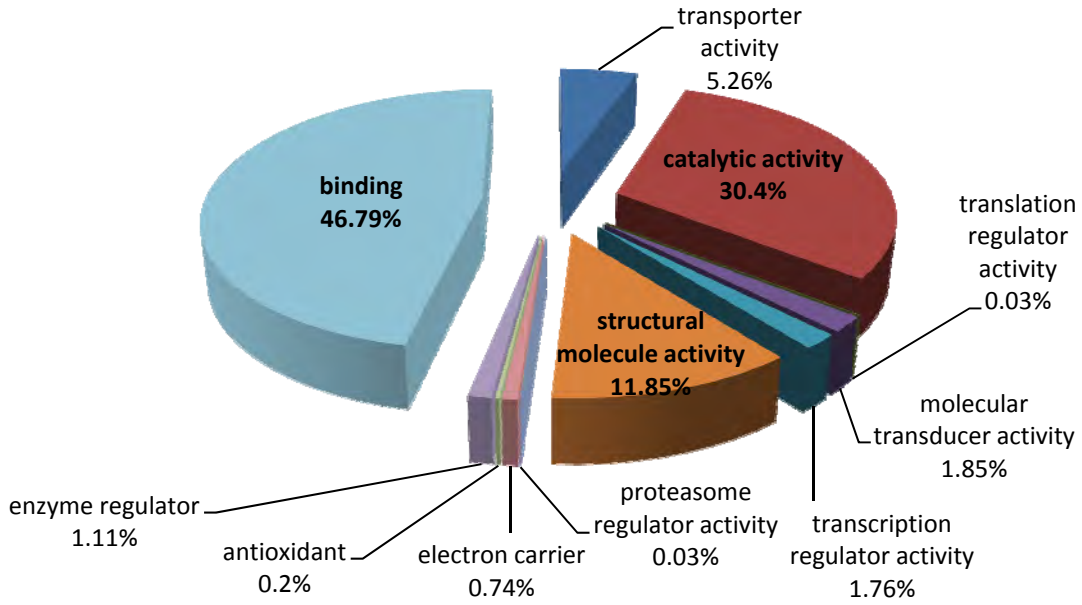

Figure 4.21 Molecular functions of the 3586 annotated genes that were actively expressed in the *F. merguiensis* muscle/cuticle tissue. The blastn function of the blast2go software was used to functionally annotate these genes. Graph displays level 2 data.

At the cellular components level, about 37 % of the annotated sequences coded for a part of the cell, and around 47 % of the sequences were a component of membrane- (~11 %) and non membrane-bound organelles (~19 %) or organelles in general (17 %) (Figure 4.22).

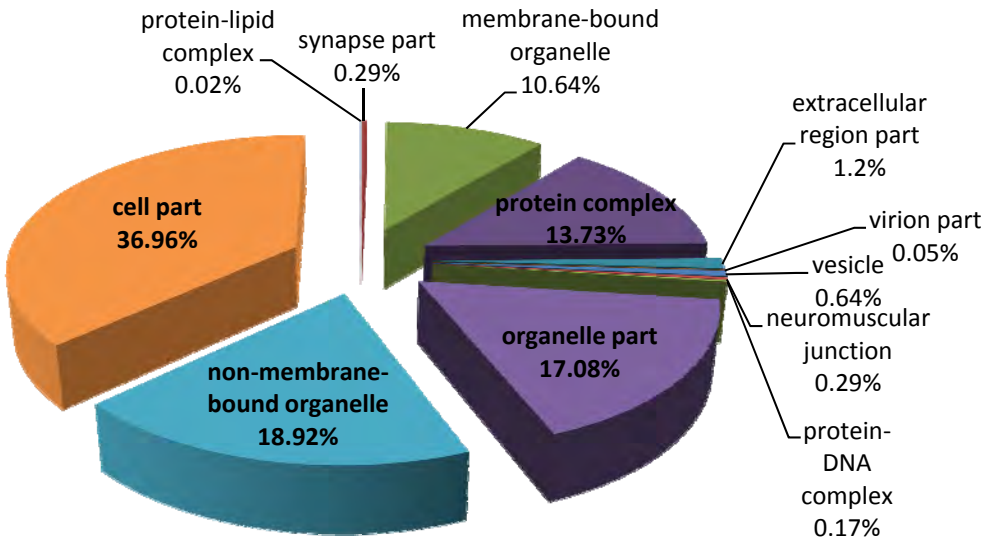

Figure 4.22 Pie chart of the cellular components, the 3586 annotated genes that were actively expressed in the *F. merguiensis* muscle/cuticle tissue coded for. The blastn function of the blast2go software was used to functionally annotate these genes. Graph displays level 3 data.

At the biological level, ~ 34 % of the sequences coded for molecules that had some function in a cellular or metabolic process (Figure 4.23). Also included in the cuticle/muscle transcriptome data were crustacyanin subunit A and subunit C, although these sequences were only represented in a very small number (0.04 % - under pigmentation) (Figure 4.23). Aligning these crustacyanin contigs and singletons from the transcriptome data by using ClustalW2 showed that they all align very closely with each other, as well as with the respective consensus sequence for crustacyanin subunit A or C that were determined through cloning. Slightly more base changes (e.g. “A” in one singleton, “C” in another) were observed for the alignment of the subunit A transcriptome contig and singletons than for the subunit C alignment. Furthermore, although the aligned transcriptome subunit A sequences were marginally shorter on the 3’ end compared to the complete cloned crustacyanin subunit A sequence, the 5’ end of the aligned sequences was extended upstream of the start codon of the cloned consensus sequence into the 5’ untranslated region. No match to the putative subunit A isoform found in the cloning experiment could be detected in the transcriptome data.

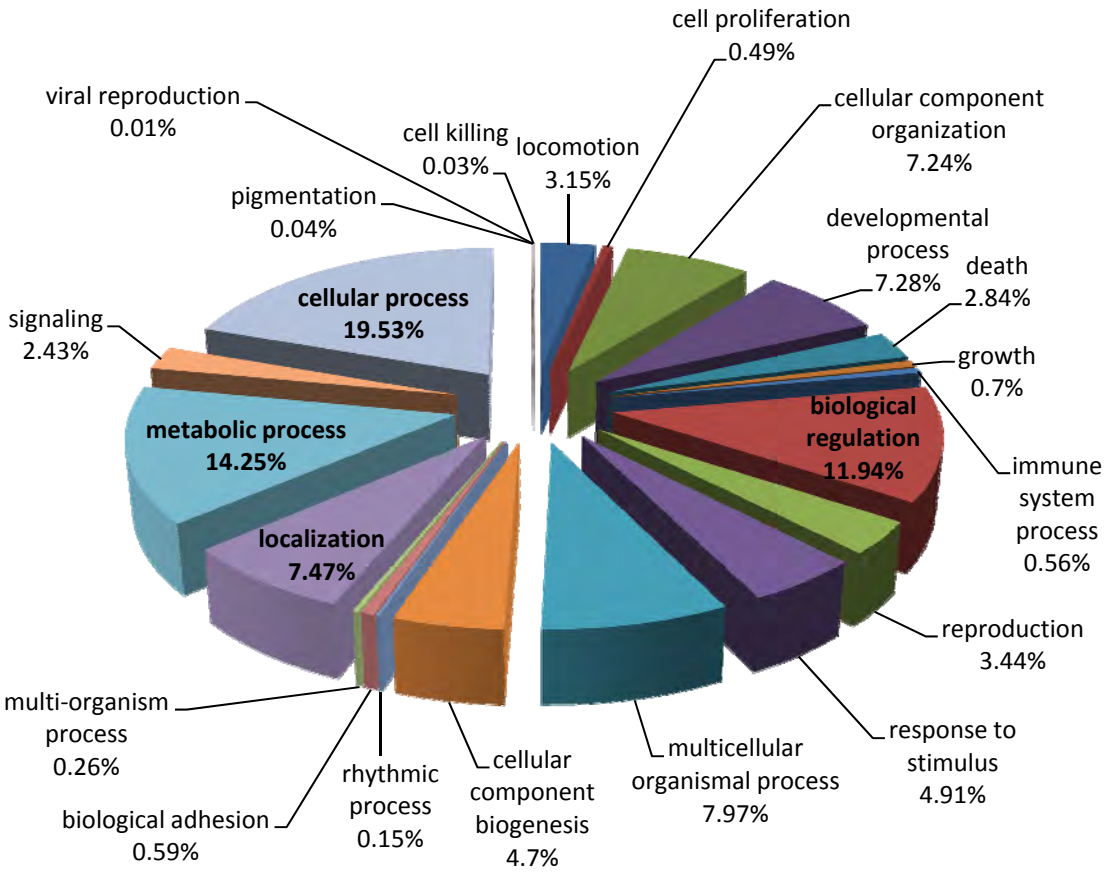

Figure 4.23 Biological processes carried out by the 3586 annotated genes that were actively expressed in the *F. merguiensis* muscle/cuticle tissue. The blastn function of the blast2go software was used to functionally annotate these genes. Graph displays level 2 data.

4.3.2 Crustacyanin transcripts expressed in other tissues

Analysis of the transcriptome data obtained from six other *F. merguiensis* tissues sent for next generation sequencing, revealed that crustacyanin subunit A and subunit C genes were also actively expressed in the hepatopancreas, eye stalk and nervous system of this prawn species. No crustacyanin gene expression was detected in the tissues of the stomach, androgenic gland and male and female gonads.

4.4 Microarray analysis

Gene expression levels in the cuticle tissue from 12 light, 12 dark and 9 albino *F. merguiensis* prawn samples were compared, using three customised microarrays and analysed with the GeneSpring GX 11.0 software. After the best possible PMT gain was chosen for each block in the three arrays (Figure 4.24), signal intensity values were corrected for the background signal in GenePix 6.0 and these corrected values imported for analysis into GeneSpring GX 11.0.

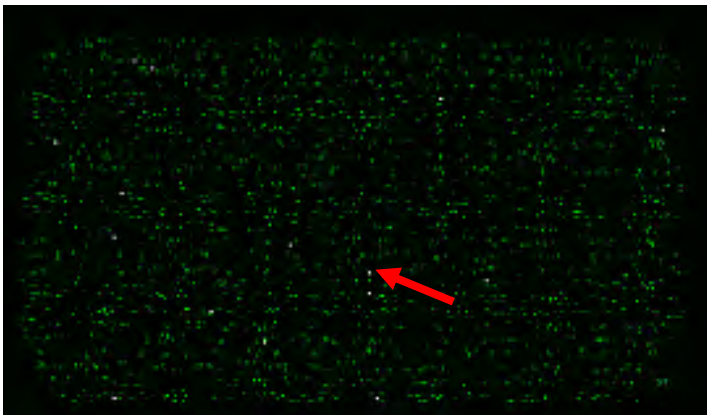

Figure 4.24 Example of a microarray scan. Optimal PMT gain for this block was set at 530 and shows the expression of genes in the cuticle tissue of one of the four light groups. A low percentage of oversaturated points can be seen (example point indicated with a red arrow).

Successful normalisation of the data by the GeneSpring GX 11.0 software was confirmed by assessing the box whisker plot of the normalised intensity values, showing that the means of the data in each of the 12 microarray blocks centre around 0 (Figure 4.25).

