JAMES COOK UNIVERSITY

College of Science and Engineering

Understanding the nutritional requirements of redclaw (*Cherax quadricarinatus*): determining the apparent digestibility of raw materials and quantifying dietary lysine requirement

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Bachelor of Science

Postgraduate Diploma of Research Methods

Submitted in fulfilment of the requirement for

the degree of Masters of Philosophy

06 November 2020

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Statement of the Contribution of Others

- Financial support was received in part of “Boosting Redclaw Industry Productivity with Improved Nutrition and Feed Management (RIRDC project PRJ-008536)
- Supervisors Dr. Igor Pirozzi and A/Prof Leigh Owens provided academic, scientific, and editorial support
- Colin Valverde (AquaVerde Redclaw Hatchery and Farm, Atherton, QLD) supplied the craylings used in Chapter 4
- Dr David Francis (Deakin University, Geelong, VIC) provided analytical support for the proximate analysis in Chapter 1

Acknowledgements

There was a time when this thesis was not going to happen. An almost impossible thought, a mountain peak that could never be reached. Even now writing the acknowledgements, it doesn't seem that the journey is at its end. There are a great number of people who have helped me along the way to reaching this end and I hope that they know every moment, no matter how small, helped me get to this point.

This thesis could not have been achieved without the continuing support and guidance of my primary supervisor, Dr Igor Pirozzi. He believed in my capabilities, even when I did not. His patience and dedication have been invaluable. There is little I can say other than a most sincere thank you. As many of us know, nothing in science ever goes as planned and my secondary supervisor, A/Prof Leigh Owens, provided much needed guidance and support when that moment came.

There are a few people have been there with me from the start: Alyssa, Adrien, Jarrod, Maria and Shannon. And a few that have joined my journey recently: Ana, Leontine, Madie, Sushmita. I will be forever grateful that our paths crossed and I am so excited to see our lives and careers develop and flourish.

She may not realise this, but I owe a great deal to Kristin Nunn. She was the instigator for an opportunity at a time when most needed and it changed the last few years of my life. It was a turning point and one that I will forever be grateful for. Thank you to Prof Colin Simpfendorfer who was a great boss, supporting my studies and giving me the time to work on my thesis when needed. At a most pivotal moment support was unexpectedly given and without question and for that I thank Prof Dean Jerry.

Thank you to the North Queensland Crayfish Farmers Association for their support, in particular John Stevenson, and Colin Valverde, and to the MARFU staff for all of their technical assistance.

I would like to thank my parents, Vickie and Morris, for the unending encouragement and support.

And lastly, I need to acknowledge and thank myself. It has been a struggle both physically and mentally to reach this point. But I did. I have. So, thank you to past me for getting me to this point. The future is bright!

ABSTRACT

Redclaw, *Cherax quadricarinatus*, is a species of freshwater crayfish native to North Queensland (Australia) and south-eastern Papua New Guinea. Redclaw are a robust species with favourable culture characteristics, which has seen them exported to countries such as Argentina, Mexico, Spain and The United States and South-East Asia and Central/South America. Commercial aquaculture of redclaw in Australia is limited to a number of farms in Queensland where 48.8 tonnes were produced in 2017-2018. Farmed in semi-intensive pond systems, naturally occurring pond biota acts as a direct feed source. It is common practice to supplement feeding with a variety of feed stuffs including commercial chicken pellets, unprocessed legumes (such as soybean, lupin and peas) and/or food scraps however this varies from farm-to-farm. To develop the industry further, farming methodologies will need to move to more intensive culture systems to increase production capacity. To do so farmers will need to rely more on quality compound feeds to provide adequate nutrition. Currently the nutritional information for redclaw is broadly limited to macronutrients such as crude protein and lipid requirements, and there is limited information on ingredient use and digestibility. The aim of this thesis was to obtain a better understanding of the nutritional requirements of redclaw to contribute towards the knowledge base from which better diet formulations can be made. This was achieved by: a) determining digestibility coefficients for a number of plant and animal-rendered ingredients; b) assessing dietary effects on overall health status; and c) determination of the dietary requirement for lysine.

Following the general introduction (Chapter 1), the digestibility of dry matter, protein and amino acids for seven protein sources (yellow pea meal (YPM), dried distillers grains (DDG, lupin meal (LM), solvent extracted soybean meal (SBM), sorghum meal (SM), poultry by-product meal (PBM) and feather meal (FM)) was determined (Chapter 2). Assessing digestibility is an essential prerequisite in evaluating ingredients for aquaculture use and enabling the formulation of a diet based on digestibility rather than a crude nutrient basis. Adult redclaw (~40g) were obtained from a farm in North Queensland. Diets contained 70% reference diet (Ridley prawn feed) and 30% test ingredient, and 0.1% yttrium oxide, Y_2O_3, as an inert marker. Redclaw were fed diets daily for a period of 12 weeks. Faecal material was collected 4 hours after feeding, by siphoning material into a collection vessel half-filled with freshwater. A mesh screen was immersed in the water allowing intact faecal strands to be carefully pipetted off the mesh, while allowing other materials to separate out. Faecal material was freeze dried and analysed for proximate composition and amino acids. Apparent

digestibility coefficients (ADCs) were then calculated for diet and ingredients. Protein ADCs among the diets were mostly similar ranging from 86.54% to 94.08%. Ingredient ADCs for dry matter ranged from 86.97% to 97.37%, while dietary protein ranged from 86.87% - 104.37%. Ingredient amino acid digestibility ranged 65.17% to 130.22% although this is including the values exceeding 100% calculated for LM, YPM and SM. The ingredient AA digestibility for key limiting amino acids were highly digestible with ADCs for Lysine (Lys) ranging from 89.67% to 94.09%, and 88.38% to 91.1% for Methionine (Met). In general, the ADC values were quite reasonable. There were no significant differences between the ADCs for plant and animal products. The results from Chapter 2 showed that redclaw are capable of digesting a variety of plant and animal ingredients which indicates a good potential for flexibility in diet formulation to achieve an adequate nutritional profile.

Chapter 3 presents a histological examination of the hepatopancreas, an important digestive organ in crustaceans, sampled from the redclaw used in Chapter 2. Initial redclaw (n=6) had been sampled prior to the digestibility study, with more redclaw sampled from each diet treatment on conclusion of the feeding trial (n = 52). The initial redclaw sample had a relatively healthy hepatopancreas as evidenced by the uniform honeycomb structure of the tubule with thick epithelium and folded inner lumen star-shaped; however, there were evidence of excess lipid storage, as indicated by numerous vacuoles, reovirus infection and granulomas. However, the redclaw fed the various diets including the reference diet all exhibited a number of structural abnormalities at the conclusion of the trial including degradation of the myoepithelial cells, sloughing of cell contents, hypertrophy of b-cells, and thinning of epithelial lumen. No sampled individuals supported a healthy hepatopancreatic structure and very few were completely pathogen free. In addition, granulomas, bacterial and viral infections (CqBV and reovirus) were also present. There were some diet effects with redclaw fed the SBM diets having significantly higher levels of CqBV than those fed the LM diet. The results were quite unexpected and counterintuitive as the ADC values obtained on Chapter 2 were quite reasonable. Histological techniques are well established and relatively straightforward to implement, yet surprisingly very little work has been done in this area with redclaw and particularly so in the context of nutritional studies. The results of Chapter 3 highlighted the importance of co-investigation of diet performance and histology when assessing nutritional requirements for redclaw.

Dietary protein is by proportion usually the most expensive component of feed formulation; however, animals don't have a requirement for protein *per se* but rather their constituent amino acids. Essential amino acids (EAAs) are those that cannot be biosynthesised *de novo* and must

be provided in the diet. There are currently no published data on the EAA requirements for redclaw. Lys is one of the first limiting amino acids in fishmeal replacement feeds, particularly when using plant proteins. The Lys requirement for juvenile redclaw was determined through a dose-response feeding trial (Chapter 4). Juvenile redclaw used in Chapter 4 were sourced as craylings from a hatchery in North Queensland as precaution against using potentially compromised farm stock as seen in Chapter 3. Redclaw were maintained in a recirculating aquaculture system until they reached ~1g. Five isoenergetic (18 MJ gross energy (GE)/kg) and isoproteic (30% CP) diets were formulated to contain one of five levels of Lys ranging from 0.5 to 2.0%. Juveniles (~1.7 g) were then fed one of five diets, twice daily, over a period of 12 weeks. Results showed a decline in growth for redclaw fed the diet with highest level of Lys (2%). Using robust segmented linear regression the optimal dietary Lys inclusion for juvenile redclaw was 1.72%, equivalent to 5.76% of dietary protein. These results provide important information towards formulating a diet based on amino acid requirements and is the first study to quantify an EAA requirement for redclaw.

This thesis has determined information on the protein and amino acid digestibility of several raw ingredients and quantified the lysine requirement for redclaw. It has also highlighted the importance of deeper investigation into the health status of redclaw used in nutritional studies through histological examination. Taken together this thesis has improved the understanding of the nutritional requirements for redclaw, providing important information towards the development of a formulated feed based on the nutritional requirements of redclaw.

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List of Abbreviations

AA/s = amino acid/s

ADCs = apparent digestibility coefficients

ANF/s = Anti nutritional factor/s

Arg = Arginine

CP = crude protein

CqBV = *Cherax quadricarinatus* bacilliform virus

CqReo = Cherax reovirus

DDG = dried distillers grains

EAA/s = Essential Amino Acid/s

ELS = excess lipid storage

FCR = food conversion ratio

FM = Fish Meal

FTM = feather meal

Hep = hepatopancreas

LM = lupin meal

Lys = Lysine

Met = Methionine

PBP = poultry by-produce meal

RAS = recirculating aquaculture system

RF = reference diet

SM = sorghum meal

SBM = soybean meal

YPM = yellow pea meal

1. CHAPTER 1 GENERAL INTRODUCTION

1.1. Background

Global aquaculture production reached 114.5 million tonnes (mt) in 2019 worth approximately USD263.6 billion (SOFIA, 2020). Of that 9.4 mt were crustacean species and while 64 crustacean species are farmed, production is largely made up of 6 species: Whiteleg shrimp (*Penaeus vannamei*), Red swamp crawfish (*Procambarus clarkia*), Chinese mitten crab (*Eriocheir sinensis*), Giant tiger prawn (*Penaeus monodon*), Oriental river prawn (*Macrobrachium nipponense*) and Giant river prawn (*Macrobrachium rosenbergii*) (FAO, 2018). Of the other crustacean species that contribute up to 10% of all crustaceans cultured, redclaw, *Cherax quadricarinatus*, is a species with great potential as demand for quality aquatic products continues to increase. However, commercial aquaculture production of redclaw remains relatively small, brought about, in part by production bottle necks associated with the availability of a commercially appropriate feed (John Stevenson, North Queensland Crayfish Farmers Association (NQCFA) –President, 2016. pers. comm).

Redclaw are an omnivorous freshwater crayfish native to North Queensland (Australia) and south-east of Papua New Guinea. Culture of redclaw was first established in Australia in the early 1980's and in the decades since has expanded rapidly to many countries including the United States, China, Mexico, Spain and Argentina (Saoud, 2013). The global production of redclaw in 2016 was reported as 305 tonnes (FAO, 2018), however not all countries where redclaw are farmed provide production reports, indicating the total production figure could be greater (FAO, 2018). The global expansion of redclaw can be attributed to the increased demand for quality seafood products, its relatively rapid growth rate, tolerance for high stocking densities, and straightforward culture and live cycle having no planktonic larval stage (Jones et al., 2002; Saoud et al., 2012; Thompson et al., 2003). In Australia, commercial aquaculture of redclaw is limited to a small number of farms in Queensland. In 2017-18 production in Queensland was 48.8 tonnes worth AUD$1.2M (DAF, 2019). While production has continued to decline over the past decade with a peak of 100 tonnes in 2004, the price has been fairly stable from $21 per/kg in 2010 (DAF, 2013) to approximately $25/kg in 2017 (DAF, 2019).

Redclaw are mostly farmed in semi-intensive pond systems with naturally occurring pond biota known to be important; periphyton, algal, other natural plant feedstuffs and naturally occurring pond organisms such as cladocerans, copepods, chironomid larvae provide direct nutrition for pond-raised redclaw (Duffy et al., 2011; Viau et al., 2012). Farmers commonly promote pond

primary productivity with the addition of fertilisers (Jones & Ruscoe, 1996) and it is common practice to supplement feeding with a variety of feed stuffs including commercial chicken pellets, unprocessed legumes (such as soybean, lupin and peas) and/or food scraps (John Stevenson, NQCFA –President, 2016. pers. comm). However, this varies from farm-to-farm with the assumption that if a good crop is produced then the feeding regime must be adequate (Jones, 1996; Saoud et al., 2012). While supplemental feeding is common, the consumption and utilisation of these feeds as a direct source of nutrition for redclaw in ponds is highly variable with the unintended consequence of supplemental feed instead likely contributing towards primary pond production of zoo- and phytoplankton (Joyce & Pirozzi, 2016). This is a rather inefficient and costly method of pond fertilisation, highlighting the need of providing a feed formulated specifically for redclaw.

1.2. Formulated Feed

Formulated feed need to be durable, water stable, and have desirable physical and textural characteristics (Cho, 1990). Diets play an important role in maintaining water quality and managing waste, with digestibility of ingredients and nutrient composition the main factors that contribute to waste outputs in aquaculture systems (Cho & Bureau, 2001). More importantly, formulated diets are necessary in providing optimal nutrition to cultured animals particularly in intensive culture systems where there is no natural food source.

Protein is the primary nutrient in formulated feed and fish meal (FM) has historically been used due to its relative affordability, high quality protein content, balanced amino acid profile, and supply of additional vitamins and minerals (Hardy, 2010; Tacon & Metian, 2015). With the expansion of aquaculture the production of aquafeed and the demand for quality ingredients has increased. However, wild fish stocks are being fished at or over capacity and the restricted supply and associated increased demand has increased the cost of FM (New & Wijkström, 2002). As such there has been increased urgency in finding alterative protein sources for use in aquaculture feeds and a great deal of research has subsequently occurred in the past few decades with plant based and animal agriculture by-products receiving particular focus (Tacon & Metian, 2008), and insect protein and algae emerging as more recent alternatives (Barroso et al., 2014).

Redclaw farmers have historically used a generic commercial redclaw feed modified from one used for other aquatic species (Saoud et al., 2012). Replacement of FM and determining the protein requirement for redclaw was a large focus in the early to mid-2000's. Muzinic et al.

(2004) reported that FM could wholly be replaced by soybean and brewer's grains with yeast in diets for juvenile redclaw with no reduction in growth or survival. Similarly, Garza de Yta et al. (2012) reported no negative effects on growth or survival of redclaw fed diets containing a mixture of plant protein alternatives such as soybean, pea meal and dried distillers grains.

Currently the redclaw industry in North Queensland uses a modified feed based on suggestions for crude protein (CP) and fat content, contains primarily wheat and soybean products (John Stevenson, NQCFA, pers. comm, 2016), with an alginate binder as recommended by Zeng et al. (2014). The diet is not produced on a large commercial scale and limited to production at a single feed mill in North Queensland (Colin Valverde, NQCFA - Northern Region President, 2019. pers. comm). Information on specific nutritional requirements for redclaw will therefore improve the diet formulation and therefore production and yield for the industry.

1.3. Feed and ingredient digestibility

Previous studies have established CP requirements of redclaw to be between 25-30% depending on life stage (0.1 – 25.6g) and culture method (Garza det Yta et al., 2012; Thompson et al., 2005; Cortes-Jacinto et al., 2004). Quantifying CP requirement establishes a baseline but does not indicate the quality or the potential availability of the protein source (Irvin & Williams, 2007). Redclaw have been shown to modify enzyme levels in response to dietary compounds, enabling them to digest a variety of feed ingredients (Lopez-Lopez et al., 2005). A very limited number of digestibility studies have been undertaken with redclaw, and these are reviewed in Chapter 2. Assessing digestibility is an important prerequisite in evaluating the quality of ingredients for aquaculture use and enabling the formulation of a diet based on a digestible rather than a crude nutrient basis (Glencross et al., 2007). Quantifying the potential availability of nutrients that can be absorbed and utilised by the animal to support metabolic process such as maintenance, reproduction and growth is vital. Digestibility of feedstuffs are typically determined through an indirect method where an indigestible marker is added to the test diet and the ratio of the marker in the feed and faeces relative to the nutrient content used to calculate the apparent digestibility coefficients (ADCs) (Wilson et al., 2002)**.** While the indirect method to determine ingredient ADCs is common it nonetheless still remains quite a challenging technique as experiments are time consuming, expensive and susceptible to variables in the methodology which are carried into the formulae (Booth & Pirozzi, 2018). Animal derived feedstuffs such as blood meal, poultry by-product and feather meal can be used to satisfy protein requirements in place of FM (Naylor et al., 2000). Plant based proteins such as canola meal, lupin meal and soybean are increasingly being included in aquafeed (Hardy,

2010); however, some plants contain compounds that can negatively affect food utilisation. Antinutritional factors (ANFs) are substances produced by plants which when consumed directly, or through secondary metabolic products, interfere with food utilisation, health, and reproduction of individuals (Hajra et al., 2013; Francis et al., 2001). ANFs can be divided into four broad groups: a) those which affect protein digestion and utilisation like protease inhibitors, b) those affecting mineral utilisation like phytates; c) antivitamins; and d) miscellaneous substances such as alkaloids and saponins (Francis et al., 2001). Treatments such as boiling, roasting, autoclaving and fermentation (Drew et al., 2007; Khattab & Arntfield 2009; Rehman & Shah 2005), are often used to reduce or eliminate such compounds. Composition of ingredients and processing conditions can also affect nutritional quality and utilisation. For example, inclusion of extracted soybean meal in diets for Atlantic salmon (*Salmo salar)* caused morphological changes in epithelial cells of the distal intestine along with reduced protein and fat absorption (Bakke-McKellep et al., 2000; Krogdahl et al., 2003). While heating, through cooking or pelleting method, can result in changes to amino acids through oxidation of sulphide bonds and Maillard reactions (Bureau et al., 1999). FM has a high nutrient digestibility (Tacon & Metian, 2015) and is often produced at low drying temperatures to reduce such changes in amino acids and maintain high digestibility coefficients (Drew et al., 2007).

Protein ADCs of feed ingredients for a number of different fish and crustacean species are presented in Table 1.1 Protein digestibility is quite variable and is largely dependent upon the target species. For example corn gluten meal is highly digestible in Australian Silver Perch (*Bidyanus bidyanus*) (95.4%) (Allan et al., 2000) and Tilapia (90.5%) (Köprücü & Özdemir 2005), while in Yellowtail Kingfish (*Seriola lalandi*) it is poorly digested (31.4%) (Dam et al., 2019). The high digestibility for Silver Perch and Tilapia could be a reflection that these species are herbivores. ADCs of ingredients can vary considerably for example Peruvian FM ADCs determined for Hybrid Nile Tilapia are reported to be 90.1% (Zhou & Yue, 2012) to 99.4% (Dong et al., 2010). Such differences between species can be a result of diet formulation, ingredient source and processing methods (Ngo et al. 2015; Francis et al., 2001; McGoogan & Riegh 1996). Similarly, the CP ADCs for tuna trimmings for Yellowtail Kingfish was 59.3%, compared to premium FM at 68.7% (Dam et al., 2019) however the ADCs for premium FM for other fish species such as Humpback Grouper, Silver Perch and Tilapia were all greater than 90% (Table 1.1). Ultimately, determining ADCs enable the formulation of a feed that will

reduce waste, provide optimal nutrition to promote good growth and survival (Allan et al., 2000; Laining et al., 2003).

Tabl.1 Protein apparent digestibility coefficients (ADCs) for various feed ingredients for aquaculture species.

	Crustaceans			Fish					
Species	Tropical Spiny Lobster (*Panulirus ornatus*	Pacific White Shrimp (*Litopenaeus vannamei*)	Chinese Mitten Shrimp *(Eriocheir sinensis)*	Australian Silver Perch *(Bidyanus bidyanus)*	Yellowtail Kingfish (*Seriola lalandi*)	Humpback Grouper (*Cromileptes altivelis)*	Nile Tilapia (*Oreochromis niloticus*)	Hybrid Nile Tilapia (*Oreochromis niloticus x Oreochromis aureus*)	
	Irwin & Williams 2007[a]	Liu et al 2013[b]	Luo et al 2008[c]	Allen et al 2000[d]	Dam et al., 2019[e]	Laining et al., 2003[f]	Koprucu & Ozdemir 2005[g]	Zhu & Yue 2012[h]	Dong et al 2010[i]
Test Ingredient	DM	As is	As Is	DM	DM	DM	DM	DM	DM
Fish Meals									
Fish Meal A	84.3	90.9	96.3	92.3	68.7	92.5	90.5	90.1	99.4
Fish Meal B	-	-	-	89.0	59.3	-	-	87.1	-
Animal Meals									
Blood Meal	-	69.1	-	90.2	50.6	55.2	-	-	-
Poultry By-Product Meal	-	83.9	-	85.4	66.5/71.3	-	-	82.4	-
Feather Meal	-	-	-	92.8	-	-	-	-	-
Mussel Meal	88.8	-	-	-	-	-	-	-	-
Shrimp Head Meal	-	78.9	-	-	-	78.0	-	-	-
Crustacean Meal	85.3	-	88.1	-	-	-	71.0	-	-
Plant Meals									
Soybean	80.6	92.3	88.3	94.8	62.5/45.3	67.2	87.4	94.9/90.9	97.8/95.7
Corn Gluten Meal	-	55.7	-	95.4	31.4	-	89.0	-	95.4
Lupin	85.9	-	-	97.1	90.0/101.3	-	-	-	-
Field Pea	-	-	-	81.0	-	-	-	-	-
Sorghum	-	-	-	77.8	-	-	-	-	-
Wheat Flour	74.2	-	-	-	-	-	-	-	-
Rapeseed Meal	-	78.3	84.7	-	-	-	-	-	-

a Fish Meal A = Peruvian; Mussel Meal = New Zealand green-lip; Soybean = solvent extracted; Lupin = dehulled
b Fish Meal A = first feed grade
c Fish Meal A = Peruvian ; Soybean = 46.7%CP; Rapeseed meal 38.6%CP; Crustacean meal = 32.8%CP (CP% as is basis)
d Fish Meal A = Australian; Fish Meal B = Peruvian; Soybean = solvent; Lupin = *L. angustifolius*; Field Pea = *P. sativum*
e Fish Meal A = prime FM; Fish Meal B = recycled tuna trims; Soybean = soy protein concentrates at two CP%; Lupin = dehulled
f Fish Meal A = sardine meal; Soybean = roasted, full fat
g Fish Meal A = Anchovy; Crustacean meal = Crayfish exoskeleton meal.
h Fish Meal A = Peruvian; Fish Meal B = local; Soybean = fermented/solvent
i Fish Meal A = Peruvian ; Soybean = unspecified /fermented

1.4. Nutritional requirements and amino acids

Nutrient requirements for redclaw were reviewed by Saoud et al. (2012) in which CP requirements were recommended to be greater than 25% for pond-raised redclaw, and 35% for redclaw grown in recirculating aquaculture systems (RAS). Lipid requirements of 4.2% was considered sufficient where natural productivity is available, however dietary lipid for female broodstock should be increased to 8.7% to promote optimal egg quality (Rodrıguez-Gonzalez et al. 2006). Requirements for cholesterol (Hernandez et al., 2004) and phospholipid (Thompson et al., 2003) have also been evaluated for redclaw. While no specific requirements for carbohydrates have been determined, optimal growth occurred when carbohydrates and lipids were provided in a 3.6:1 ratio (Zhu et al., 2013). Since the review by Saoud et al. (2012) there has been very little further published work conducted on redclaw. There are still many gaps in the nutritional information for the various life-stages of redclaw including information on vitamin and minerals, carotenoids and essential amino acid requirements (Harlıoğlu & Farhadi 2017).