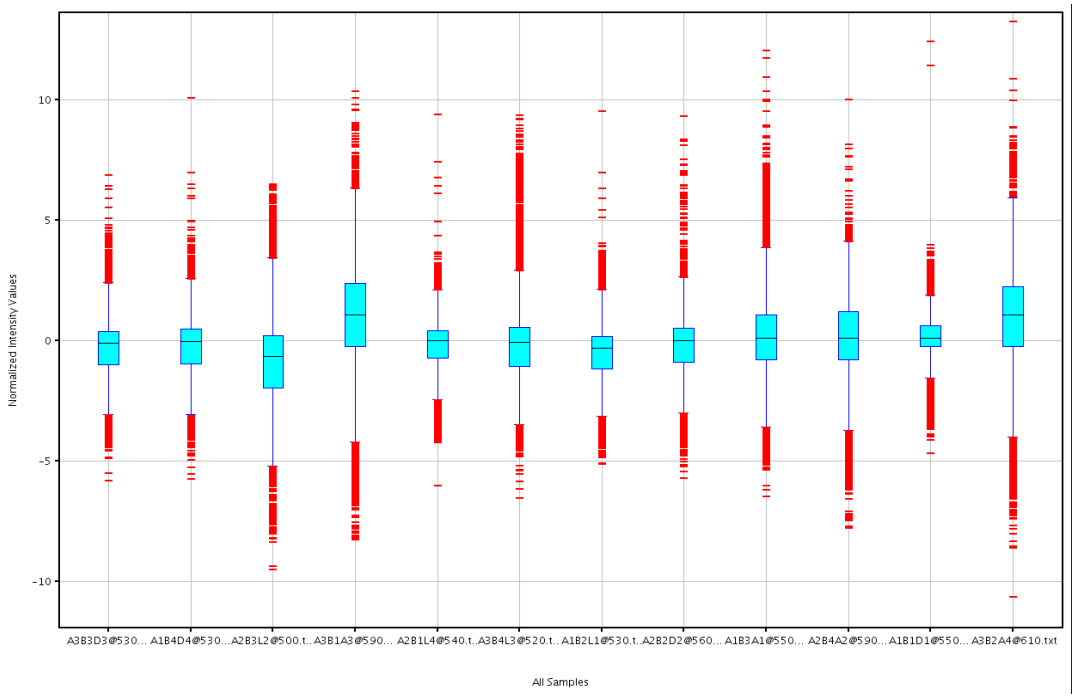

Figure 4.25 Box Whisker plot of the normalised data for all albino, light and dark samples of the 12 microarray blocks. Y- axis shows normalised intensity values, x- axis shows the 12 samples.

Furthermore, as albino samples of different labelled cRNA amounts (825 ng, 1050 ng and 1650 ng) were hybridised to the three microarrays, the validity of the normalisation on the albino samples was assessed with a cluster analysis (k=20) (Figures 4.26 – 4.32). The 20 clusters were grouped into seven main clusters, each cluster sharing a similar expression pattern. Visual analysis of the main clusters indicated that normalisation was appropriate across the different amounts of labelled cRNA used for the albino samples. Therefore, any variation observed in the cluster groups is considered to be the response to genuine differences in the gene expression level of the different albino groups and was not attributed to the different amounts of labelled cRNA hybridised to the three arrays.

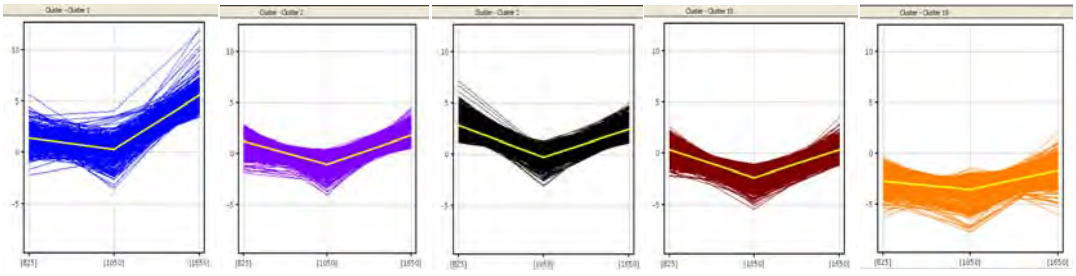

Figure 4.26 Graphs showing cluster group 1, displaying microarray probes that were found to be similarly expressed across the three different, labelled cRNA amounts (825 ng, 1050 ng, 1650 ng) of the albino samples. Data was obtained through K-mean cluster analysis of the normalised data (k=20).

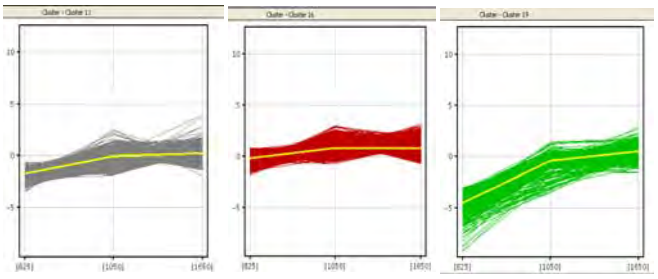

Figure 4.27 Graphs showing cluster group 2, displaying microarray probes that were found to be similarly expressed across the three different, labelled cRNA amounts (825 ng, 1050 ng, 1650 ng) of the albino samples. Data was obtained through K-mean cluster analysis of the normalised data (k=20).

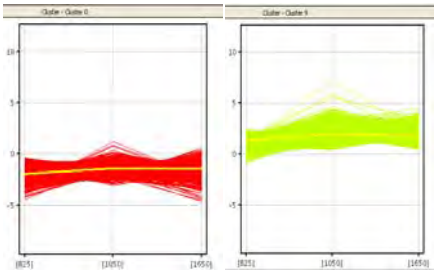

Figure 4.28 Graphs showing cluster group 3, displaying microarray probes that were found to be similarly expressed across the three different, labelled cRNA amounts (825 ng, 1050 ng, 1650 ng) of the albino samples. Data was obtained through K-mean cluster analysis of the normalised data (k=20).

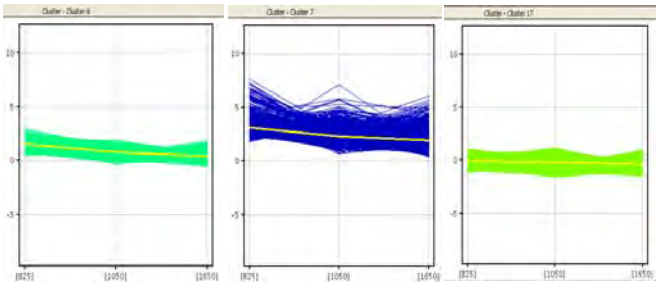

Figure 4.29 Graphs showing cluster group 4, displaying microarray probes that were found to be similarly expressed across the three different, labelled cRNA amounts (825 ng, 1050 ng, 1650 ng) of the albino samples. Data was obtained through K-mean cluster analysis of the normalised data (k=20).

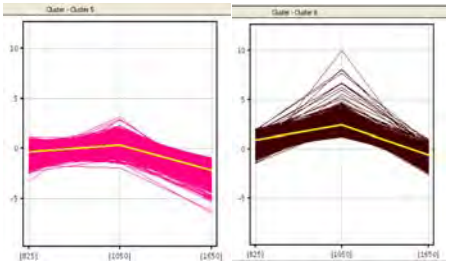

Figure 4.30 Graphs showing cluster group 5, displaying microarray probes that were found to be similarly expressed across the three different, labelled cRNA amounts (825 ng, 1050 ng, 1650 ng) of the albino samples. Data was obtained through K-mean cluster analysis of the normalised data (k=20).

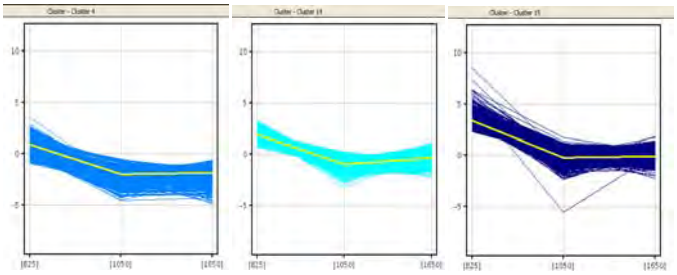

Figure 4.31 Graphs showing cluster group 6, displaying microarray probes that were found to be similarly expressed across the three different, labelled cRNA amounts (825 ng, 1050 ng, 1650 ng) of the albino samples. Data was obtained through K-mean cluster analysis of the normalised data (k=20).

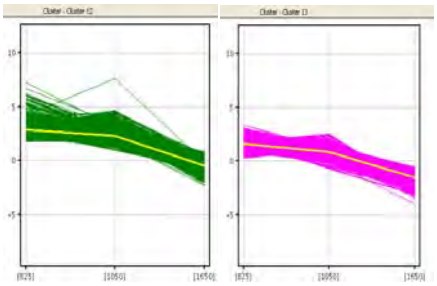

Figure 4.32 Graphs showing cluster group 7, displaying microarray probes that were found to be similarly expressed across the three different, labelled cRNA amounts (825 ng, 1050 ng, 1650 ng) of the albino samples. Data was obtained through K-mean cluster analysis of the normalised data (k=20).

A principal component analysis (PCA) was performed on the normalised microarray data in order to visualize broad trends in the microarray data according to colour category (albino, light and dark). The 3-dimensional scatter plot of the PCA scores showed that overall gene expression in the albino colour groups was fairly consistent across the four groups (all four clustered closely together) and distinctly different to the light and dark groups (Figure 4.33). Overall gene expression of the dark colour groups also seemed to be consistent across all four groups (all four groups are closely

clustered), whereas the four light groups appeared to be more broadly distributed relative to either the dark or the albino groups, possibly indicating that gene expression was more varied in this colour groups. (Figure 4.33).

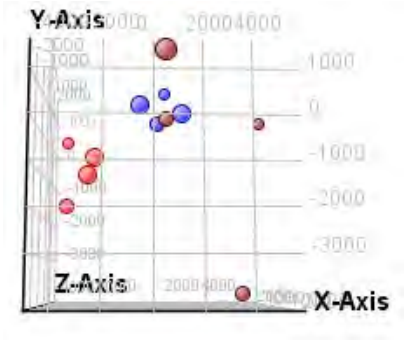

Figure 4.33 3-dimensional principal component analysis (PCA) plot for the 4 albino, 4 light, and 4 dark colour groups. PCA scores were calculated and visualised by the GeneSpring GX 11.0 software, where red dots = albino colour groups, blue dots = dark colour groups, and brown = light colour groups.

Statistical analysis of the normalised microarray data, using unpaired t-tests with Benjamini and Hochberg FDR multiple testing corrections enabled the determination of genes that were significantly differentially expressed between the albino and light, albino and dark, and light and dark groups. The following diagrams (Figures 4.34 - 4.36) show the results of this analysis. For the individual comparisons between albino and light, albino and dark, and light and dark, genes that were significantly expressed in at least three out of four probes, using a significance value of $p < 0.05$ and a 4-fold change in expression level, were determined. No significantly differentially expressed genes were found between the light and dark group. However, data of the probes with a 4-fold change in expression level – although not significant ($p > 0.05$) – was included in this section, to give an indication as to which genes could have the potential to be biologically relevant as signifying differences between samples of a more pronounced light and dark body coloration (Figure 4.36). Pie-charts for the albino and light, and albino and dark comparisons both show that 55 % (albino and light) and over 70 % (albino and dark) of the significantly differentially expressed probes represented some form of actin (e.g. beta-actin, actin 2) or were unannotated. Probes coding for sarcoplasmic calcium-binding proteins and arginine kinase/allergen Pen m comprised the next highest group of differentially expressed probes in the albino and light and albino and dark groups. Both comparisons also showed probes that coded for troponin I and to a lesser amount myosin light chains (Figures 4.34 and 4.35). The only differences found between the albino and light, and albino and dark comparisons were probes coding for tropomyosin (total of 14.8 % in albino versus light) and two types of

elongation factors (total of 5.6 % in albino versus dark). Probes coding for some form of actin, sarcoplasmic calcium-binding proteins, arginine kinase/allergen Pen m, troponin I, myosin light chains, tropomyosin and elongation factor 1α and 2 were all found to be significantly down regulated in the albino samples compared to the light and dark samples.