Redclaw, like all animals, do not have a requirement for protein *per se*, but rather the constituent amino acids that they are comprised of. Amino acids are involved in numerous metabolic processes from DNA transcription/translation to protein deposition. Essential amino acids (EAAs) are those that cannot be biosynthesised *de novo* and must be provided in the diet (Wu, 2009). Knowledge of EAA requirements are critically important to formulating appropriate diets using fishmeal replacement proteins, yet there are currently no published studies on amino acid nutrition for redclaw. However, the EAAs for redclaw are likely to be the same as that of other crustaceans which includes arginine (Arg), histidine, isoleucine, leucine, lysine (Lys), methionine (Met), phenylalanine, threonine, tryptophan, and valine (Chen, 1998; Saoud et al., 2012). EAA requirements for a number of crustacean species is presented in Table 1.2. Lys and Arg supplemented diets showed improved growth for *P. monodon,* although the authors noted a marked decline in growth beyond the optimal dietary requirements (Millamena et al., 1998). The Lys requirement is quite similar across species (2.05-2.55%) with the exception of Kurama Shrimp (*Marsupenaeus japonicas)* (3.22%).

Obtaining an understanding of EAA requirements is important as an imbalanced profile or provision of protein beyond that required for maintenance and growth can result in AAs being oxidised and deaminated for energy, consequently reducing the relative contribution to protein synthesis and increasing metabolic wastes such as ammonia (Bureau & Hua, 2010; Green & Hardy, 2008; Wu, 2009). Increases in metabolic waste not only affect water quality, increase

burden on infrastructure for waste management (Bureau & Hua, 2010) but also represents an economic loss as feed accounts for a significant portion of a farms operating costs (Rana et al., 2009). EAA requirement is further influenced by life stage, reproduction, environmental condition, and digestibility and AA profile of the dietary protein (Furst & Stehle 2004).

In general, dietary EAA content is higher in animal sources with Lys and/or Met often limiting in plant-based proteins (Gorissen et al., 2018). In broad terms, cereal grains tend to be deficient in Lys, while legumes are often deficient in Met and cysteine (sulphur containing amino acids) (Hardy 2010; Gorissen et al., 2018). The CP content of common feed ingredients used in aquaculture diets are presented in Figure 1, and the EAA profile for these ingredients shown in Table 1.3. The differences in plant and animal products is apparent, for example FM contains 1.76% Met and 4.71% Lys; while soybean (dehulled, solvent extracted) has 0.64% Met and 2.76% Lys, and pea protein concentrate 0.41% Met and 3.75% Lys. The slight difference in EAA proportions of two soybean meals (expeller vs solvent extracted) in Figure 1.1 illustrates how processing of raw ingredients can impact the chemical composition i.e. de-hulling, solvent extracted soybean meal slightly increases the overall EAA profile, highlighting why feed manufacturers combine ingredients to achieve the required nutritional profile for the target species. Ultimately understanding the dietary requirement for essential amino acids facilitates the formulation of more efficient diets, reducing operational costs and improving yields (Millamena et al., 1999; Wilson et al., 2002)

1.5. Nutrition and Health

Determining specific nutritional requirements is critical for the formulation of an optimal diet that promotes good growth, health, and survival. Physical deformities can occur as a result of nutritional deficiencies, for example, Met deficient diets resulted in Rainbow Trout (*Oncorhynchus mykiss*) developing cataracts (Cowey et al., 1992), while tryptophan deficiency induced scoliosis in salmonids (Cahu et al., 2003). Nutritional deficiency in crustaceans also results in deformities such as duplication or absence of appendages (Okada et al., 1997), malformed uropods and rostrum (Béguer et al., 2008) and abdominal segment deformity disease (Santander-Avancena et al., 2017).

In crustaceans, the site of digestion and nutrition absorption is the hepatopancreas (Hep). Epithelial cells are differentiated into four main cell types (E-, F-, R- and B-cells) which line the tubules which branch throughout the organ (Barker & Gibson, 1977; Loizzi, 1971). The Hep has often been used as an indicator of health for crustaceans with changes in Hep and cell

structure reported to occur from environmental influences such as chemical toxicity (Boudet et al., 2015; Frías-Espericueta et al., 2008), salinity (Li et al., 2008), feeding practices (Calvo et al., 2011) and vitamin deficient diets (Fernandez Gimenez et al., 2004). Growth, feed intake, food conversion ratio (FCR), whole body and tail muscle composition are commonly measured parameters in redclaw studies assessing the suitability of ingredients or dietary requirements. Of the publications highlighted in the review by Saoud et al. (2012) none reported sampling of the Hep. This is surprising as studies on other crustacean species have clearly demonstrated that the structure of the Hep to be a sensitive indicator of nutritional value of diets in *P. monodon* (Anger et al., 1985; Vogt et al., 1985) Southern Rock Lobster (*Jasus edwardsii*) (Johnston et al., 2003) and *Cherax destructor* (Jones & Obst, 2000).

1.6. Research Aim and Objectives

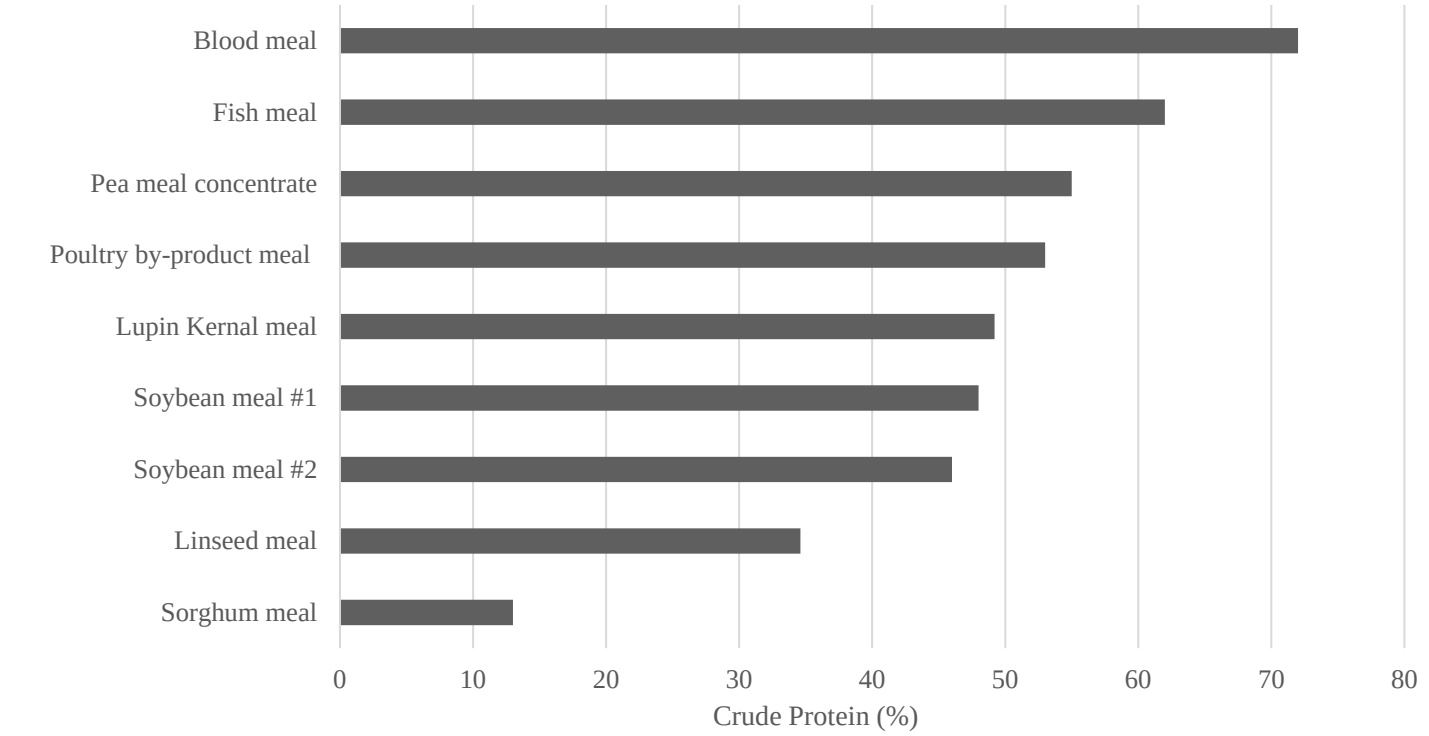
The aim of this thesis was to obtain a better understanding of the nutritional requirements of redclaw to aid in the improvement of a species-specific diet formulation. To achieve this there were three key objectives:

1. Determine the digestibility coefficients for dry matter, protein, and AAs for a number of plant and animal-rendered protein sources (Chapter 2)
2. Examine the potential impact of these feed ingredients on the health status of redclaw through a histopathological survey of the Hep (Chapter 3); and
3. Determine the optimal dietary Lys requirement for redclaw (Chapter 4)

Table 1.2 Estimated amino acid requirements for various crustacean species (% of diet)

Species	Amino Acid										Ref
	Life stage	Arg	Lys	His	Isoleu	Leu	Met	Phe	Thr	Trp	Val	
Atlantic Ditch Shrimp (*Palaemonetes varians)*	Postlarvae, 0.17g)	2.05	2.42	-	-	-	0.96	-	-	-	-	Palma et al., 2013
Swimming Crab (*Portunus trituberculatus)*	Juvenile (7.86g)	-	2.17*	-	-	-	-	-	-	-	-	Jin et al., 2015
Pacific White Shrimp (*Litopenaeus vannamei*)	Juvenile (0.52g)	-	1.64*	-	-	-	-	-	-	-	-	Xie et al., 2012
	Juvenile (0.50g)	1.96*	-	-	-	-	-	-	-	-	-	Zhou et al., 2012
	Juvenile (0.48g)	-	-	-	-	-	-	-	1.36	-	-	Huai et al., 2009
	Juvenile (0.43g)	-	-	-	1.59	-	-	-	-	-	-	Liu et al., 2014
Kurama Shrimp (*Marsupenaeus japonicas*)	Juvenile (0.79g)	2.89	3.22	1.11	2.33	3.44	1.33	2.56	2.33	0.63	2.44	Teshima et al., 2002[a]
	Juvenile (0.25g)	2.45[e]	-	-	-	-	-	-	-	-	-	Alam et al., 2004
Tiger Shrimp (*Penaeus monodon*)	Postlarve (0.21g)	1.85	2.08	-	-	-	-	-	-	-	-	Millamena et al., 1998
	Postlarvae (0.14g)	-	-	-	-	-	-	-	-	-	1.35	Millamena et al., 1996
	Postlarvae, (0.20g)	-	-	0.8	1.01	1.7	-	1.4	-	0.2	-	Millamena et al., 1999
	Postlarvae, (0.05g)	-	-	-	-	-	-	-	1.4	-	-	Millamena et al., 1997
	Juvenile (0.32g)	2.50	-	-	-	-	-	-	-	-	-	Chen et al. 1992
Chinese Mitten Crab *(Ericheir sinensis)*	Megalope; (0.68g)	2.00	2.55	-	-	-	-	-	-	-	-	Jiang et al., 2005
	Juvenile (2.1g)	3.62	2.34	-	-	-	1.12	-	-	-	-	Ye et al., 2010

* calculated based on leaching rate of pellets
[a] based on EAA increment in whole body when fed diets high nutritive value (based on 50%CP)



Int feed #

Blood meal 5-00-380; Fish meal 5-02-000; Poultry-by-product meal 5-03-800; Pea meal n/a; Soybean meal #1 5-04-612 (dehulled, solvent extracted); Soybean meal #2 = expeller; Linseed meal 5-02-048 (solvent extracted); Sorghum meal = 4-04-444

Adapted from AFFRIS (http://www.fao.org/fishery/affris/feed-resources-database/ingredient-analysis/en)

Figure 1.1 Crude protein (%) profile of various feed ingredients

Table 1.3 Essential Amino Acid (EAA) profile of various feed ingredients (% CP)

	Sorghum meal	Linseed meal[a]	Soybean meal[b]	Soybean meal[c]	Lupin Kernal meal	Poultry by-product meal	Pea meal concentrate	Fish meal	Blood meal
Arginine	0.45	2.88	3.22	3.45	2.96	3.5	4.83	3.68	2.87
Histidine	0.27	0.69	1.19	1.21	0.81	1.42	1.3	1.53	3.56
Isoleucine	0.49	1.71	2	2.09	1.37	2.1	2.15	3.03	0.81
Leucine	0.52	2.01	3.33	3.53	2.24	3.95	3.75	4.82	9.11
Lysine	1.8	1.14	2.71	2.76	1.65	2.25	3.75	4.81	5.39
Methionine	0.26	0.52	0.56	0.64	0.24	0.91	0.41	1.9	0.82
Phenylalanine	0.39	1.47	2.23	2.36	1.15	1.6	2.56	2.66	4.92
Threonine	1.16	1.21	1.62	1.72	1.14	1.88	1.85	2.69	0.92
Tryptophan	0.39	0.5	0.73	0.73	0.31	0.5	0.49	0.72	1.56
Tyrosine	0.29	1.09	0	1.73	1.1	1.41	1.58	2.13	2.1
Valine	0.13	1.7	2.33	2.15	1.26	2.32	2.35	3.34	5.77
Int. Feed #	4-04-444	5-02-048	-	5-04-612	-	5-03-800	-	5-02-000	5-00-380

a Solvent extracted; b expeller; c dehulled, solvent extracted

Adapted from AFFRIS (http://www.fao.org/fishery/affris/feed-resources-database/ingredient-analysis/en)

2. CHAPTER 2: PROTEIN AND AMINO ACID DIGESTIBILITY OF SELECTED FEED INGREDIENTS FOR *CHERAX QUADRICARINATUS*

2.1. Introduction

Feed is one of the major operating costs in extensive and semi-intensive aquaculture operations and it is therefore important to provide a cost-effective feed that provides optimal nutrition to promote growth (Oliva-Teles, 2012). As protein levels influence diet costs it is important that diets containing different protein sources be evaluated to ensure that the AA profile meets the organisms nutritional requirements (Thompson et al., 2005). FM is the most common source of protein in aquaculture diets due to its high-quality protein content, balanced AA profile, essential fatty acids and palatability. However, FM is one of the most expensive ingredients in aquaculture diets due to decreases in wild caught fishery stocks and increasing global demand (Han et al., 2018). As such there has been increased need for alternative, low-cost protein rich sources to substitute FM. Products such as feather meal, poultry by-product meal and meat and bone meal have successfully been included in diets for a number of aquaculture species including malabar grouper (*Epinephelus malabricus)* (Wang et al., 2008), *P. vannamei* (Forster et al., 2003) and *M. nipponense* (Yang et al., 2004).

Plant proteins are increasingly being used as alternative protein sources (Tacon et al., 2015, Hua et al., 2019). As redclaw are omnivorous, plant proteins have the potential to be good FM substitutes. Grain legumes have the potential to provide good energy and moderate protein to manufactured diets. In recent years there has been increased interest in the use of plant meals. Due to its favourable cost and consistent availability soybean meal is widely used as a FM replacement (Taher et al., 2017). **S**oybean meal, lupin and pea protein concentrate have the potential to replace 33% of FM in extruded salmon feeds (Carter & Hauler, 2000); while, Gomes et al. (1995) indicated that up to 66% of FM could be replaced by plant proteins in rainbow trout diets. Studies investigating the suitability of plant-based and other alternative protein sources for crustaceans have increased in recent years and include soybean (Garza de Yta et al., 2012; Muzinic et al., 2004; Thompson et al., 2005), pea protein isolate (Fuertes et al., 2013), poultry-by-product meal (Cruz-Suárez et al., 2007; Saoud et al., 2008), and meat and bone meal (Forster et al., 2003; Ye et al., 2010).

However, feed that provides optimal nutrition, promotes good growth and survival requires an understanding of an ingredients digestibility (Allen et al., 2006; Laining et al., 2003). Determining digestibility quantifies the potential availability of nutrients that can be absorbed and utilised to support reproduction and metabolic process such as maintenance and growth

(Reigh et al., 1990). Digestibility of a feedstuff depends on the physical and biochemical characteristics of the feed, but environmental conditions, such as where the crop is farmed and how it is processed**,** can also have an influence on the digestibility of the feed (Vasagam et al., 2007). Further, diets are often evaluated in terms of growth, not on the digestibility and effective assimilation of nutrients (Thompson et al., 2005, Glencross et al, 2007).

There are very few published reports that have considered digestibility of diets and ingredients for redclaw. A search of the primary literature resulted in only seven publications from 2005 – 2020 with digestibility in the title, abstract or a keyword. Of those articles three determined ADCs (DM, CP, Lipid, Carbohydrates and Energy) for a combined total of 12 ingredients (Table 2.1). The remaining four studies did not assess digestibility of ingredients, rather the effects of nutritional compounds in diets on digestive enzymes and digestibility. From the primary literature there has been no published research on the digestibility of feed ingredients for redclaw since 2008.

The purpose of this study was to determine the apparent digestibility coefficients for a number of plant and animal-based protein sources to aid in the formulation of a nutritionally complete feed for redclaw.

Table 2.1 ADCs determined for redclaw fed plant and animal products based on search of the literature

			Diet ADCs					Ingredient ADCs					
Size	**Inclusion**	**Feed type**	**Dry Matter**	**Crude Protein**	**Lipid**	**Carbs**	**Energy**	**Dry Matter**	**Crude Protein**	**Lipid**	**Carbs**	**Energy**	**Reference**
Juvenile 3.6g	15%	Plant	86.0 ± 0.3	91.3 ± 0.5	94.5 ± 0.7	91.7 ± 0.5	-	87.9 ± 1.9	84.8 ± 9.2	91.4 ± 4.1	90.1 ± 4.1	-	Campaña-Torres et al., 2005, 2006[a]
		Animal	83.9 ± 1.6	89.0 ± 1.0	92.6 ± 1.4	88.8 ± 0.8	-	72.2 ± 11.8	55.7 ± 11.6	75.6 ± 19.7	24.5 ± 6.0	-	
Pre-adult 10.1g	15%	Plant	-	-	91.6 ± 1.2	90.1 ± 1.1	-	-	-	89.6 ± 8.7	86.4 ± 5.2	-	Campaña-Torres et al., 2008 [a]
		Animal	-	-	89.2 ± 2.7	88.9 ± 1.1	-	-	-	68.9 ± 24.6	25.8 ± 12.1	-	
Adult 94.5g	30%	Plant	82.4 ± 1.9	93.4 ± 0.7	-	-	89.3 ± 1.6	74.4 ± 6.5	86.8 ± 2.6	-	-	82.1 ± 5.4	Pavasovic et al., 2007 [b]
		Animal	79.3 ± 2.4	90.0 ± 1.2	-	-	87.7 ± 1.6	83.3 ± 5.5	93.1 ± 1.4	-	-	87.8 ± 4.7	

All studies used chromic oxide as inert marker.
[a] Plant = Soy paste, textured wheat, sorghum meal; Animal = 58% and 67% CP sardine meal, squid meal, red crab meal
[b] Plant = Soybean meal, canola meal, lupin meal, brewer's yeast; Animal = Fish meal, meat and bone meal, poultry meal,

2.2. Materials and methods

2.2.1. *Animals and experiment design*

Adult redclaw were sourced from a commercial redclaw farm in the Atherton Tableland region in North Queensland. Current farming practices have remained fairly similar to when the industry began in the late 1980s. Redclaw sampled had been cultured in outdoor earthen ponds, where growth is largely attributed to the consumption of naturally occurring pond biota. Grow-out ponds contain artificial shelters often provided in the form of stacked PVC pipes, synthetic mesh and old car tyres, with ponds often prepared with inorganic fertilizers and forage material such as hay to promote a plankton bloom (Jones 1995). Experiments were conducted at the Marine and Aquaculture Research Facility (MARF) at James Cook University (JCU), Townsville, Australia. Redclaw were weighed individually (40.1 ± 6.35 g) prior to the commencement of the experiment and randomly assigned to treatments. Six crayfish were assigned per replicate (n=6) with three replicates per diet treatment (diet n total = 18). To prevent cannibalistic events occurring after moulting, 60L tanks (597 x 362 x 266 mm) were partitioned into six compartments with PVC dividers. Tanks were integrated within a RAS (1000L sump, UV clarifier (18W) and two bag filters (45 µm)) in which optimal water quality was maintained for the duration of the experiment. The U.V treated freshwater was supplied to each compartment, within each tank at a rate of 0.8L/min. Water temperature was maintained at 27.0 ± 1°C, pH between 7 - 8.5 (Jones & Ruscoe, 1996). All tanks were individually aerated to maintain dissolved oxygen concentrations above 8 mg/l and compartments were regularly tested to ensure adequate oxygenation and water quality (YSI, Ohio, USA, API Freshwater Master Test Kit, Mars Fishcare Inc.). Photo-period was set on a 12h day/night cycle with start of the light cycle at 0700 h each day. Crayfish were acclimated to the system and treatment diets for 14 days prior to faecal collection occurring.