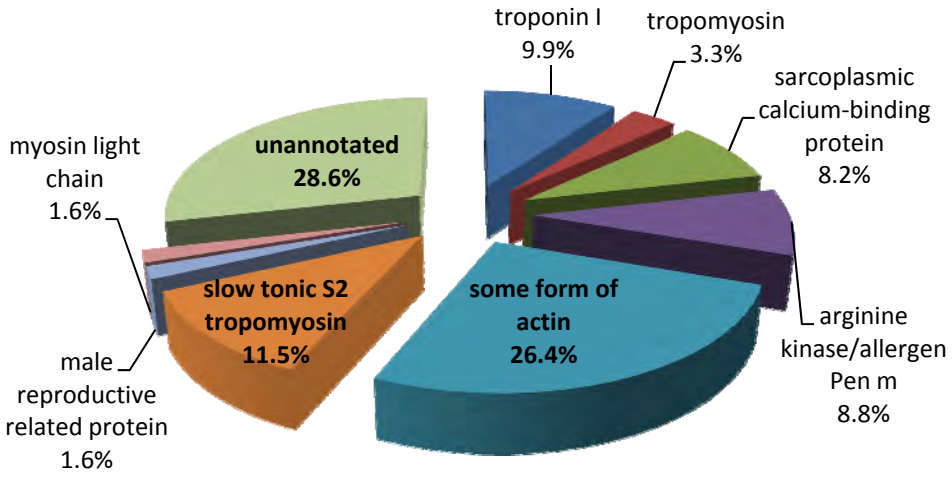

Figure 4.34 Diagram of the percentage of probes significantly ($p < 0.05$) differentially expressed between the albino and light groups. Pie-chart shows the probes that were 4-fold differentially expressed between the two groups and had at least 3 probes per gene sequence expressed in this pattern.

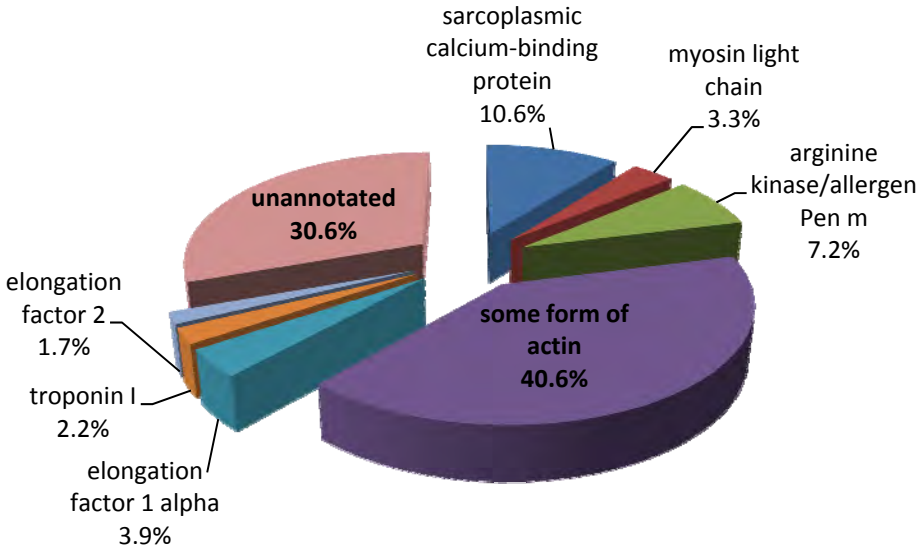

Figure 4.35 Diagram of the percentage of probes significantly ($p < 0.05$) differentially expressed between the albino and dark groups. Pie-chart shows the probes that were 4-fold differentially expressed between the two groups and had at least 3 probes per gene sequence expressed in this pattern.

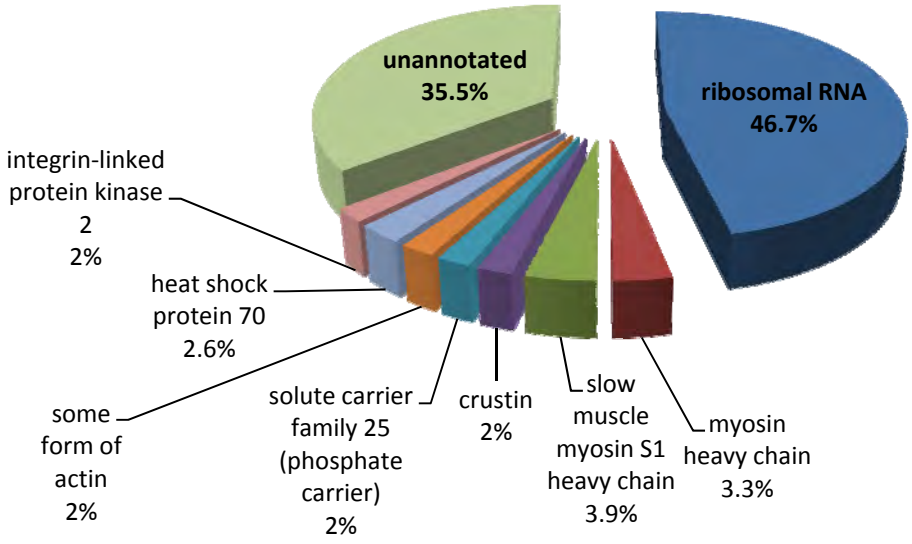

Figure 4.36 Pie chart diagram of the percentage of probes that were 4-fold differentially expressed between the light and dark groups and had at least 3 probes per gene sequence expressed in this pattern. Differences were not statistically significant ($p > 0.05$).

Although the probes determined to be 4-fold differentially expressed between the light and the dark group also contained a large amount of unannotated sequences, nearly half of the probes found coded for ribosomal RNA. Smaller percentages of probes coded for some form of actin, crustin, heat shock protein, solute carriers, myosin and integrin-linked protein kinase. Neither crustacyanin subunit A nor subunit C was found to be 4-fold differentially expressed between any of the three groups.

After separately analysing the data for the probes that were significantly differentially expressed between two groups, the data from all comparisons were combined into a Venn-diagram (Figure 4.37). The overlap areas of the Venn-diagram were then analysed. For this analysis, at least two of four probes per gene sequence had to be significantly differentially expressed (no significance observed in the comparison between light and dark), with an observed 2-fold change in expression level in these probes between the three colour groups.

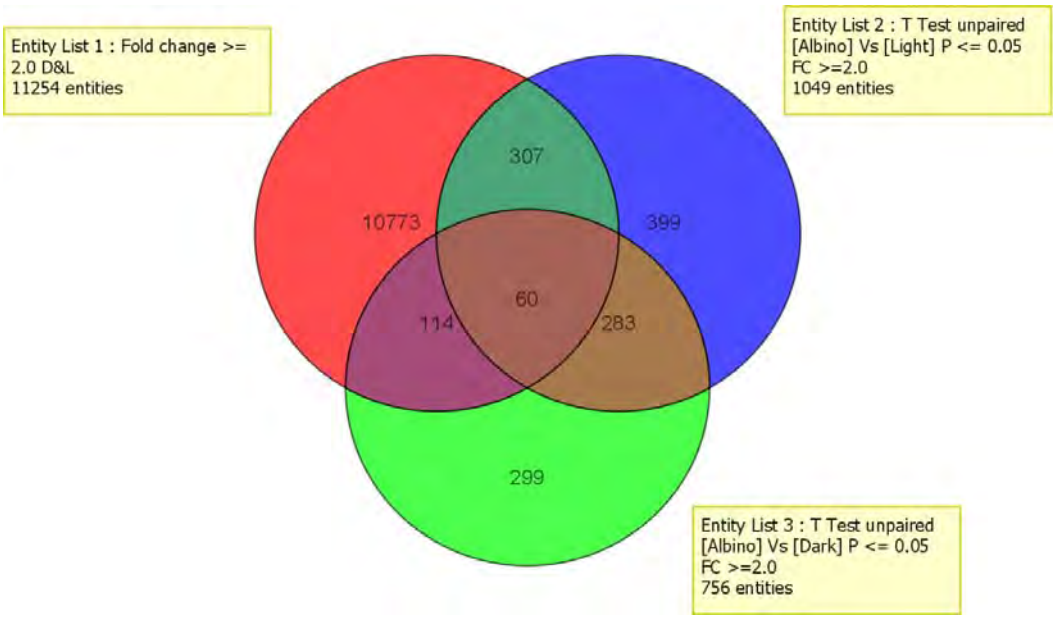

Figure 4.37 Venn-diagram of all three comparisons between the different prawn groups. Probes included in this diagram had to be 2-fold differentially expressed, with at least 2 probes present per gene sequence. The full red circle shows the genes that are 2-fold (not significantly) differentially expressed between the light and dark groups, the blue and green circles stand for 2-fold significantly differentially expressed genes between albino and light groups, and albino and dark groups, respectively.

Closer examination of the probes detected in the overlap areas of the Venn-diagram (e.g. between light and dark and albino and light) showed that the majority of probes found in these areas were again either unannotated or coded for some form of actin or for sarcoplasmic calcium-binding protein. Other probes found in the overlap areas coded for QM protein, crustacyanin subunit A, cytochrome C oxidase, crustin or arginine kinase/allergen Pen m. Probes coding for crustacyanin subunit C were not observed in any of the four overlap areas (Figures 4.38 – 4.41).

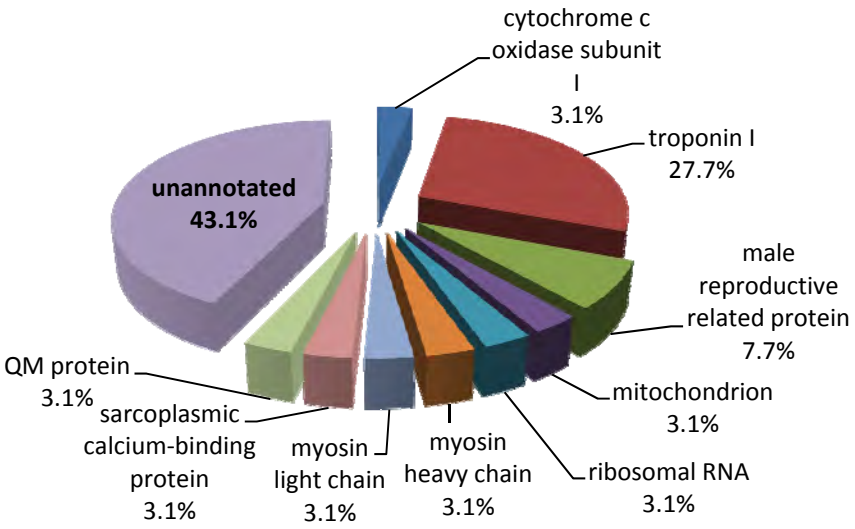

Figure 4.38 Pie chart of 307 gene probes detected in the comparison between albino and light, as well as in the comparison between light and dark groups (dark green overlap in Venn-diagram 4.37).

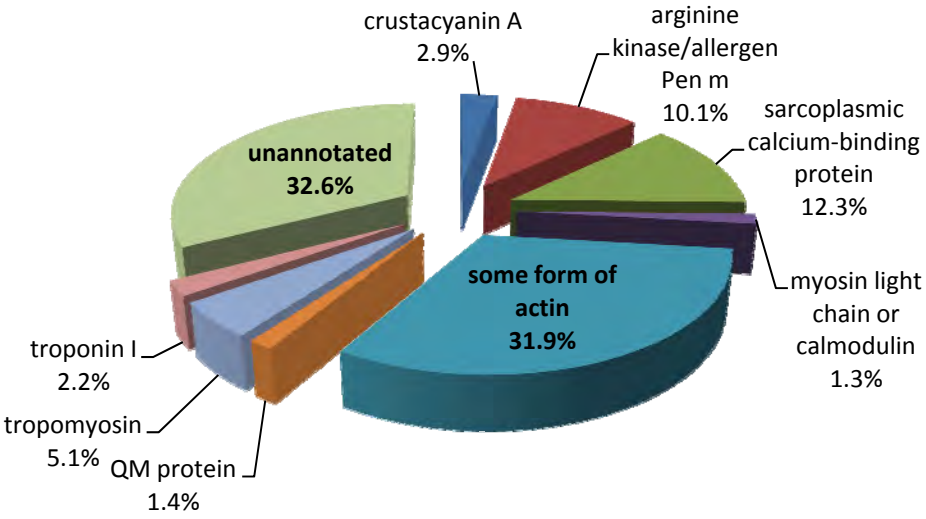

Figure 4.39 Pie chart of 283 gene probes detected in the comparison between albino and light, as well as in the comparison between albino and dark groups (brown overlap in Venn-diagram Figure 4.37).

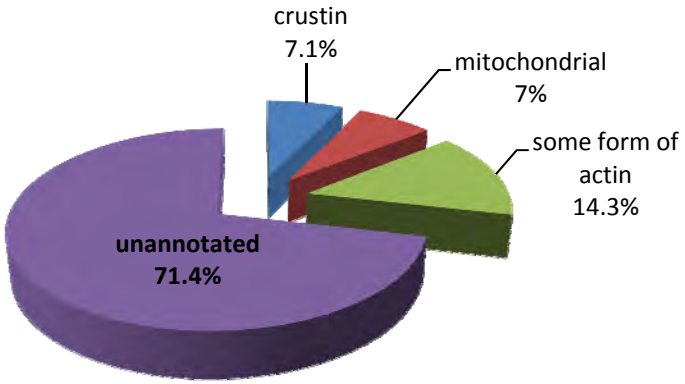

Figure 4.40 Pie chart of 114 gene probes detected in the comparison between albino and dark, as well as in the comparison between light and dark groups (purple overlap in Venn-diagram Figure 4.37).

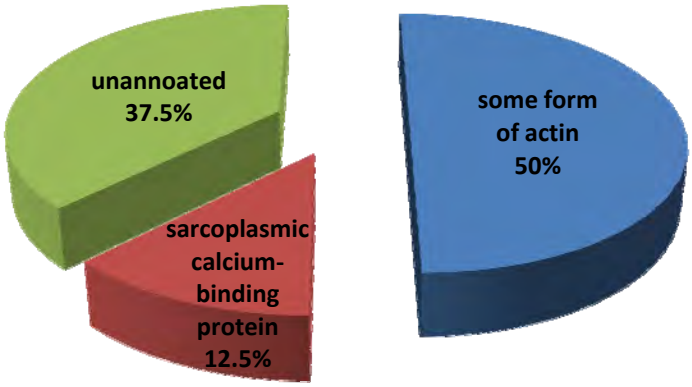

Figure 4.41 Pie chart of 60 gene probes detected in the comparison between albino and light, as well as in the comparisons between albino and dark, and light and dark groups (red-brown overlap in Venn-diagram Figure 4.37).

Unannotated probes that were detected in every comparison in a relatively high percentage were found to be probes designed from 56 gene sequences. As no matches existed on the NCBI database for these gene sequences, the 56 genes were analysed with the Pfam (http://pfam.sanger.ac.uk/) and InterProScan programs for signal peptides and protein domains. 23 of these genes did not have a known domain or a signal peptide, and 12 did have a signal peptide but no domain. The 21 gene sequences that contained a functional domain are listed in Table 4.1. Domain functions of these 21 gene sequences varied, with the main functions appearing to be either binding, transport or some regulatory or catalytic purpose.

Table 4.1 List of protein domains and functions of 21 unannotated gene sequences, found to be significantly ($p < 0.05$) 2- or 4-fold differentially expressed between the albino and light and albino and dark groups and not significantly between the light and dark groups.

Transcriptome ID	Signal peptide	Domain type*	Domain function
A86K5	absent	cytochrome b	~ electron carrier activity ~ oxidoreductase activity
ANCWJrev	absent	Peptidase_M14	~ metallocarboxypeptidase activity ~ zinc ion binding
AQEONrev	present	MFS_general_subst_transpt	secondary membrane transporter
AY47U	present	myosin head	~ motor activity ~ ATP binding
B11UPrev	absent	fibrillar collagen	extracellular matrix structural constituent
BXWCQrev	absent	WAP-4-diS_core	peptidase inhibitor activity
C0173	absent	actin	protein binding
C95Y3rev	present	ATPase_P-typ_ion-transptr	~ ATP binding ~ ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism
CFFRCrev	absent	MFS_general_subst_transpt	secondary membrane transporter
CGC11rev	absent	pyruvate kinase	~ magnesium ion binding ~ pyruvate kinase activity ~ potassium ion binding
CIQIV	present	Chitin_bind_4	structural constituent of cuticle
CJ2P2	present	actin zf-C2H2	protein binding ~ zinc ion binding
CMRY6rev	present	zf-C2H2	zinc ion binding
Contig 1137rev	absent	ATP-gua_Ptrans	~ kinase activity ~ transferase activity, transferring phosphorus-containing groups
Contig 1413rev	absent	RNA-binding S4 Blue (type 1) copper protein	RNA binding ~ copper ion binding ~ electron carrier activity
Contig 227rev	absent	zf-CCHC	~ nucleic acid binding ~ zinc ion binding
Contig 905rev	present	MFS_general_subst_transpt	secondary membrane transporter
CP026	absent	ATP-gua_Ptrans	~ kinase activity ~ transferase activity, transferring phosphorus-containing groups
DFP6W	absent	Destabilase	lysozyme activity
EBSXErev	present	Ser/Thr_prot_kinase-like	~ protein serine/threonine kinase activity ~ ATP binding
EY7U2	absent	AA_TRANSFER_CLASS_2 Lipocalin like	transferase activity binding

* Abbreviations for the domain type

Abbreviations for domain types in Table 4.1

ATP-gua_Ptrans	ATP:guanido phosphotransferase, C-terminal catalytic domain
zf-CCHC	Zinc finger, CCHC-type
WAP-4-diS_core	Whey acidic protein, 4-disulphide core
MFS_gen_subst_transpt	Major facilitator superfamily, general substrate transporter
Chitin_bind_4	Insect cuticle protein
AA_TRANSFER_CLASS_2	Aminotransferase, class-II, pyridoxal-phosphate binding site
Peptidase_M14	Peptidase M14, caboxypeptidase D unit 2
ATPase_P-typ_ion-transptr	ATPase, P-type, K/Mg/Cd/Cu/Zn/Na/Ca/H-transporter
zf-C2H2	Zinc finger, C2H2-type
Ser/Thr_prot_kinase-like	Serine/threonine-protein kinase-like

5. Discussion

In *F. merguiensis*, one of the three commercially important prawn species in Queensland, a darker body colouration is perceived to be more favourable by the customer, resulting in an up to AU$ 2-4 per kilo higher sales price. Therefore, this study has been carried out to gain an understanding of the factors involved in creating the colour variation observed between different individuals of *F. merguiensis*, by pursuing five aims. The first aim in this study was to isolate the crustacyanin subunit A and C sequences in *F. merguiensis*. These sequences were then used for the development of gene specific primers for the quantitative assessment of the expression levels of these genes, facilitating the second aim, which was to determine the crustacyanin subunit A and C gene expression level in *F. merguiensis* prawns that showed different body colour intensities, by using quantitative PCR. The third aim of the project was to determine the levels of the pigment astaxanthin in the same individual prawns for which gene expression levels were determined. As the fourth aim in this project was to gain an understanding of the genes that were actively expressed in the muscle/cuticle tissue of *F. merguiensis* prawns, whole transcriptome sequencing and analysis was carried out. Lastly, the fifth aim was to use the data obtained from the transcriptome sequencing to develop customised microarrays that were used to find novel genes in the cuticle tissue that could potentially be involved in colouration in *F. merguiensis*.

5.1 Isolation of the crustacyanin cDNA sequences

Crustacyanin subunit A and subunit C gene sequences were successfully isolated for *F. merguiensis* and the consensus sequences for both published on GenBank (Accession number HM370278 for subunit A and HM370278 for subunit C). The full length consensus sequence for subunit C was slightly longer (597 bp) than the sequence observed for subunit A (573 bp), with both sequences representing the only complete crustacyanin transcripts reported for prawns to date, to my knowledge. Analysis of the deduced protein sequences showed that both subunits had the three SCRs and motifs with the conserved core amino acids (GxW, TDY and R) (Salier 2000) typically found in proteins belonging to the kernel lipocalin family (Figures 4.6 and 4.7). Furthermore, the overall pattern of cysteine residues shared by proteins of the lipocalin family can also be found in the *F. merguiensis* crustacyanin subunit A and subunit C sequences, with the observed six cysteine residues potentially forming three disulphide bridges. This is consistent with the literature that groups proteins that show one, two or all three SCRs and motifs into the lipocalin superfamily, with the kernel lipocalins encompassing proteins such as crustacyanin or retinol-binding protein that contain all

three SCRs in their sequence (Flower 1996; Grzyb et al. 2006; Wade et al. 2009). Next to the SCRs and motifs, the conserved core amino acids of the three motifs can be found across the lipocalins and are considered an important characteristic of this superfamily (Charron et al. 2002; Flower et al. 1993; Wade et al. 2009; Wang et al. 2007). Another characteristic of the lipocalin family is the presence of conserved cysteine residues (up to six residues) in the protein sequence (Grzyb et al. 2006; Wang et al. 2007), which form the disulphide bridges and have been found in the crustacyanin subunits isolated in this study and in the subunits found in other crustacean species by Wade et al. (2009).

Alignment of the deduced crustacyanin subunit A and C protein sequences with each other showed a similarity of 35 % between the two sequences (alignment not shown). This is consistent with other studies, which report that protein sequence similarity in lipocalins is often not more than 30 % (Grzyb et al. 2006). Furthermore, Wang et al. (2007) detected lipocalins that showed a similarity in protein sequence to other lipocalins (e.g. crustacyanin) of between 24 % to 59 %, and Cianci et al. (2002) observed a 35 % identity between the crustacyanin subunit A and C protein sequences isolated from a lobster species. Incidentally, Cianci et al. (2002) also found that the protein sequence of their subunit A gene was slightly shorter than the one for their subunit C gene, as was observed for the subunits from *F. merguiensis* in this project. These findings further confirm that the two crustacyanin subunits isolated in this project belong to the lipocalin superfamily, and more importantly, show the same characteristics of crustacyanin that have been observed in other studies.

Additional to the two consensus sequences for the crustacyanin subunit A and C genes, a potential isoform of subunit A was detected among the sequences cloned from *F. merguiensis*. This gene sequence was only a partial cDNA clone, with most of the nucleotide sequence aligning very closely to the cDNA consensus sequence of subunit A, with the exception of a 108 bp insertion that showed no alignment at all with the consensus sequence (Figure4.8). As this insertion carries the GT-AG nucleotide sequence typical for intron-exon boundary, it is likely that this segment is an unprocessed intron. This could mean that the sequence mentioned above represents an isoform of the crustacyanin subunit A, produced through alternative splicing, and as the insertion does not disrupt the reading frame, it could in fact be a functional variant of this gene. It cannot be ruled out that the segment cloned was isolated before the intron could be spliced out, and thus has no functional significance.

Analysis of the crystal structure and protein sequence of crustacyanin in lobster species showed that two types of crustacyanin subunit (type1 or crustacyanin subunit C and type 2 or crustacyanin subunit A) exist in the lobster species, with type 1 comprised of 3 different C subunits or isoforms and type 2 containing two different A subunits or isoforms (Cianci et al. 2002; Grzyb et al. 2006; Ilagan et al. 2005). Of these 5 isoforms, C1 (subunit C) and A2 (subunit A) are considered to be the two predominant forms (Chayen et al. 2003; Keen et al. 1991), which suggests that the subunit A and subunit C forms found in this study could be the equivalents of A2 and C1, respectively. Moreover, from such findings in other crustacean species, it is likely that other crustacyanin isoforms would exist in *F. merguiensis*. Wang et al. (2007) indicated that other lipocalins such as salivary lipocalin have been found to have different isoforms, which bind differently to their ligand. The authors suggest that these variances in the binding properties could have a role in the regulation of different processes such as coloration or moulting. This could also be the case for the crustacyanin subunit A and C isoforms in crustaceans, as intron retention can lead to a different protein sequence and thus folding and potentially a different function or binding capacity. In the case of the subunit A isoform found in this project, the included sequence resulted in a 36 amino acid insert into the deduced crustacyanin subunit A protein sequence, making it likely that this addition could cause changes in the protein folding and binding properties as had been suggested by Wang et al. (2007) for the different isoforms of the salivary lipocalin.