2.2.2. *Ingredients and diet preparation*

The reference diet substitution procedure (Glencross et al., 2007) was used to determine the apparent digestibility of seven feed ingredients: yellow pea meal (YPM), dried distillers grains (DDG), lupin meal (LM), soybean meal (solvent extracted) (SBM), sorghum meal (SM), poultry by-product meal (PBP) and feather meal (FTM). The reference diet (RF) was a commercial diet for *P. monodon* (Ridley Aquafeed, Brisbane). Experimental diets were formulated by combining the test ingredient and the RF at a 30:70% ratio, on a dry weight basis. Yttrium oxide (Sigma Aldrich) was used as the inert digestibility marker (Irvin & Williams, 2007), included in all diets at 1g kg^{-1}. Individual ingredient proximate composition

is presented in Table 2.2 and proximate composition of test diets in Table 2.3. Dry ingredients were milled and sieved to <630 ɥm using a commercial spice grinder (Model TS-08SF, Federal Hospitality Equipment Pty Ltd, Australia). Dry ingredients were thoroughly mixed using a Hobart A200N mixer (Hobart Manufactures, Brition, UK) for 45 minutes prior to the addition of sufficient water to form a dough. The dough was screw-pressed through a 2 mm die plate using a pasta maker (La Monferrina, Italy), with a blade attachment to cut into 20 mm. Pellets were oven dried at 60^{o}C for 24 hrs to a moisture content of <10% and then stored at -20 ^{o}C until required. Eight diets were randomly assigned to 24 tanks, with each diet fed to three tanks containing six individuals. Redclaw were fed once daily to satiation with uneaten feed removed after 30 minutes. Average pellet weights (0.02 g) (n = 200) for each diet had been pre-determined and feed intake data adjusted accordingly.

2.2.3. *Faecal collection*

Collection of faecal samples began 4 hrs after feeding as it was established during the acclimation phase that little faeces were produced prior. Faecal matter was collected via siphoning following modified protocols of Jones & De Silva (1997) and Campana-Torres et al. (2006). The faecal collection vessel was half filled with freshwater, with a mesh screen immersed in the water to allow intact faeces to settle allowing other materials and faces indiscernible from feed waste to separate out. The intact faecal strands were then carefully pipetted off the mesh screen with great care taken to prevent breakage, avoiding potential nutrient losses and to ensure the integrity of the faecal strands. During periods in which moulting occurred faeces were not collected from that or surrounding crayfish for 24hrs. Faecal collection continued for 125 days until sufficient faecal material was collected for chemical analyses, with samples pooled within each replicate. Faecal samples were kept at -20 ^{o}C prior to being frozen at -80 ^{o}C in preparation for freeze drying.

On conclusion of the feeding trial three redclaw were randomly sampled from each diet treatment for histology purposes and this data is presented in Chapter 3.

Table 2.2 Nutritional composition and amino acid profile of test ingredients (DM basis)

	YPM[1]	FTM[2]	DDG[3]	LM[4]	PBPl[2]	SBM[5]	SM[1]
Dry Matter (%)	87.88	92.89	87.39	92.27	92.03	90.47	88.33
Protein (%)	27.72	90.76	25.38	48.54	70.55	50.75	12.08
Crude lipid (%)	2.49	6.77	6.50	8.48	14.34	2.00	4.22
Ash (%)	2.96	2.47	5.36	3.50	15.11	9.30	1.86
Carbohydrates* (%)	66.82	0	62.76	39.47	0	37.95	81.83
Energy (kJ g-1)	19.01	24.14	19.34	21.61	22.36	19.3	18.57
Amino Acids (%)							
Alanine	1.08	3.93	1.17	1.57	4.21	2.19	1.12
Arginine	2.56	5.97	1.43	5.52	5.02	3.64	0.60
Aspartic Acid	2.70	5.58	1.53	4.43	5.18	5.52	0.86
Cystine/Cystine	0.39	4.31	0.58	0.59	1.16	0.83	0.27
Glutamic Acid	4.31	9.20	3.97	9.65	8.18	8.83	2.52
Glycine	1.04	6.75	1.09	1.84	6.15	2.04	0.42
Histidine	0.56	0.71	0.49	1.27	1.34	1.26	0.27
Isoleucine	1.06	4.26	0.87	1.87	2.51	2.22	0.61
Leucine	1.87	7.01	1.71	3.18	4.62	3.88	1.66
Lysine	1.49	1.83	0.51	1.74	3.80	2.67	0.29
Methionine	0.23	0.57	0.34	0.30	1.23	0.66	0.22
Phenylalanine	1.14	4.10	0.97	1.76	2.83	2.48	0.74
Proline	1.29	8.77	1.91	2.59	5.12	3.51	1.35
Serine	1.30	10.87	1.16	2.40	3.52	2.82	0.68
Taurine	0.01	0.04	0.01	0.01	0.26	0.01	0.01
Threonine	0.99	4.27	0.87	1.68	2.75	2.09	0.45
Tyrosine	0.91	2.58	0.79	1.80	1.94	1.89	0.58
Valine	1.10	5.75	1.09	1.71	3.00	2.32	0.67

[1]Broken River Ingredients, Benella, Australia [2]Ridley Agriproducts Pty Ltd, Narangba, Australia, [3]Manildra Group, Gladesville, Australia, [4]Corrow Seeds, Corrow, Australia, [5]Riverina Australia Pty Ltd. West End, Australia

*Calculated as 100 – (protein+lipid+ash)

2.2.4. **Analytical methods and calculations**

2.2.4.1. Ingredients and Diets

Moisture content was determined gravimetrically by oven-drying to a constant weight at 105°C. Dried samples were milled to a fine powder consistency and were analysed for crude protein, crude fat and nitrogen-free extract using standard laboratory methods (AOAC no. 950.46B, JAOAC 33, 749 (1950), JAOAC 36, 279 (1953) respectively) (Deakin University, Geelong, VIC). Gross energy was calculated using the conversion factors for protein 23.65; lipid 39.55; and carbohydrate 17.16 kj/g (NRC, 2011). Amino acid content was determined by HPLC method (Chemcentre, Bentley, WA).

2.2.4.2. Faecal samples

Faecal samples were freeze-dried to -80^{o}C prior to being ground. Where enough material was collected for analysis of dry matter, protein and amino acid profile were conducted using standard laboratory methods (AOAC no. 950.46B, JAOAC 33, 749 (1950), JAOAC 36, 279 (1953); Conlan et. al, 2014). Crude protein was calculated as N x 6.25. Gross energy was calculated using the following conversion ratios protein 23.65; lipid 39.55; and carbohydrates 17.16 kj/g (NRC). Amino acid content was determined HPLC method with yttrium oxide content determined by Inductively Coupled Plasma (ICP) Spectroscopy. All chemical analysis (from each tank) was done in triplicate where enough material was available (Chemcentre, Bentley, WA). There was only enough matter to determined dry matter of faecal material from three replicates and these values were used as the average dry matter for all faecal material.

2.2.5. *Apparent digestibility and performance parameters*

The ADCs for the nutrients and energy of the test and reference diets were calculated using the following formula (Cho et al., 1982).

$$\text{ADC}\ (\%) = 100 \times [1 - \left(\frac{\text{F}}{\text{D}} \times \frac{D_{Y_2O_3}}{F_{Y_2O_3}}\right)]$$

Where F = % nutrient in faeces; D = % nutrient in diet; $D_{Y_2O_3}$ = % yttrium oxide in diet; and $F_{Y_2O_3}$ = % yttrium oxide in faeces

$$\text{ADC}_{\text{ING}}\ (\%) = [\text{Nutr}_{\text{TD}} \times \text{AD}_{\text{TD}}) - (\text{PRD} \times \text{Nutrient}_{\text{RD}} \times \text{AD}_{\text{RD}})]/[(\text{P}_{\text{ING}} \times \text{Nutr}_{\text{ING}})]$$

Where ADC_{ING} = apparent digestibility of nutrient or gross energy in the test ingredient; $Nutr_{TD}$ = the nutrient or gross energy concentration in the test diet; AD_{TD} = the apparent digestibility of the nutrient or gross energy in the test diet; PRD = proportional amount of reference diet; $Nutr_{RD}$ = the nutrient or gross energy concentration in the reference diet; AD_{RD} = the apparent digestibility of nutrient or gross energy in the reference diet; P_{ING} = proportional amount of test ingredient; and $Nutr_{ING}$ = the nutrient or gross energy concentration in the test ingredient. Digestibility values over 100% were not adjusted and are reported as determined.

While the assessment of performance of redclaw fed the various diets was not the main objective of the study, the following indices were nonetheless considered:

Weight gain (g)	$W_f - Wi$
Percent Weight Gain (WG, %)	$100 \times (W_f - W_i) / Wi$
Specific growth rate (SGR, % day)	$[(\ln W_f - \ln W_i) / t] \times 100$
Feed Conversion Ratio (FCR)	Total feed intake / total weight gain
Survival	(final no. crayfish/initial no. crayfish) x 100

Where W_f is the final body weight (g), W_i is the initial body weight (g), t is duration

2.2.6. **Statistical Analysis**

All data are presented as mean ± SE. Limited faecal material resulted in lower replicates for the YPM (n = 1) and SM (n = 2) diets, except for DM in which n = 3 for all diets.

Data was analysed for homogeneity of variance by Levene's test prior to being analysed with a one-way analysis of variance (ANOVA). Data that was not normally distributed and could not be transformed was analysed with a non-parametric test (Kruskil-Wallis). This occurred for the growth and feed intake data, and individual AA data at both diet and ingredient level. Levels of significance were determined using Tukey's highly significant difference (HSD) test. Limits for significance set at $P < 0.05$. Linear regression was used to determine the correlation between CP and DP content. Analysis was conducted in SPSS (v25*)* and GraphPad Prism (8.1.0).

Table 2.3. Proximate composition (% dry matter), essential amino acid content (% diet) of the reference and test diets) (70:30 ratio of reference diet to test ingredients)

	YPM	FTM	DDG	LM	PBP	SBM	SM	RF
Dry Matter (%)	96.82	97.32	99.70	97.34	97.78	97.59	96.90	97.04
Protein (%)	42.80	63.53	41.35	49.12	54.73	50.29	37.81	49.58
Crude lipid (%)	5.97	6.63	7.63	8.77	9.82	6.66	7.03	8.60
Ash (%)	7.63	7.84	8.59	7.82	11.40	9.10	7.30	9.95
Carbohydrates (%)	43.60	22.00	42.43	34.28	24.05	33.95	47.86	31.88
Energy (kJ g^{-1})	19.97	21.42	20.08	20.97	20.95	20.35	19.94	20.59
Amino Acids (%)								
Alanine	1.95	2.65	1.99	2.22	3.00	2.39	2.02	2.42
Arginine	2.75	3.57	2.49	3.82	3.71	3.22	2.25	2.99
Aspartic Acid	3.59	4.20	3.28	4.35	4.51	4.67	3.14	4.12
Cystine	0.56	1.70	0.62	0.67	0.84	0.69	0.55	0.68
Glutamic Acid	6.97	7.93	6.88	8.91	8.46	8.70	6.66	8.42
Glycine	1.87	3.30	1.89	2.23	3.47	2.27	1.71	2.31
Histidine	0.91	0.82	0.89	1.13	1.20	1.18	0.87	1.07
Isoleucine	1.79	2.56	1.69	2.02	2.30	2.24	1.67	2.14
Leucine	2.96	4.19	2.91	3.49	3.92	3.71	2.99	3.56
Lysine	2.01	2.18	2.01	2.55	2.91	2.91	1.80	2.72
Methionine	0.93	0.97	0.86	0.87	1.19	0.96	0.87	1.10
Phenylalanine	1.80	2.50	1.71	2.04	2.37	2.22	1.70	2.09
Proline	2.78	5.35	2.88	3.09	3.97	3.36	2.86	3.26
Serine	1.87	4.35	1.86	2.36	2.62	2.42	1.75	2.23
Taurine	0.14	0.15	0.15	0.16	0.25	0.16	0.15	0.23
Threonine	1.61	2.44	1.60	1.94	2.22	2.03	1.50	1.96
Tyrosine	1.45	1.82	1.39	1.76	1.80	1.76	1.37	1.72
Valine	1.82	3.04	1.74	1.96	2.54	2.31	1.70	2.11

2.3. Results

2.3.1. *Dietary ADCs*

Dietary ADCs for dry matter, protein and amino acids are presented in Table 2.4. DM among the diets were mostly similar (86.54% to 94.08%) but there were significant differences ($p<0.05$) between YPM (94.98%) and all but the basal and LM diets, and further between PBP (86.54%) and SM (91.38%). Protein digestibility were also similar (87.89% to 93.22%) but the ADCs for LM (93.22%) were significantly different ($p<0.05$) than FTM (89.13%), DDG (89.58%) and the basal diet (88.60%). Dietary ADCs for amino acids was good and ranged from 83.57% to 97.08%, with glutamic acid and taurine digestibility quite high across all diets 91.44% to 96.18%, and 96.38% to 97.08% respectively. Protein digestibility was independent of CP content (Figure 2.1)

2.3.2. *Ingredient ADCs*

Ingredient ADCs for protein, DM and AAs are presented in Table 2.5. DM ADCs for LM and YPM had the same value of 94.98%. ADCs values for ingredient DM were significantly different ($p<0.05$) between LM (94.98%) and PPB (81.50%), and PBP (81.50%) and SM (97.37%) diets. There were no differences for protein digestibility although values exceed 100% for LM, YPM and SM (although the latter two were not included in statistical analysis as not enough dry matter was available for replicates).