The putative intron detected in the crustacyanin subunit A isoform was found 67 amino acids downstream of the first core amino acid sequence (GxW), but only 9 amino acids upstream of the second core amino acid sequence (TDY). According to Salier and Sánchez et al. (2000; 2003), GxW is found at the start of exon 2 and TDY in exon 4 (Figure 5.1). Considering the placement of the putative intron of the *F. merguiensis* isoform, it is likely that the *F. merguiensis* putative intron observed is intron C.

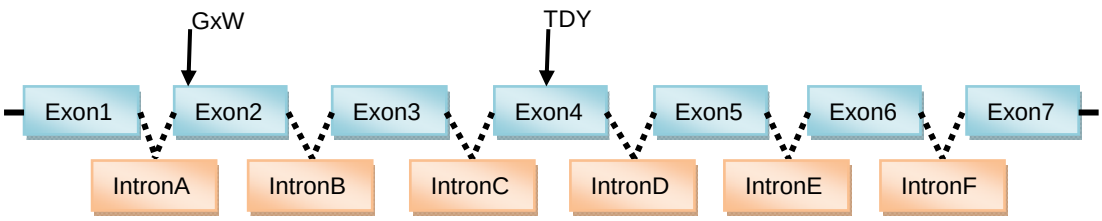

Figure 5.1 Typical exon-intron structure of lipocalins (adapted from Salier and Sánchez et al. (2000; 2003)) with the location of the core amino acid sequences marked.

Close assessment of the first six nucleotides of this putative intron also revealed that the 5' nucleotide splice sequence (GTATAG) was different to the consensus GTAAGT

sequence generally found in introns. This deviation could potentially weaken the splice signal due to reduced base pairing between the small nuclear RNA molecules and the consensus sequence, resulting in alternative splicing (e.g. intron inclusion) of the crustacyanin subunit A gene (Ast 2004; Izquierdo & Valcárcel 2006). Similar to the potential isoform observed in this project, Mechaly et al. (2009) detected an isoform of the kisspeptin-1 receptor in *Solea senegalensis* in which the 5' splice sequence deviated from the consensus GTAAGT sequence, leading to intron inclusion, again possibly due to weakening of the splice signal. However, while the authors found that the intron insert into the kisspeptin-1 receptor disrupted the reading frame of the isoform, resulting in a truncated protein, the crustacyanin subunit A isoform isolated from *F. merguiensis* maintained the correct reading frame to form the full length protein.

The findings of this part of the project contributed new knowledge, as neither the crustacyanin subunit A nor the subunit C gene sequence had been identified for *F. merguiensis*. Additionally, a putative second crustacyanin subunit A form was isolated, a novel finding which has not been reported for this prawn species before.

5.2 Colour analysis with absolute qPCR, spectrophotometer and visual observation

Quantitative PCR, a highly sensitive molecular method, was used to determine the crustacyanin subunit A and C gene expression levels in the cuticle tissue of 50 *F. merguiensis* prawns presenting albino, light and dark body colouration. For this, gene specific primers were developed from the consensus sequences of crustacyanin subunit A and C, which were isolated in the scope of this project. Results of the quantitative PCR experiment revealed that both, crustacyanin subunit A and C showed significantly higher expression in prawns classified light (copy numbers of 206,998.9 ± 63,971.3 and 265,687.7 ± 105,295.7, respectively) compared to the prawns classified albino and dark (Figures 4.16 and 4.17). The lowest crustacyanin subunit A and C expression level was observed in the prawns classified albinos (copy number of 1000.6 ± 463.9 for subunit A and 525.4 ± 305.8 for subunit C), as could be expected. However, it was expected to find a significantly higher crustacyanin subunit A and C expression level in the prawns classified dark, yet a significantly lower crustacyanin expression level was observed in the dark prawns (copy number of 40,661.6 ± 15,020.2 for subunit A and 30,185 ± 12,860.7 for subunit C) compared to the light prawns. Furthermore, while crustacyanin subunit A showed higher expression levels in the albino and dark prawns than crustacyanin subunit C, in the light prawns the reverse was observed, with

subunit C transcripts present at a higher copy number than subunit A. Correlation analysis on the crustacyanin subunit A and C gene expression levels of individual prawns revealed a significantly positive relationship between the expression levels of the two subunits. To my knowledge, the only other study that quantitatively analysed the expression of a gene similar to crustacyanin was carried out in white and red western rock lobster (*Panulirus cygnus)* and found that there was no difference in gene expression between the two colour groups (Wade 2006). This suggests that there might be different physiological processes involved in the colouration of the white and red lobsters, compared with the *F. merguiensis* prawns of different body colouration analysed in this study.

Two potential factors might be responsible for the unexpected lower crustacyanin subunit A and C gene expression levels in the dark prawns. Firstly, as crustacyanin gene expression levels were measured in the cuticle (outer epithelium and endocuticle), it is possible that most of the crustacyanin responsible for the darker colour in dark prawns has been transcribed, translated and already secreted into the hard exoskeleton by the time of intermoult, when the prawns were collected, with a lower level of gene expression maintained in the cuticle. While in the lighter coloured prawns, translation of the crustacyanin subunits could be slower, and transcript levels therefore remain higher in the cuticle. Another possible explanation, taking into consideration that two forms of crustacyanin subunit A and three forms of crustacyanin subunit C have been reported in the literature, and that the β-crustacyanin heterodimer is always formed by one crustacyanin A and one crustacyanin C (different combinations of the forms for each subunit) (Cianci et al. 2002; Ilagan et al. 2005), is that the subunit that is higher expressed (e.g. more copy numbers of subunit A than C in the dark prawns) could bind not only to the respective subunit C analysed in this study, but to other forms of this subunit, not yet identified. The same rational could apply to the light prawns, where crustacyanin subunit C was higher expressed than subunit A, suggesting that some of the subunit C's might be bound to other, as of yet not isolated, forms of subunit A. This would lead to different combination of β-crustacyanin heterodimers in the dark, compared to the light. Since the association of the keto groups of the astaxanthin molecules (Figure 2.3) with the His residues of the crustacyanin protein subunits are important for the formation of the β-crustacyanin dimer (two protein subunits bound to two astaxanthin molecules) (Chayen et al. 2003; Helliwell 2010), slight changes in the position of these His residues across the crustacyanin subunits through, for example, differences in the nucleotide and putative amino acid sequence in the different forms of crustacyanin subunits A and C, could

have an effect on the interaction between the protein and astaxanthin and thus on the expression of colour in the respective prawn.

In addition to the determination of crustacyanin subunit A and C gene expression levels in albino, light and dark prawns, levels of the pigment astaxanthin were also assessed in the same 50 *F. merguiensis* prawns. Results of the total astaxanthin extraction and spectrophotometric analysis showed that the highest amount of total astaxanthin (14.6 µg/g of wet weight ± 0.5) was found in the prawns classified dark by Seafarm (Figure 4.18). These levels were significantly higher than the levels of total astaxanthin found in the light (12.8 µg/g of wet weight ± 0.6) and albino (4.5 µg/g of wet weight ± 1.4) prawns.

Similar to the findings of this study, Wade et al. (2005) correlated the total amount of carotenoids found in the western rock lobsters shell and epithelium with the colour of the lobster shell, with lower amounts of carotenoid found in the white lobster and higher levels in the red lobster. Tume et al. (2009), on the other hand determined that the quantity of astaxanthin extracted from *P. monodon* prawns could not be closely associated with the visually observed colour of these prawns. These results differ from the results of this study, where albino prawns showed a significantly lower amount of total astaxanthin than light prawns, and light prawns a significantly lower amount of total astaxanthin than dark prawns. This discrepancy could be due to the way prawn samples were prepared in the study by Tume et al. (2009). Firstly, Tume et al. (2009) used pre-cooked commercial prawns, which were thawed and directly assessed for colour, before the prawns were dissected and astaxanthin extracted. In comparison, the *F. merguiensis* prawns used in this study were cooked, photographed and directly frozen at -20 °C for not more than 20 h before the prawns were dissected and astaxanthin extracted. Furthermore, dissection and astaxanthin extraction was performed at 4 °C and the segments used for extraction kept in the dark as much as possible to protect the photosensitive pigment. Secondly, while prawns in this study were only frozen for a short time, Tume et al. (2009) made no comment regarding the amount of time the commercial cooked prawns were kept frozen before they were used in the study, the freezing temperature or how long the prawns were held thawed for colour assessment and dissection. As prolonged freezing of the cooked prawns can result in a loss of pigment (R Smullen 2010, Ridley Aquafeeds pers. comm., 20 October), it is possible that a part of the variation in results observed between the two studies could have been caused by a loss in pigment in the prawns Tume et al. (2009) used in their study. Also, it appears that Tume et al. (2009) based their analysis of the amount of astaxanthin versus visual colour on groups of samples that had received the

same ranking or score (e.g. a score or ranking of 5), whereas the analysis of this study was broader with the samples grouped into albino, light and dark. Both studies used acetone as solvent for the astaxanthin extraction and similar segments of the prawns were chosen for the extraction process, therefore variation in results caused by the extraction process were expected to be minimal.

Visual analysis and ranking of the colour intensity of the cooked prawns used for astaxanthin extraction, was performed by USC staff and the ranking values compared to the pre-cooked colour classifications given to the prawns by Seafarm staff. Analysis of the results of this comparison indicate that the colour classifications given by Seafarm staff to the pre-cooked prawns represented fairly closely the perceived colour intensity of the cooked prawns, suggesting that the colour of the cooked prawns can be predicted from the colour of the uncooked prawn. More importantly, results obtained in this comparison indicate that after follow-up tests, future research on this prawn species could potentially be carried out on either the pre-cooked animal with colour classifications done by Seafarm staff or on the cooked animal by grouping them according to their individual ranking values.

To my knowledge, this is the first study that compared the amounts of total astaxanthin across *F. merguiensis* prawns displaying different colour intensities with the expression levels of crustacyanin subunit A and C in the same prawns.

5.3 Next generation sequencing and analysis

Next generation sequencing, a relatively new molecular method that allows the sequencing of whole transcriptomes in a short time-frame, was used in this study to assess the genes actively expressed in the muscle/cuticle tissue as well as six other *F. merguiensis* tissues (androgenic gland, hepatopancreas, stomach, nervous system, eye stalk and male and female gonads). Until recently, next generation sequencing has been mainly applied to obtain genetic information about model organisms, gain insight into human health (e.g. cancer research) or into metagenomics (studying genetic diversity directly in environmental samples without the need for culture methods in the laboratory). The use of next generation technology in research pertaining non-model organism has only started recently and is still considered a novel approach in a field, for example, such as aquaculture (Elmer et al. 2010; Mardis 2008; Wyman et al. 2009).

In this study, 54928 total sequence reads, with a read length of ~ 30 to 540 bp were obtained from the muscle/cuticle tissue of *F. merguiensis* through Roche 454 next generation sequencing, with the majority of the sequences having a length of

approximately 250 to 450 bp. These results are quite typical of 454 next generation sequencing, that has been reported to generate a large amount of sequence reads of roughly 300 to 400 bp length on average (MacLean et al. 2009; Parchman et al. 2010).

From the 54928 total sequence reads, 1455 contigs and 4535 singletons were produced with the CLC Workbench, and those were analysed using blast2go. No sequence matches to sequences stored on the NCBI database were found to around 40 % of the contigs and singletons analysed with blast2go. This was expected, as there does not appear to be an extensive gene library available in the NCBI database for crustaceans. Therefore, it is likely that genes, unique to crustaceans/*F. merguiensis* or with fairly low sequence similarity to their counterparts in other species, could not be matched to other sequences with blast2go. As only sequences with matches in the NCBI database were functionally annotated with blast2go, these 2404 sequences remained unannotated. Further analysis of these sequences, for example by assessing the genes for functional domains, will be necessary to determine their potential functions in *F. merguiensis*.