AA ADC values ranged 81.5 – 130.2% with the exception of glutamic acid (65.17%) and threonine (69.38%) in the PBP diet. ADCs of AAs for the LM diet exceed 100% for all but taurine. Values over 100% were also calculated for a number of amino acids for the SM and YPM diets.

2.3.3. *Performance parameters*

Performance parameters of redclaw fed the different dietary protein sources and basal diet are presented in Table 2.6. Statistical analysis showed there were no significant differences between redclaw fed the different diets. However, individuals fed the FTM diet had weight gain of 25.60% compared to the other diets which ranged from 8.29 - 19.32%. Survival for redclaw fed PBP diet was 88.89% with survival of the redclaw in the remaining diets ranging 77.78 – 83.33%. Individuals fed DDG diet had a growth rate of 8.29% and an FCR of 3.32.

Table 2.4 Diet ADC's for dry matter, protein and amino acids of redclaw fed a range of raw ingredients. Pea meal (n=1) and Sorghum (n=2) were removed from data analysis. Different letters in columns show statistically significant differences (P < 0.05).

	YPM	FM	DDG	LM	PBP	SBM	SM	RF	SEM
Dry Matter*	94.98^{c}	88.33^{ab}	89.06^{ab}	90.70^{ab}	86.54^{b}	89.80^{ab}	91.38^{ab}	88.92^{a}	0.75
Protein	91.49	89.13^{a}	89.58^{a}	93.22^{b}	87.89^{a}	90.57^{ab}	89.97^{ab}	88.60^{a}	0.68
Alanine	88.98	87.48^{ab}	87.71^{ab}	91.67^{a}	87.77^{b}	88.92	87.59^{ab}	87.86	0.83
Arginine	91.78	88.97^{b}	90.34^{b}	94.73^{a}	89.85^{b}	91.86^{ab}	91.71	90.39^{b}	0.64
Aspartic acid	90.49	87.55^{a}	88.18^{a}	93.13^{a}	87.25$^{\mathbf{b}}$	90.49^{a}	90.00	88.27^{ab}	0.73
Cysteine	88.54	85.73^{a}	89.90^{ab}	92.59$^{\mathbf{b}}$	83.57^{a}	88.18^{ab}	90.91	89.27^{ab}	1.10
Glutamic acid	94.30	91.44^{a}	93.51^{ab}	96.18^{b}	91.73^{a}	94.14^{ab}	92.69	93.47^{ab}	0.51
Glycine	90.08	88.34^{a}	89.24^{ab}	93.09^{b}	89.55^{ab}	89.87^{ab}	90.66	89.14^{ab}	0.79
Histidine	92.85	89.00^{a}	90.88^{ab}	94.16^{b}	89.05^{a}	91.76^{ab}	91.89	90.28^{ab}	0.80
Isoleucine	88.55	86.76	87.06	91.76	86.03	88.70	88.65	86.86	0.84
Leucine	89.58	87.10^{ab}	88.41^{b}	92.69^{b}	86.52^{a}	89.48^{ab}	87.91	88.04^{ab}	0.83
Lysine	91.85	89.80^{a}	90.93^{a}	94.09^{b}	89.67^{a}	91.97^{ab}	92.35	90.68^{a}	0.60
Methionine	90.60	89.10	89.60	91.38	88.39	88.38	91.10	88.38	0.88
Phenylalanine	89.21	87.23^{ab}	88.04^{ab}	92.40^{b}	86.39^{a}	89.18^{ab}	88.58	87.28^{ab}	0.81
Proline	92.48	88.41^{ab}	91.76^{ab}	94.28^{a}	88.84^{b}	91.93^{ab}	90.66	91.03^{ab}	0.85
Serine	90.42	87.45^{a}	89.39^{ab}	93.67^{b}	86.89^{a}	90.58^{ab}	90.37	89.35^{ab}	0.89
Taurine	97.05	96.38	96.48	96.67	96.78	97.00	97.08	96.96	0.25
Threonine	89.22	86.74^{a}	87.76^{a}	92.27^{b}	86.45^{a}	88.84^{ab}	89.43	87.53^{a}	0.85
Tyrosine	89.61	87.46^{a}	88.11^{a}	92.83^{b}	86.25^{a}	89.34^{ab}	89.00	88.04^{a}	0.79
Valine	89.10	86.91	87.17	91.49	86.53	89.13	89.28	87.16	0.87

Table 2.5 Ingredient ADCs for and dry matter, protein and amino acids for redclaw fed a range of raw ingredients. Pea meal (n=1) and Sorghum (n=2) were removed from data analysis. Different letters in columns show statistically significant differences (P < 0.05).

	YPM	FTM	DDG	LM	PBP	SBM	SM	SEM
Dry Matter	94.98^{ab}	86.97^{ab}	89.39^{ab}	94.98^{b}	81.50^{a}	91.89^{ab}	97.37^{b}	2.06
Protein	103.70	91.23	94.10	104.37	86.87	95.06	103.44	3.75
Alanine	94.99	86.92^{a}	87.25^{a}	105.80^{b}	87.77^{a}	91.71^{ab}	86.15	6.87
Arginine	95.69	87.23^{a}	90.18^{a}	100.39^{b}	89.04^{a}	94.76^{ab}	107.86	7.40
Aspartic acid	98.63	86.24	87.90	104.00	85.46	94.45	110.26	7.96
Cysteine	85.44	84.36^{a}	91.56^{ab}	101.88^{b}	81.99^{a}	86.03^{a}	100.98	7.56
Glutamic acid	98.20	86.91^{ab}	93.64^{ab}	101.87^{b}	65.17^{a}	95.66^{ab}	86.31	6.82
Glycine	95.16	87.67^{a}	89.53^{a}	105.02^{b}	89.80^{a}	91.85^{a}	111.14	8.03
Histidine	104.75	84.29^{a}	92.62^{ab}	102.07^{b}	88.60^{ab}	94.77^{ab}	107.45	8.29
Isoleucine	96.83	86.64^{a}	87.69^{a}	105.25^{b}	83.22^{a}	92.96^{ab}	104.01	7.98
Leucine	96.65	85.94^{ab}	89.50^{ab}	105.23^{a}	82.01^{b}	92.64^{ab}	87.26	6.90
Lysine	97.02	86.60^{a}	91.73^{a}	106.88^{b}	88.41^{a}	95.11^{ab}	130.22	9.36
Methionine	116.49	92.50^{ab}	93.36^{ab}	117.65^{b}	88.40^{a}	88.35^{a}	125.15	11.24
Phenylalanine	97.76	87.18^{ab}	90.27^{ab}	107.08^{a}	83.17^{b}	93.01^{ab}	97.64	7.47
Proline	101.34	86.03^{a}	93.76^{ab}	104.09^{b}	83.52^{a}	93.92^{ab}	88.46	6.96
Serine	94.82	86.50^{a}	89.50^{a}	103.35^{b}	84.64^{a}	92.89^{a}	98.49	7.16
Taurine	101.65	88.99	94.73	82.25	96.75	99.30	102.91	10.77
Threonine	97.29	85.86^{ab}	88.43^{ab}	105.58^{a}	69.38^{b}	91.79^{ab}	109.50	9.24
Tyrosine	96.78	86.51^{a}	88.33^{a}	103.88^{b}	83.88^{a}	92.17^{ab}	96.02	7.29
Valine	98.06	86.68	87.22	104.39	85.08	93.43	105.70	7.95

Table 2.6 Performance parameters of redclaw fed plant and animal-based protein sources. +/- SE. No significant differences were found among any of the diet treatments for each of the performance indices tested (p>0.05).

	Initial BW (g crayfish^{-1})	**Final BW (g crayfish^{-1})**	**Weight gain (%)**	**Growth (g crayfish^{-1})**	**Feed intake (g crayfish^{-1})**	**FCR**	**Survival (%)**
YPM	40.05 ± 1.52	47.82 ± 2.22	19.32 ± 1.09	7.77 ± 0.72	9.95 ± 0.23	1.30 ± 0.17	77.78 ± 11.1
FM	39.72 ± 1.44	50.1 ± 5.56	25.60 ± 9.42	10.44 ± 4.12	11.97 ± 1.70	1.57 ± 0.81	83.33 ± 9.6
DDG	42.21 ± 0.91	45.70 ± 0.85	8.29 ± 1.04	3.49 ± 0.41	11.11 ± 0.79	3.32 ± 0.77	72.22 ± 5.6
LM	38.71 ± 1.11	44.31 ± 2.07	14.37 ± 2.05	5.61 ± 0.96	8.72 ± 0.85	1.61 ± 0.26	77.78 ± 5.6
PBP	39.33 ± 0.48	44.41 ± 1.59	12.91 ± 3.74	5.08 ± 1.49	9.80 ± 0.91	2.48 ± 1.38	88.89 ± 11.1
SBM	40.44 ± 1.46	47.26 ± 1.96	16.82 ± 1.42	6.81 ± 0.67	10.66 ± 1.64	1.64 ± 0.52	77.78 ± 14.7
SM	39.19 ± 1.09	46.29 ± 0.76	18.35 ± 4.44	7.10 ± 1.60	9.49 ± 1.77	1.50 ± 0.59	77.8 ± 5.6
RF	41.38 ± 2.68	47.38 ± 3.13	14.56 ± 2.97	5.99 ± 1.20	11.74 ± 2.22	2.10 ± 0.56	77.8 ± 14.7
P Value	0.05	0.05	0.05	0.05	0.05	0.05	0.05