Of the 3586 annotated sequence reads (contigs and singletons), only 0.04 % coded for crustacyanin subunit A and subunit C, with all subunit A and all subunit C segments of the transcriptome aligning very closely with the consensus sequences of subunit A and subunit C obtained in the first part of this study. Only one form was found for either subunit, strengthening the argument made in section 5.1 that the cloned consensus sequences are likely to represent crustacyanin subunit A2 and C1, the two most prominent forms of the crustacyanin protein. The fact that no other forms of subunit A or C were found in the transcriptome data could be due to a number of factors. Firstly, the other crustacyanin isoforms mentioned in the literature (Cianci et al. 2002; Grzyb et al. 2006; Ilagan et al. 2005) appear to be less prevalent than subunits A2 and C1. As some genes are more abundant in the muscle/epithelial tissue of *F. merguiensis* or are longer gene sequences, it is likely that the next generation sequencing amplified and sequenced these genes over the shorter or less abundant genes (Bullard et al. 2010; Wheat 2010; Zagrobelny et al. 2009). Therefore it is possible that the less prevalent isoforms were not sequenced in the transcriptome sequencing project, as the coverage of the muscle/cuticle genes was not complete. Secondly, it is possible that potential other crustacyanin subunit A and C forms have sufficiently diverse gene sequences that no match could be found on GenBank and thus they were not annotated. However, considering that crustacyanin belongs to the lipocalin superfamily (Grzyb et al. 2006), the three SCRs, motifs and conserved amino acids found in the kernel lipocalins of this family should have been matched at least with other lipocalins during the annotation

process. Thirdly, it cannot be ruled out that the crustacyanin subunit A clone with the putative intron insert, observed in the first part of the study, might have been the product of an uncomplete splice event in which case it would not have a functional significance and is not expected to necessarily be found again in the transcriptome. All these could account for the absence of other crustacyanin subunit A or C isoforms in the *F. merguiensis* muscle/cuticle tissue and would need to be further analysed to be clearly answered.

Analysis of the functional annotation performed on the 3586 annotated muscle/cuticle transcriptome sequences was carried out to obtain three divisions (molecular function, cellular component and biological process) (Figures 4.18 – 4.20). Results showed that "binding" and "catalytic activity", followed by "structural molecule activity", were the three main categories observed in the molecular function division. In the cellular component division, the major part of the annotated sequences coded for a "cell part", "non-membrane-" or "membrane-bound organelle", whereas the prevalent functions of the annotated genes in the biological process division were "cellular process", "metabolic process" and "biological regulation". The same main categories across all three divisions were reported in a study carried out by Elmer et al. (2010) that analysed the transcriptome of two cichlid fish species (*Amphilophus spp*.), using the tissue of newly hatched and up to 1 month post-hatched fish. Although Elmer et al. (2010) appeared to have analysed mostly whole hatchlings, their results also showed, for example, "binding" and "catalytic activity", followed by "structural molecule activity" as the three main categories observed in the molecular function division. However, while the large categories in each of the three divisions detected in the muscle/cuticle tissue of *F. merguiensis* showed the same pattern of gene expression as the findings of Elmer et al. (2010), the gene expression pattern of the less prevalent sequences obtained in this study varied from those found by Elmer et al. These results were expected, as the tissue used in this experiment was very specific (muscle/cuticle tissue of *F. merguiensis* prawns), compared to the mixed tissue used by Elmer et al. (2010). As each tissue has to perform specific functions in an animal, it is very likely that, for example, genes coding for enzymes, transport or developmental processes are more common or specific in one tissue compared to another. Furthermore, while general metabolic processes (e.g. catabolism) and functions (e.g. transport of nutrients, cell signalling, binding) (Martini 2006) would be expected to be the same across different animal species, animal specific genes (e.g. crustacyanin in crustacean body coloration) are expressed in one species but not another, resulting in a different gene expression pattern. Overall, these results indicate that the transcriptome data obtained for the

muscle/cuticle tissue of *F. merguiensis* is most likely a true representation of the genes actively expressed in this tissue, although probably not all of the genes were covered.

Additionally to crustacyanin subunit A and C being expressed in the muscle/cuticle tissue of *F. merguiensis*, both subunits were also detected in the transcriptome data of the eye stalk, nervous system and hepatopancreas of this prawn species. These results are novel, as the only other study that examined the expression of crustacyanin in a variety of tissues was the study of Wade et al. (2009) that observed crustacyanin subunit A in the eye stalks, gonads, muscle and epithelial tissue of the lobster *Panulirus cygnus*. Interestingly, Stepanyan et al. (2006) also detected crustacyanin in the olfactory epithelium of the lobster *Homarus americanus*, however the authors did not comment on the function of the protein in this species of lobsters. The role of crustacyanin in other tissues, as well as the differences observed in the tissue distribution of crustacyanin between the *F. merguiensis* prawns of this study and *P. cygnus* is unclear and would need to be functionally analysed in detail.

Crustacyanin subunit A and subunit C transcript sequences found in the *F. merguiensis* transcriptome data of the eye stalk, nervous system and hepatopancreas were all closely matching to the two cloned consensus sequences for subunit A and C, as well as to their shorter 3' and 5' clone segments. No matches were found to the potential crustacyanin subunit A isoform in any of these three transcriptomes.

There does not appear to be any information in the literature as to why crustacyanin subunit A and C would be expressed in the eye stalk, nervous system or hepatopancreas. However, crustacyanin belongs to the lipocalin superfamily, a family of proteins that generally binds small, hydrophobic molecules, such as steroids, carotenoids or pheromones (Charron et al. 2002; Flower et al. 1993). Other functions of members of this family are, for example, cryptic coloration, functions in immune regulation, cell growth, olfaction, cell homeostasis, development of the nervous system and binding to receptors on the surface of cells (Charron et al. 2002; Flower 1996; Sánchez et al. 2003; Wang et al. 2007). While some of the lipocalins are believed to be very specific in the ligands they bind, most lipocalins can bind a wide range of ligands (Sánchez et al. 2000), therefore crustacyanin might not be limited to binding the carotenoid astaxanthin, but could also bind other molecules such as steroids, for example. For instance, apolipoprotein D, a lipocalin related to the insect bilin binding protein and crustacyanin subunit A1, is synthesised by cells in the central nervous system, where it is believed to function in regeneration of nerves, signalling, metabolism and the transport of arachidonic acid (Eichinger et al. 2007; Flower 1996).

Other lipocalins expressed in the developing nervous system of *Drosophila melanogaster* and grasshoppers are *Lazarillo* and two *Lazarillo*-like lipocalins that aid in the development of the axon and guide the direction of their growth (Flower 1996; Grzyb et al. 2006; Sánchez et al. 2000; Wang et al. 2007). Considering this information, it is possible that crustacyanin has a similar role to apolipoprotein D or *Lazarillo* in the nervous system of *F. merguiensis* prawns.

Crustacyanin detected in the transcriptome data of the eye stalk of *F. merguiensis* could be involved in similar functions as that of lipocalins purpurin or lipocalin-type prostaglandin D synthase (L-PGDS). L-PGDS has been found in a variety of tissues, such as the brain, heart, testis and the retinal epithelial (pigmented) as well as iris and ciliary body epithelial cells (non-pigmented) (Beuckmann et al. 1996; Urade & Hayaishi 2000), and is the enzyme responsible for the production of the prostaglandin PGD_2 in the eye which is believed to have an active role in retinal function. Furthermore, L-PGDS is thought to function as a transporter for retinoids and other lipophilic molecules that transports these molecules from the retinal pigment epithelium to the photoreceptor cells in the eye (Beuckmann et al. 1996). Purpurin, like L-PGDS binds and transports retinol; however purpurin has also been observed to have a function in cell differentiation and adhesion, as well as the survival of the retinal epithelium and neurons. Furthermore, purpurin was expressed in the photoreceptor cells of the developing and regenerating fish retina, with an increase in the protein expression level observed between the second and fifth day of optic nerve regeneration and a higher purpurin protein expression level in the retina of fish during early embryogenesis compared to the mature fish (Ganfornina et al. 2000; Matsukawa et al. 2004; Tanaka et al. 2007). As lipocalins L-PGDS and purpurin appear to share a function as transporters, it is possible that the crustacyanin subunits detected in the eye stalk tissue of *F. merguiensis* have a similar role.

In the hepatopancreas, crustacyanin potentially serves a similar purpose as it does in the prawns' cuticle tissue, for instance, by binding to the carotenoid astaxanthin. Astaxanthin is believed to have an additional role to crustacean pigmentation, by playing a part in crustacean and fish health due to the pigments' antioxidant abilities (Anderson 2000; Tume et al. 2009). These antioxidant properties are thought to, for instance, aid crustacean and fish in the resistance to stress caused by hypoxia (Pan et al. 2010). As the hepatopancreas of decapods not only functions in food digestion, nutrient absorption, storage and metabolism, but also as an initial layer of immune defence (Jiang et al. 2009; Shih et al. 1997), Jiang et al. (2009) analysed the proteins expressed in the hepatopancreas of *Fenneropenaeus chinensis* prawns exposed to

short-term hypoxia. Proteins that were observed to be up-regulated in their study were, for example, carboxypeptidase B, chitinase and crustacyanin subunit C1. While the authors were unable to explain the importance of the finding that crustacyanin subunit C1 was up-regulated in the hepatopancreas of their experimental species (Jiang et al. 2009), their findings are consistent with the ones presented later by Pan et al. (2010), who assessed the antioxidant capacity of fish fed with dietary carotenoids (astaxanthin and β-carotene) during a short-term hypoxia event and found that fish fed with a diet supplemented with carotenoids had a 56 % lower superoxide dismutase level, as well as a decreased glutathione peroxidise and alanine transaminase activity than the control fish without the carotenoid supplements. These findings suggest that the carotenoids fed to the fish had an increased antioxidant capacity, potentially leading to a higher resistance level to hypoxic stress events in these animals. Combined with the above study by Jiang et al. (2009), this suggests that the crustacyanin proteins expressed in the hepatopancreas could serve as astaxanthin collectors or transporters of astaxanthin and β-carotene to the tissues most affected by a hypoxic stress event, thus contributing to the overall wellbeing of the prawn.

While the main reason for the next generation transcriptome analysis of the *F. merguinesis* muscle/cuticle tissue was to gain an understanding of the genes actively expressed in this tissue, the data was also used to create a customised microarray that would enable the determination of genes that were differentially expressed across the cuticle tissue of light, dark and albino *F. merguiensis* prawn individuals. Furthermore, the next generation sequencing data obtained for the muscle/cuticle and six other tissues of *F. merguiensis* prawns will be a valuable tool for further research into the gene expression profile of this important commercial aquaculture species.

5.4 Microarray analysis

The transcriptome data gave an overview of the genes that were actively expressed in the muscle/cuticle tissue of *F. merguiensis* prawns at the time of sampling. In the next step, microarrays were deployed to identify genes that are differentially expressed in the cuticle tissue of *F. merguiensis* individuals that displayed different body colouration (albino, light and dark). By employing microarrays, the vast transcriptome data obtained in this study for the *F. merguiensis* prawns was scanned for potentially novel genes involved in the colouration process of these prawns.

Normalised microarray data were analysed and gene probes that were significantly differentially expressed between albino, light and dark *F. merguiensis* groups identified.

Gene probe analysis was restricted to probes that were 2- or 4-fold differentially expressed in the cuticle tissue, when albino & light, albino & dark, and light & dark groups were compared with each other, while probes that were significantly different, but less than 2-fold differentially expressed between the three colour groups were excluded. This cut-off was chosen to increase the likelihood that the identified gene probes had a genuine role in the animals of one colour group, compared to those from another colour group and to keep the number of genes that were further investigated to a manageable level. Furthermore, to ensure that results observed in this study were due to genuine differences in gene expression between animals of the three colour groups, emphasis was put on the normalisation of the data as the hybridisation efficiency can vary between different microarrays and even between the individual blocks of each microarray. Normalisation was especially important for the albino samples as the amount of labelled cRNA for the four albino groups varied from 850 ng (two groups) to 1050 ng (one group) and 1650 ng (one group), and it was essential to confirm that these variations in the amounts of labelled cRNA did not influence the results.

Initial analysis of the normalised microarray data, using a PCA plot (Figure 4.33), showed that the four albino groups were all closely clustered together, further confirming that results observed for the albino groups indeed reflected the expression differences between albino and light or dark prawns. The PCA plot also indicated that the albino cluster (the four albino sample groups) was distinctly different in its gene expression from the clusters of the light and dark animals, as it was found to be separate from the light and dark cluster, which appeared to be closer to each other in their gene expression. The four light groups seem to be more loosely clustered than the albino or dark samples, which have a tighter clustering. This is consistent with the results obtained in the qPCR experiment, where albino, light and dark prawns were analysed for their crustacyanin subunit A and C gene expression level (Figures 4.16 and 4.17) in the cuticle of the *F. merguiensis* prawns. Similar to the cluster pattern observed in the PCA plot, gene expression levels of crustacyanin subunit A and subunit C in albino prawns were distinctly separate from the results of the light and dark prawns, with an overlap in gene copy number observed between the light and dark samples when gene expressions of the individual prawns were analysed (Appendix 1). Additionally, standard errors for the crustacyanin subunit A and C gene expression levels were observed to be higher for the light prawns, compared to the dark prawns (SE of 63,971.3 and 105,295.7 for the light and 15,020.2 and 12,860.7 for the dark prawns for subunit A and C, respectively), confirming that there was a larger

variation in the expression levels of the two subunits in the light prawns compared to the dark prawns.

Closer analysis of the normalised microarray data showed that the individual comparisons between albino & light, albino & dark, and light & dark, resulted in a somewhat similar distribution of differentially expressed genes. All three comparisons of the 4-fold differentially expressed genes had a large amount of probes that were unannotated (between 28.6 % and 35.5 %), as well as probes that coded for some form of actin (from 2 % to 40.6 %). No clear distinction could be made regarding the actin genes found in this study, due to highly significant matches with a variety of actin forms (e.g. beta-actin, actin 2), thus the term “some form of actin” was given to these genes. Further analysis would need to be carried out to determine the exact form of actin these genes code for.

Probes differentially expressed in the comparison of the light & dark groups showed that the major part of the probes were either unannotated or coded for ribosomal RNA. Between 2 % and 3.9 % coded for some form of actin, heat shock protein 70, crustin, myosin, phosphate carrier and integrin-linked protein kinase 2 (Figure 4.36). Expression patterns observed in this light & dark comparison, differ from the albino & light, and albino & dark single comparisons (Figures 4.34 and 4.35) and could be due to the fact that the four light and four dark groups were found to be quite closely clustered together, while the albino cluster was observed to be distinctly separate to the light and dark cluster (Figure 4.33). Furthermore, although the 24 prawns that scored the highest and lowest colour ranking of the dark and light groups were chosen, the colour difference between these light and dark samples could still have been potentially too small to observe genes significantly differentially expressed between these two colour categories. It should be noted that as not all albinos used in this experiment were in the intermoult stage (some were pre- and postmoult) and some showed a slightly more degraded total RNA profile than the light and dark samples, differences between these albino groups might in fact be more extreme than what has been observed. As albinos are a rare event in the aquaculture pond environment, it was not possible to eliminate these variations to the experimental set up in the scope of this experiment.

Focusing on the probes that were found to be significantly (or non-significantly for the light & dark comparison) differentially expressed in each of the single group comparisons, gene probes that code for some form of actin, sarcoplasmic calcium-binding protein, arginine kinase/allergen Pen m and troponin I, were identified to be

down regulated in the albino samples compared to the light and dark samples and appear to play an important role in all three colour groups. This finding was confirmed, when the three single comparisons were combined into a Venn-diagram (Figure 4.37) and the same gene probe groups (e.g. sarcoplasmic calcium-binding protein) were found to be 2-fold differentially expressed across more than one individual comparison.

The detection of actin in the cuticle tissue of the *F. merguiensis* prawns in this study was consistent with a study by Mykles et al. (2000), who observed the presence of actin in the epithelium and membranous and endocuticular layer of the crab and lobster exoskeleton. The authors suggest that the epithelium of these crustaceans secretes actin, together with tubulin to enable the incorporation of these proteins into the extracellular matrix. While they question the role of these proteins in the extracellular matrix, they indicate that the proteins could function as stabilizers or organizers of this matrix. Other studies, however, suggest a different function for these two proteins. Fingerman et al. (1975), for instance, suggested that microtubules and microfilaments found in the fiddler crab *Uca pugilator* could play a role in the movement of pigment granules in the chromatophores of the animal. In their study, they used colchicines and cytochalasin B to disrupt the microtubules and microfilaments and observed the inhibition of pigment concentration and dispersion in ovarian erythrophores of the fiddler crab. Further studies appear to result in similar findings, as, for example, Beckerle & Porter (1983) analysed granule movement in the erythrophores of the squirrelfish *Holocentrus ascensionis* which the authors mainly ascribed to microtubules, however, there also appeared to be microtubule-independent movement that was believed to be due to actin microfilaments. Tuma & Gelfand (1999) stated in their review of pigment granule movement in melanophores that both, microtubules and actin microfilaments play an important role in aggregation and dispersion of the pigment granules. Furthermore, they note that especially the dispersion of the pigment granules and maintaining this dispersed state requires actin microfilaments. Research on pigment movement in retinal pigment epithelial cells coincides with the results obtained in chromatophores, as a study by Burnside et al. (1983) suggested that dispersion of pigment granules in the retinal pigment epithelial cells was dependent on actin for the translocation of the pigment. Also, research carried out by McNamara & Ribeiro (1999) indicates that actin could have a role in the slower phase of pigment aggregation.

As the literature indicates that actin is necessary for the dispersion of pigment and for holding this state in the animal (Tuma & Gelfand 1999), it is possible that the 2- and 4-fold change in expression level observed in probes coding for some form of actin in

F. merguiensis prawns of this project reflected the colour, as well as the state of pigment dispersion or aggregation in the prawns used in this experiment, as actin was found to be down regulated in albinos when compared to light or dark prawns. If this is the case, it is unclear why no change in expression level was found for the gene coding for tubulin, though it could be possible that the amount of microtubules in the epithelium and exoskeleton of the *F. merguiensis* prawns did not change or only slightly changed, so that it was not detected in the analysed 2-fold and 4-fold significant changes in expression level, or that tubulin levels are regulated at the post transcriptional level. These findings of the involvement of microtubules and actin microfilaments in the movement of pigment could also explain the light and dark adaptation Tume et al. (2009) observed in their study on the black tiger prawn *Penaeus monodon*, where the pigment was found to be aggregated in lighter environments, resulting in a lighter prawn than the prawns found in darker environments, where the pigment was shown to be dispersed.

Other *F. merguiensis* gene probes that coded for troponin I, tropomyosin, sarcoplasmic calcium-binding protein, QM protein, arginine kinase and to a smaller percentage, for myosin light and heavy chain showed a 2- or 4-fold change in gene expression level across the three different colour groups in the microarray analysis. The function of these proteins might be linked to the action of actin in the cuticle tissue of *F. merguiensis* prawns of different body colouration. For example, additional to the microtubules and microfilaments, different motors are thought to be important for the movement of granules in melanophores. Research has directed its focus so far on dynein and kinesin II as motors for pigment aggregation and dispersion, respectively. Also, myosin V is believed to be the motor that enables the transport of pigment across actin microfilaments, functioning in pigment dispersion (Kotz & McNiven 1994; McNamara & Ribeiro 1999; Tuma & Gelfand 1999). Although neither dynein nor kinesin II were found to be 2- or 4-fold differentially expressed in either of the three *F. merguiensis* microarrays, small amounts of probes that gave matches to myosin light and heavy chain, where detected to be 2- and 4-fold differentially expressed across the three prawn colour groups. As myosin V, the motor protein associated with actin microfilaments (Tuma & Gelfand 1999), belongs to the myosin superfamily (Coureux et al. 2003; Espreafico et al. 1992), it is possible that the 60 bp long probes of the microarray shared sequence homology with other members of the superfamily that have already been annotated or are novel members of the family.

Arginine kinase, which was also determined to be significantly differentially expressed between the three colour groups (expression was down regulated in albinos, compared

to light and dark prawns), belongs to the phosphagen kinase family (Boyd-Kimball et al. 2006; Kinsey & Lee 2003). Arginine kinase functions as an enzyme, using arginine phosphate as a substrate to produce ATP from ADP. The produced ATP has a buffering effect by allowing maintenance of cell function over a short period of time when energy demand is higher than what oxidative phosphorylation can provide (Canonaco et al. 2003; Ellington 2001; García-Orozco et al. 2007; Holt & Kinsey 2002). As energy in form of ATP is needed for the transport of pigment (dispersion and aggregation), and potentially to maintain dispersion of the pigment granules, arginine kinase could have a role in providing temporally the ATP necessary for this function.

QM protein, also found to be 2-fold differentially expressed across the albino, light and dark colour groups, is thought to play a role in the immune system of invertebrates, but could also have a function in the actin cytoskeleton, as deletion of GRC5/QSR1, a homolog of the QM protein, resulted in an abnormal actin cytoskeleton (Xu et al. 2008). Troponin I and tropomyosin, two proteins that are directly associated with actin, were also identified among the probes that were 2- and 4-fold differentially expressed across the three prawn colour groups. Both proteins are known to bind to actin filaments, where they are two of four proteins that regulate actin-myosin interactions, with the type of interaction dependent on the presence or absence of calcium (McArdle et al. 1998; Untalan et al. 2005). The sarcoplasmic calcium-binding protein detected in the microarray analysis is also linked to actin and potentially to pigment motility, as this protein functions as calcium carrier, moving the protein to where it is needed, mainly to stimulate contraction by permitting actin-myosin interaction (Healy et al. 2003). Although speculative, the observed significant fold down regulation of sarcoplasmic calcium-binding protein expression in the albino prawns compared to the light and dark prawns, could indicate that there are differences in calcium levels across the three colour groups that make it necessary to have increased or decreased amounts of this calcium carrier. This finding could be linked to reports in the literature that indicate a potential calcium involvement in the movement of pigment. For instance, Tuma & Gelfand (1999) stated that increased intracellular calcium levels cause pigment aggregation, a pigment movement that was observed to be inhibited when no extracellular calcium was present. This finding was confirmed by Kotz & McNiven (1994) that observed that an increase in calcium levels triggered the aggregation of pigment in erythrophores and an inhibition of aggregation when extracellular calcium was absent. Furthermore, the authors indicate that pigment dispersion relied on a decrease in calcium level as well as an increase in cAMP levels. Considering the link between calcium levels and pigment aggregation, as well as dispersion, differential

expression of the sarcoplasmic calcium-binding protein in the three different colour groups of the *F. merguiensis* prawn samples suggests that the movement of pigment in the chromatophore could function in a similar way as was observed for retinal pigment epithelial cells, melanophores and erythrophores.

Two elongation factors, elongation factor 1α and elongation factor 2 were other interesting genes found to be significantly down regulated in the albino groups compared to the dark groups. While elongation factor 2 is specific in its function of regulating the elongation step in protein synthesis (Qiu et al. 2008), elongation factor 1α appears to have a variety of functions. For instance, elongation factor 1α was indicated to have a role in cell proliferation along with zinc finger proteins, which were also found to be differentially expressed between the three *F. merguiensis* colour groups (Table 4.1). Elongation factor 1α has also been shown to have an actin bundling, as well as a microtubule severing ability, however the signals or mechanisms that trigger this switch between bundling and severing action have not been established (Ejiri 2002). Whether or not the different elongation factors, particularly elongation factor 1α, have any function in prawn colouration remains to be determined, however, as actin microfilaments and microtubules have been shown to facilitate pigment transport, it is possible that elongation factor 1α could be directly or indirectly involved in or associated with pigment transport.