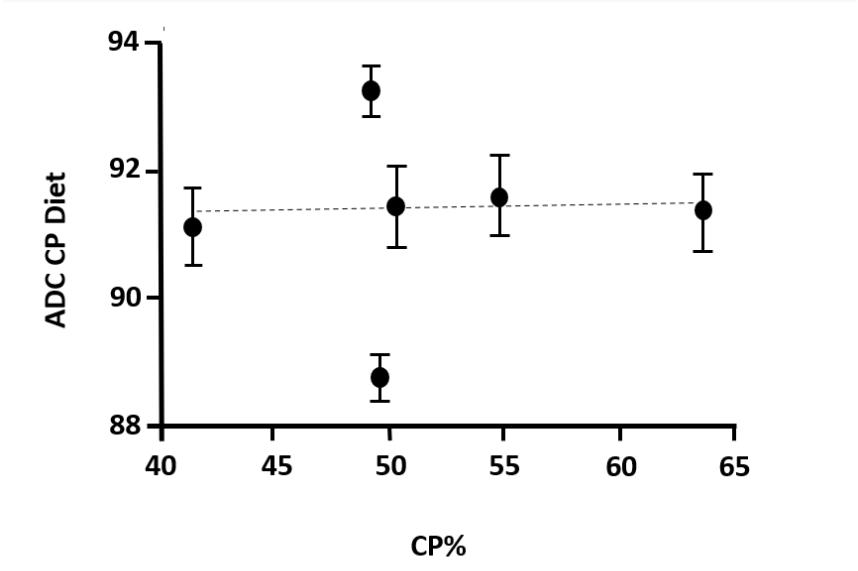


Figure 2.1 Relationship between the apparent digestible protein and the crude protein content of a range of ingredients fed to redclaw

2.4. Discussion

The results in the present study show that the ADCs for both plant-based and animal ingredients were quite reasonable. The ADCs for DM and protein at both the diet and ingredient level for LM, PBP and SBM diets are similar to those reported by Pavasovic et al. (2007), and results for SM similar to those reported by Campaña-Torres et al., (2005). AA digestibility is more representative of the protein digestibility overall and this study reports the digestibility coefficients of AAs for the first time. In general, the AA digestibility of both plant and animal test ingredients were very good, with SBM in particular consistently above 90% (Table 2.5). Results from the present study show redclaw have the capacity to digest a wide range of protein sources, which provides greater flexibility in formulating a feed using both plant-based and animal ingredients.

ADC values in the present study did exceed 100% for a number of ingredients, particularly AA. Positive values are potentially a result of endogenous losses such as sloughing of gut epithelial cells, enzymatic secretions, interactions among ingredients of the diet, or the nutritive value of the ingredient e.g. low content of specific amino acids in an ingredient leading to a higher uptake from the basal diet, resulting in ADC values above 100% in the ingredient (Campos et al., 2018). Such observations for digestibility parameters have been reported previously (Ngo et al., 2015) and is a common occurrence in digestibility studies (Booth & Pirozzi, 2018). Glencross et al. (2007) recommended including such erroneous ADC values but rounding to 100% for use in any feed formulation. The 30% inclusion rate for ingredients is common in digestibility studies but is quite extreme for a commercial diet. However, the high inclusion is useful to examine the effect of raw ingredients. The ADCs indicate that for redclaw the high inclusion rates do not appear to negatively influence the digestibility of ingredients.

While ADCs values were generally good, ingredient source and processing are factors known to influence the digestibility of ingredients. Germination of cow pea and mung bean increased protein content by 18.3% and 15.6% respectively, with digestibility for DM and protein higher in *P. monodon* feed the legumes which were progressed by germination in combination with autoclaving (Vasagam et al., 2007). Similarly, the nutritional composition of canola meals are affected by processing methods. Expeller extracted canola meal had higher lipid levels and reduced protein content compared to solvent-extracted canola meal. Protein digestibility for barramundi (*Lates calcarifer*) was subsequently affected with lower values for expeller extracted canola meal (63.1%) compared to solvent-extracted meal (74.5 - 84.1%) (Ngo et al.,

2015). Canola meals in Ngo et al. (2015) were sourced from four different processing plants with variations between solvent-extracted meals thought to be related to growing conditions or the crushing methods employed by the processing plants. While the SBM in the current study was solvent extracted, the only additional processing that the raw ingredients underwent was milling to <630 ɥm. It is possible that additional processing could alter the digestibility of some ingredients for redclaw.

Processing of ingredients can also mitigate the effects of ANFs that are found in plant-based ingredients. ANFs include compounds which reduce nutrient utilisation of plant products such as saponins, which may interfere with lipid and protein digestion, and protease inhibitors which inhibit the activity of digestive enzymes (Krogdahl et al., 2010). Methods such as extrusion of feeds can inactivate or destroy heat sensitive ANFs (Nunes et al., 2014). Higher digestibility coefficients for crude protein and dry matter were obtained in *P. monodon* fed germinated and autoclaved grain legumes (Vasagam et al., 2007). Autoclaving raw and germinated grain legumes resulted in a reduction of tannins, phytic acid and trypsin inhibitors, with the simple act of dehulling raw legumes significantly reducing the level of tannins (Vasagam et al., 2007). Similarly, phytic acid and trypsin inhibitors were reduced in fermented soybean meal, with authors reporting the treated soybean meal could replace up to 28% of FM in diets of Indian prawn (*Penaeus indicus*) (Sharawy et al., 2016). Caution is required when incorporating plant-proteins due to the presence of ANFs however, redclaw are known to consume a variety of plant and animal ingredients, with adults showing a clear preference for plant ingredients. When given the choice redclaw (~25 g) chose corn, pea and soybean over seafood products such as prawn and squid **(**Hiep Le & Pirozzi, RIRDC Project No PRJ-008536**,** *unpublished data, 2013*). Redclaw have the necessary digestive enzymes to utilise cellulose as a nutrient source (Xue et al., 1999), with cellulase present in much higher levels in redclaw than the carnivorous mud crab (Truong et al., 2009). It is unclear as to what extent ANFs would affect redclaw given their natural predisposition to plant ingredients, but additional processing of ingredients is worth considering in the future.

Core to measurements of digestibility is the inclusion of an inert marker in the feed. Markers need to not alter the passage of nutrients through the gastrointestinal tract, be indigestible, not affect the metabolism of the animals, and be easily and accurately analysed at low concentrations (Austreng et al., 2000). Historically, chromic oxide (Cr_2O_3) has been used but its use has been called into question. A reduction in ingredient digestibility was shown to be related to the level of Cr_2O_3 in tilapia feeds (Shiau & Liang, 1995), while partitioning and

preferential elimination of Cr_2O_3 was reported for *C. destructor* (Jones & De Silva, 1997). As such yttrium oxide (Y_2O_3) was used as the inert marker in present study as it is a suitable alternative that is non-toxic, does not influence absorption of metabolism of ingredient and is commonly used in other digestibility studies (Austreng et al., 2000; Hansen et al., 2007; Wan et al., 2017).

In addition to the inert marker, leaching of nutrients from submerged feed or faeces can influence results with estimated values being higher than actual values. Leaching rates were not determined in the present study, however this not unusual with only four of fifteen studies, which assessed digestibility of ingredients for crustaceans, calculating leaching rates (Chapter 1; Table 1.2). Moreover, the feeding and faecal collection protocol in the present study were implemented to mitigate leaching effects with feed removed after 30 minutes. Like other crustaceans, the faecal material from redclaw is encased in a peritrophic membrane, a thin film that coats the faecal material, reducing nutrient leaching and uptake of moisture (Luo et al., 2008). Only intact faecal strands were collected in the present study as the modified faecal collection protocol was designed to allow intact faeces to settle while separating out other material and damaged faeces. This method required that the feeding trial continued for 125 days until enough faecal material was collected to undertake protein and amino acid analyses. As the volume of faecal material produced was low, this precluded further proximate analyses of fat, ash and gross energy.

Crustaceans are chemosensory feeders with feeding behaviour stimulated by small, water soluble, nitrogen-bearing compounds released from potential food items which include free amino acids (taurine, glycine, arginine, glutamic acid and alanine), peptides and organic acids (Smith et al., 2007; Tantikitti, 2014). Suresh et al. (2011) reported that petfood grade PBP was only slightly inferior to fishmeal based on its chemical composition, attractability and palatability assessments for Blue Shrimp (*Litopenaeus stylirostris*). The feeding preferences of Red Swamp Crayfish (*Procambarius clarkia)* on macrophyte assemblages were altered when difficulties imposed by plant structure were removed, with plants with higher protein becoming more favourable (Cronin et al., 2002). Feed intake data in the present study did not indicate there were any such concerns with the diets used, but palatability of ingredients is important and needs to be considered when formulating a feed for redclaw.

While the performance parameters (Table 9) are not the target values from digestibility studies, they can inform on the usefulness of ingredients. Both PBP and DDG were highly digestible

however FCRs were greater than the other diets. Redclaw fed DDG had the lowest weight gain of all treatments but one of the highest feed intakes. Survival in the present study ranged 72.2% - 88.9% which is lower than anticipated given individuals were cultured in compartments. However, survival ranged 40.2% - 79.2% in Barki et al., (2006) due to individuals escaping, or movement of individuals into compartments resulting in mortality through fighting. Mortalities are not unexpected, particularly with cannibalistic or opportunistic-cannibalistic species such as redclaw as shown in Barki et al., (2006). The compartment style system used in the present study was implemented to limit competition over feed, reduce antagonistic feeding interactions that might result in harm, reduce reproduction, and mitigate instances of cannibalism, particularly during moulting. Mortalities did occur in the present study, but there was no evidence of individuals causing mortalities in neighbours.

It is uncommon for nutrition studies to consider broader health implications, particularly so for studies on redclaw. Bacterial and viral diseases exist in the current population of farmed redclaw in north Queensland and have been present for some time. On-farm surveys in the late 90's reported bacterial infections were quite common (Edgerton & Owens, 1999). Recently two separate RNA virus were identified in redclaw farmed in North Queensland: Chequa iflavirus, associated with increased mortalities after stress events (Sakuna et al., 2017) and a second, yet unassigned negative-sense ssRNA virus (Sakuna et al., 2018). Both have no obvious histological pathology unlike *C. quadricarinatus* bacilliform virus (CqBV) which is a common infection found in farmed redclaw (Saoud et al., 2013). Redclaw used in the present study were from a commercial farm in North Queensland and the possibility of individuals being infected is explored more thoroughly in Chapter 3.

Conclusion

Redclaw are clearly able to consume a wide variety of plant and animal ingredients based on the ADCs results for DM, protein, and AAs. The results from this study will better inform ingredient used in formulated feed for redclaw particularly plant based. Importantly, diets will be formulated based on digestibility coefficients ensuring optimal availability of nutrients that can be absorbed and utilised. Palatability of ingredients, processing methods and ingredient origin are all aspects that need to be considered when formulating a feed and ideally these factors would be determined to provide a comprehensive database on ingredients used. The role of ANFs and the relationship between nutrition and health needs to be explored more thoroughly for redclaw. Providing a diet that negatively impacts the health of farmed stock is

not ideal and the unexplained mortalities in the present study indicates this potential. An understanding of diet effects on the Hep and health status of redclaw is therefore explored more thoroughly in Chapter 3.

3. CHAPTER 3: HISTOPATHOLOGICAL SURVEY OF REDCLAW FED A VARIETY OF DIETARY PROTEIN SOURCES

3.1. Introduction

A well-established and effective method to investigate the health and condition of crustaceans is through histopathology of the Hep. As the primary site for digestion and nutrient absorption, the Hep is comprised of numerous branching tubules, and the epithelium contains four cell types (E-, F-, B-, and R-) (Al-Mohanna & Nott 1986, Al-Mohanna & Nott 1987, Anger et al., 2985). The appearance of certain structures and cells can provide detailed insights into responses to experimental treatments that might not be obvious from chemical analysis or performance based data e.g. growth, survival, FCR etc. For example, *L. vannamei* exposed to different salinities demonstrated an increase in abundance of B-cells at a higher salinity, with the resulting structural and cellular changes providing evidence of physiological adaptations to salinity changes (Li et al., 2008). Also, severe structural changes and cellular death observed in Argentine Red Shrimp (*Pleoticus muelleri*) fed Vitamin E deficient diets, indicating that Vitamin E is necessary to maintain normal hepatopancreatic structure (Fernandez Gimenez et al., 2004).