While a variety of genes were found to be differentially expressed between the three colour groups, crustacyanin subunit A was only detected once in 2.9 % of the probes that were 2-fold significantly down regulated in the albino prawns compared to the light and dark prawns (Figure 4.39). These findings correlate with the results of the qPCR experiment of this study, where significantly lower crustacyanin subunit A gene copy numbers were found for the albino samples than for light or dark prawns. No crustacyanin subunit C was detected in the 2-fold and 4-fold differentially expressed genes, however, this could be due to the stringent cut-off. If only significantly differentially expressed genes would have been analysed for the single comparisons (e.g. albino and light) and the amount of probes the gene has to be expressed in (e.g. from being expressed in at least three probes to at least two probes), it is quite likely that crustacyanin subunit A and C could have been found to be differentially expressed between all three comparisons (light and dark, albino and light, albino and dark). Analysis of the genes at different fold change levels gives an indication as to how strongly differentially expressed the genes were. Considering that crustacyanin subunit A and C are known to be important for colouration in crustacean, it is interesting to observe that there was just a 2-fold and lower change in expression level of the

crustacyanin subunit A and C genes between the three *F. merguiensis* colour groups, compared to genes that code for, for instance, some form of actin, troponin I or arginine kinase. These large differences in expression levels could be caused by two factors, a) already slight changes in the expression of crustacyanin subunit A and C cause a change in prawn colouration by either creating lighter or darker coloured prawns, or b) crustacyanin subunit A and C genes are only responsible for a small change in prawn colour, with one or more other factors having a stronger influence on prawn colouration than the crustacyanin genes. Another interesting observation was that while crustacyanin subunit A probes were found to be 2-fold differentially expressed, no crustacyanin subunit C probes were detected among these probes. This confirms the results obtained in the qPCR experiment, where crustacyanin subunit A showed a higher expression level (higher copy numbers for subunit A than C per *F. merguiensis* individual) than subunit C per animal.

Although this project concentrated on analysing only genes that were 2- or 4-fold differentially expressed, additional information could be gained by assessing the genes that were only significantly differentially expressed, irrespective of the fold difference of expression. This analysis was outside the scope of this project due to the sheer volume of data that it included. Furthermore, re-analysis of the unannotated genes at a later point in time is likely to allow further annotation of these genes with new information deposited on GenBank on a daily basis and thus assess their function to potentially determine other genes that could be involved in *F. merguiensis* body colouration.

6. Conclusion

In conclusion, this project has achieved the aims that were set, in that the sequences encoding for the crustacyanin subunits A and C were isolated from the cuticle tissue in *F. merguiensis* and their expression levels characterised in prawns displaying different colouration patterns. The levels of astaxanthin in the same group of prawns were also analysed. A surprising outcome was that gene expression levels of crustacyanin subunit A and C in darker coloured prawns (which showed high astaxanthin levels) were found to be lower than in the lighter coloured prawns, suggesting that the predominant forms of crustacyanin isolated in this study are not solely responsible for the colour phenotypes found in *F. merguiensis*.

The transcriptome analysis of the muscle/cuticle tissue of *F. merguiensis* revealed the profile of genes which are actively expressed in these tissues and enabled the construction of customised microarrays for the assessment of gene expression of prawns displaying different colour phenotypes. These array experiments identified a suite of genes that were significantly differentially expressed between albino, light and dark prawns, highlighting sequences possibly involved in the movement of pigment that represent genes which were not previously thought to play a role in prawn colouration.

Albino prawns, analysed in this study showed that in addition to having a low level of astaxanthin (as expected), they also displayed a low crustacyanin subunit A and C gene expression level. In addition, genes such as arginine kinase, some form of actin, sarcoplasmic calcium-binding protein and troponin I were also down regulated in the albino prawns. This suggests that the albino phenotype is associated with changes acting upstream of these genes.

Further research, focusing on the genes that were identified in this study will contribute to basic knowledge concerning the biology and regulation of colouration in crustaceans, and through this can assist the industry in selecting for genetic lines that display the commercially important darker colour.

7. Reference list

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8. Appendix

Appendix 1: Raw data of the quantitative analysis of the crustacyanin subunit A and C gene expression levels in albino, light and dark prawns.

Prawn sample number	crustacyanin subunit A mean copy number	crustacyanin subunit C mean copy number
1	139792.25	220908.18
2	913729.18	1450004.72
3	19313.58	4860.87
6	8010.94	4259.68
7	97635.17	40863.07
8	101730.96	48056.13
9	96024.49	51182.71
11	10394.22	4925.39
13	532799.84	680071.61
14	850713.24	1528701.76
15	409605.28	387252.11
16	564518.18	714414.54
19	98596.07	43276.73
20	85276.46	47778.11
22	60722.85	27085.94
23	5556.94	1609.14
24	2983.59	426.63
25	13029.20	4084.96
26	16610.19	6890.81
30	112935.13	47100.01
86	213302.46	164836.00
87	69928.03	25548.32
88	76.42	below detectable limit
89	14507.66	11772.63
90	2990.79	675.86
91	6792.99	3468.03
93	9958.08	4050.85
94	5470.92	2119.87
95	52375.09	26328.11
97	6832.55	3760.31
100	235039.68	209486.66
101	5318.76	2193.14
104	69892.34	82550.67
105	7060.53	3980.14
106	12690.46	3547.44
108	66039.95	37329.11
109	5912.72	1505.03
110	20340.89	14601.47
111	6497.93	5433.23
112	2203.04	498.43

group classified light by Seafarm
group classified dark by Seafarm
group classified albino by Seafarm

Prawn sample number	crustacyanin subunit A mean copy number	crustacyanin subunit C mean copy number
173	1282.96	687.62
174	134.45	102.32
175	144.94	64.68
176	below detectable limit	below detectable limit
177	3563.18	422.02
178	1085.23	774.89
180	below detectable limit	below detectable limit
181	3717.68	3147.76
182	below detectable limit	below detectable limit
183	below detectable limit	below detectable limit

Appendix 2: Raw data of total astaxanthin extracted from albino, light and dark prawns. Colour coding as Appendix 1.

Prawn sample number	Absorbance 1 (A)	Absorbance 2 (A)	Segment weight (g)	m (µg/g wet weight)
1	0.241	0.240	1.387	16.0
2	0.185	0.185	1.395	12.2
3	0.165	0.165	0.964	15.8
6	0.199	0.199	1.504	12.2
7	0.182	0.182	1.835	9.13
8	0.215	0.206	1.382	14.0
9	0.150	0.150	1.090	12.7
11	0.219	0.221	1.686	12.0
13	0.182	0.182	1.016	16.5
14	0.140	0.140	1.457	8.85
15	0.184	0.184	1.202	14.1
16	0.151	0.152	1.792	7.78
19	0.164	0.164	1.211	12.5
20	0.159	0.160	1.246	11.8
22	0.169	0.169	1.366	11.4
23	0.233	0.235	1.437	15.0
24	0.163	0.163	1.276	11.8
25	0.152	0.152	1.281	10.9
26	0.193	0.193	1.107	16.1
30	0.206	0.206	1.295	14.6
86	0.202	0.202	1.263	14.7
87	0.153	0.153	1.406	10.0
88	0.226	0.226	1.778	11.7
89	0.234	0.234	1.257	17.1
90	0.250	0.250	1.361	16.9
91	0.197	0.197	1.253	14.5
93	0.187	0.187	1.410	12.2
94	0.207	0.207	1.504	12.7
95	0.206	0.206	1.485	12.8
97	0.353	0.353	1.921	16.9
100	0.161	0.161	0.863	17.2
101	0.266	0.268	1.725	14.3
104	0.306	0.306	1.733	16.3
105	0.219	0.219	1.212	16.6
106	0.223	0.224	1.320	15.6
108	0.207	0.211	1.170	16.4
109	0.201	0.202	1.403	13.2
110	0.225	0.225	1.657	12.5
111	0.307	0.307	1.597	17.7
112	0.258	0.258	1.827	13.0
173	0.052	0.052	1.036	4.62
174	0.019	0.019	0.934	1.87
175	0.029	0.029	1.416	1.89

Prawn sample number	Absorbance 1 (A)	Absorbance 2 (A)	Segment weight (g)	m (µg/g wet weight)
176	0.058	0.058	0.737	7.25
177	0.017	0.017	1.520	1.03
178	0.061	0.061	0.969	5.80
180	0.194	0.195	1.166	15.4
181	0.053	0.053	1.208	4.04
182	0.019	0.019	1.166	1.50
183	0.012	0.012	0.794	1.39

Appendix 3: Raw data of of the ranking values given to albino, light and dark prawns by USC staff. Colour coding as Appendix 1. m refers to male ranker, f refers to female ranker.

Prawn sample number	Ranker 1 (m)	Ranker 2 (f)	Ranker 3 (f)	Ranker 4 (m)	Ranker 5 (m)	Ranker 6 (m)	Mean	SD
1	3	3	2	1	2	2	2.2	0.8
2	4	2	4	2	2	2	2.7	1.0
3	2	2	2	1	1	1	1.5	0.5
6	3	3	3	2	2	1	2.3	0.8
7	3	3	3	2	2	2	2.5	0.5
8	4	3	3	3	2	2	2.8	0.8
9	3	3	4	3	2	1	2.7	1.0
11	4	4	5	4	2	2	3.5	1.2
13	3	3	4	3	2	2	2.8	0.8
14	3	3	3	2	2	2	2.5	0.5
15	2	3	2	1	1	1	1.7	0.8
16	4	3	3	3	1	2	2.7	1.0
19	2	2	2	1	1	1	1.5	0.5
20	3	3	3	2	2	1	2.3	0.8
22	3	3	3	2	2	2	2.5	0.5
23	4	3	3	3	2	1	2.7	1.0
24	3	2	2	1	1	2	1.8	0.8
25	4	2	2	2	2	2	2.3	0.8
26	4	3	4	2	2	1	2.7	1.2
30	4	3	3	3	3	2	3.0	0.6
86	4	4	4	3	3	3	3.5	0.5
87	4	4	4	4	2	3	3.5	0.8
88	4	4	5	4	2	3	3.7	1.0
89	3	4	4	2	2	2	2.8	1.0
90	4	4	5	4	3	2	3.7	1.0
91	3	3	3	2	2	2	2.5	0.5
93	4	4	5	4	3	3	3.8	0.8
94	4	4	3	3	2	2	3.0	0.9
95	4	5	4	7	3	3	4.3	1.5
97	3	4	4	3	3	2	3.2	0.8
100	3	3	3	3	2	2	2.7	0.5
101	4	4	5	3	3	3	3.7	0.8
104	5	4	6	4	3	4	4.3	1.0
105	3	3	3	3	2	2	2.7	0.5
106	4	3	4	3	2	2	3.0	0.9
108	3	4	2	2	2	3	2.7	0.8
109	4	4	4	4	3	3	3.7	0.5
110	3	4	3	3	2	2	2.8	0.8
111	4	4	3	3	2	2	3.0	0.9
112	3	4	3	3	2	2	2.8	0.8
173	1	1	1	1	1	1	1.0	0.0
174	1	2	1	1	1	1	1.2	0.4

Prawn sample number	Ranker 1 (m)	Ranker 2 (f)	Ranker 3 (f)	Ranker 4 (m)	Ranker 5 (m)	Ranker 6 (m)	Mean	SD
175	1	1	1	1	1	1	1.0	0.0
176	1	2	1	1	1	1	1.2	0.4
177	1	1	1	1	1	1	1.0	0.0
178	2	2	1	1	1	1	1.3	0.5
180	1	1	1	1	1	1	1.0	0.0
181	1	1	1	1	1	1	1.0	0.0
182	1	2	1	2	1	1	1.3	0.5
183	1	1	1	1	1	1	1.0	0.0