Results from the digestibility study (Chapter 2) indicated that plant and animal by-product ingredients are well accepted by redclaw. This is beneficial in reducing reliance on FM in formulated diets. Plant-based protein sources such as those from the legume family, and those often used as supplemental feed sources in redclaw pond culture, including peas and lupin, have been assessed as alternatives to FM (Hardy, 2010; Vasagam et al., 2007). Pea protein concentrate can replace 35% FM for the Signal Crayfish (*Pacifastacus leniusculus*) (Fuertes et al., 2013), while lupin kernel meal has been shown to be a suitable replacement of 40% for *P. monodon*) (Smith et al., 2007). PBP meal has been shown to be a suitable replacement *L. vannamei*) (Cruz-Suárez et al., 2007) and redclaw (Saoud et al., 2008). Indeed, the digestibility data from Chapter 2 showed PBP to be highly digestible (81.50% DM and 86.87% CP). However, the FCR for redclaw fed the PBP diet was quite high (2.48), and weight gain was the second lowest amongst the experimental diets. As discussed previously in Chapter 2, ingredient source, processing and ANFs can influence digestibility of feed ingredients and such responses are likely to be represented in the Hep. Indeed, cells and tissues of the Hep in *P. monodon* were demonstrated to be sensitive to different diets as evidenced by the loss of tubule and lumen structure, sloughing of cell lining and enlargement of B-cell vacuoles evident (Vagasam et al., 2007).

While histopathology can provide valid insights, the use of such methodology is limited in redclaw diet related studies. A search of the primary scientific literature using Scopus (https://www-scopus-com) on 20 February 2020 for relevant publications on redclaw nutrition was conducted and is available in Appendix A. Only 22 of 132 published studies considered histopathology. Of those studies only eight conducted histology of the Hep, with the remaining 14 conducting analysis using the Hep including: biochemical, digestive and enzymatic analysis, biochemical composition and RNA extraction. Further, of those eight that conducted histology, four used a tropical fish flake (Tetra®) as the primary dietary source for redclaw, and none of the 22 articles corresponded with studies on redclaw digestibility included in the review by Saoud et al. (2012). Histology is an effective way of investigating the potential effects of a diet/ingredient and is information that is not necessarily reflected in performance parameters, chemical analysis or digestibility coefficients (Masson et al., 2012; Ong & Johnston 2006).

The Hep plays a major role in digestion and nutrient absorption, and structural and cellular changes can be observed in relation to dietary inputs (Calvo et al., 2011, Xiao et al., 2014, Cervellione et al., 2016), The aim of this chapter was to conduct a histopathological survey on redclaw fed plant-based and rendered animal by-products, and examine the potential impact of feed ingredients on the health status of redclaw.

3.2. Materials and Methods

3.2.1. *Source Tissue*

Hepatopancreatic tissues for the histopathological survey were obtained from redclaw used in Chapter 2. A detailed description of the experimental design, diet formulation and crayfish husbandry is presented in that chapter. Briefly, diets were formulated on a 70/30 ratio in which 30% of a RF (commercial diet for *P. monodon)* was substituted with the test feed ingredient and included YPM, DDG, LM, SBM, SM, PBM and FM. Redclaw were from stock that had been held at the Marine and Aquaculture Research Facility, James Cook University, for ~3 months, and were originally sourced from a commercial farm on the Atherton Tablelands, Queensland.

3.2.2. *Histology Preparation*

Six redclaw were sampled as an initial reference (41.8 ± 4.0 g, n = 6). All diet treatments had n=6 redclaw sampled, except for YPM diet where n = 5. The average body weight of sampled redclaw at the conclusion of the digestibility trial (Chapter 2) was 45.04 ± 3.0 g. Redclaw were

anaesthetised in an ice water slurry and their spinal cord cut prior to being injected with 10% body weight of Davidson's fixative (Bell & Lightner, 1988). Redclaw were kept in Davidson's fixative for 24 hrs and then transferred to 70% ethanol. After the fixative process, a section of the mid-cephalothorax was sampled and processed for histology, with haematoxylin and eosin-stained sections prepared. Tissues were examined by light microscopy (Fernández Gimenez et al., 2004) and were also converted to digital SCN files (Queensland University of Technology, Brisbane) and examined digitally using slide imaging software (Aperio ImageScope, Leica Biosystems).

3.2.3. *Histopathological survey – method development: transect vs whole tissue*

Initially, one slide from each dietary treatment was compared against the initial reference slides to determine the best method for obtaining sufficient information to confirm health status of the redclaw based on hepatopancreatic structure and cell types. On examination under light microscopy, initial redclaw presented with moderate bacterial load. Further, both bacterial and viral infections were present in the three of the diet treatments, PM, FM and DDG. Eosinophilic intranuclear inclusions were observed and determined to be CqBV (Image 3.1) (Anderson & Prior, 1992; Edgerton & Owens, 1999), similarly, inclusions in hepatopancreocytes and inflammatory cells were observed in tubules which are characteristic of Cherax reovirus (CqReo) (Edgerton et al., 2000; Hayakijkosol & Owens, 2011).

To determine which method provided sufficient information on the health status of the redclaw, two methods were compared: i) viral infection along a transect; or ii) viral infection across all tissue. Using measurement tools in the slide imaging software Aperio ImageScope) (Leica Biosystems) the hepatopancreatic tissue was divided into four quadrants. A transect was determined by drawing a diagonal line through the quadrant. Each Hep was examined for CqBV due to its obvious pathology of displaced nucleoli and marginated chromatin. The number of tubules along this line/transect infected with CqBV were recorded, and repeated for each quadrant. For whole tissue the entire area of each quadrant was examined with the number of tubules with CqBV recorded. The two methods were applied to the three diet treatments that were initially examined, PM, FM and DDG. Two slides from each diet treatment were examined. Using these two methods it was determined that transects were not an accurate representation of the health status of redclaw. Insufficient information was obtained as CqBV was either not recorded, thus indicating no infection, or the level of infection not as severe as recorded when analysing whole tissue.

3.2.4. *Histopathological survey – method development: determining what cells and structural components to survey*

As the whole tissue was to be analysed, identifying the important cell types, structural differences and viral pathology present was required. There are numerous structural changes and observable pathology that can provide insights into the health of the redclaw. Granulomas are typically produced in response to an infection or inflammation and an overabundance can indicate an overwhelmed immune system. Structural disorganisation of tubules, enlarged tubular lumen and hypertrophy of B-cells were observed upon preliminary examination. Similarly, other abnormalities and lesions observed in the Hep included: unknown precipitation in lumen (thought to be protein precipitation), overabundance of lipid storage vacuoles, which could also cause structural disintegration (referred to as excess lipid storage (ELS) within this chapter; refer Image 3.2); inclusion of iron deposits (distinctive crystal-like structures in F-cells (Edgerton Romero & Jimenez, 2002)); and abundance of enlarged and hypertrophic B-cells. Degradation of tubule structure was observed and classified as: disorganisation of the tubule/lumen structure, shrinkage of lumen away from the basal lamina, sloughing of epithelial cells and increase in haemal sinuses between tubules. Images related to pathology above are presented in Appendix B.

3.2.5. *Histopathological survey - qualitative grading scheme*

Based on information available in the literature a breakdown and classification of particular characteristics were determined and a qualitative grading scheme was developed following that of Edgerton & Owens (1999) and Lightner (1996) and is presented in Appendix B. Levels defined were reflective of the pathology in question. For structural characteristics the grading scheme levels related to the impact on health e.g. in healthy individuals each tubule has a distinct star shape lumen when cross-sectioned surrounded by a thick epithelium with the impact on health low, whereas a lumen that is ovular with thin epithelium has negative effects on the functioning of the Hep and would be graded as high. An example of this is presented in Table 3.1 and 3.2. For infections and viruses known to exist in redclaw populations the grades reflected the severity of the pathology. The total number of tubules infected with lesions for each sample were also recorded, as were instances of co-infections. Any other pathology present outside the Hep i.e. in the reproductive organs, was also noted.

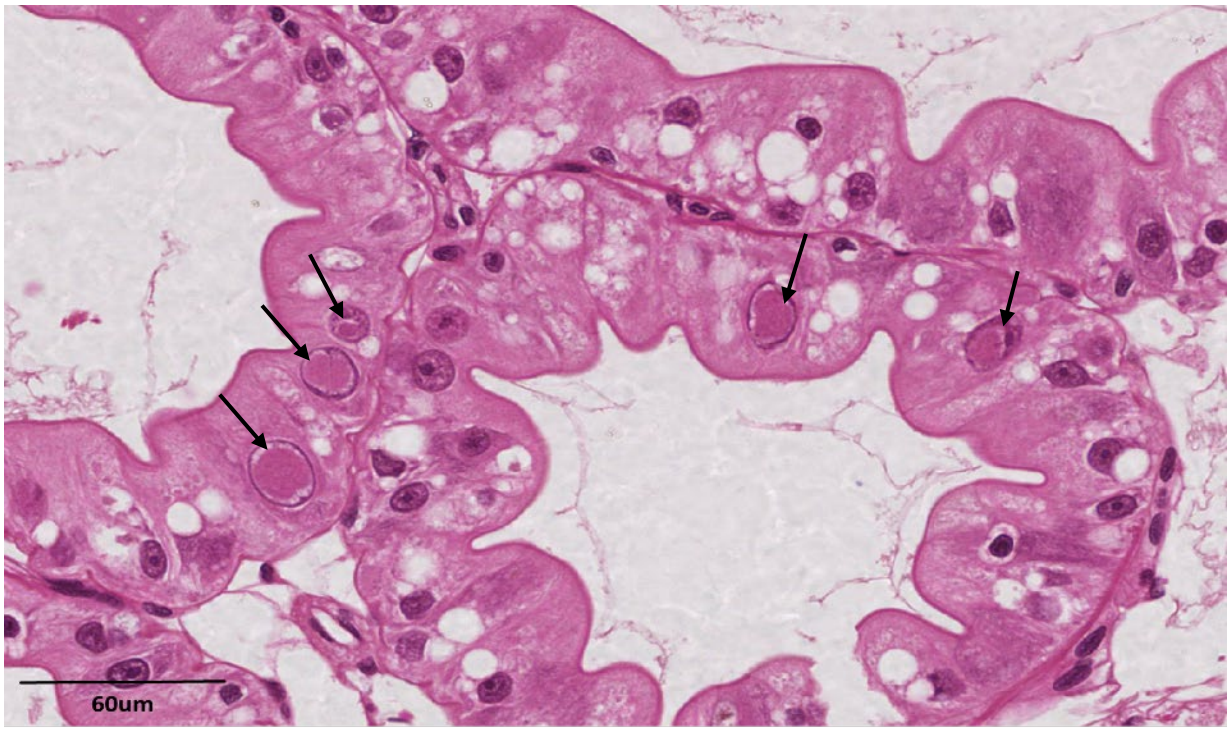


Image 3.1. Tubule infected with C. quadricarinatus bacilliform virus (CqBV) (arrows). Noteinfection occurring in adjacent tubule.

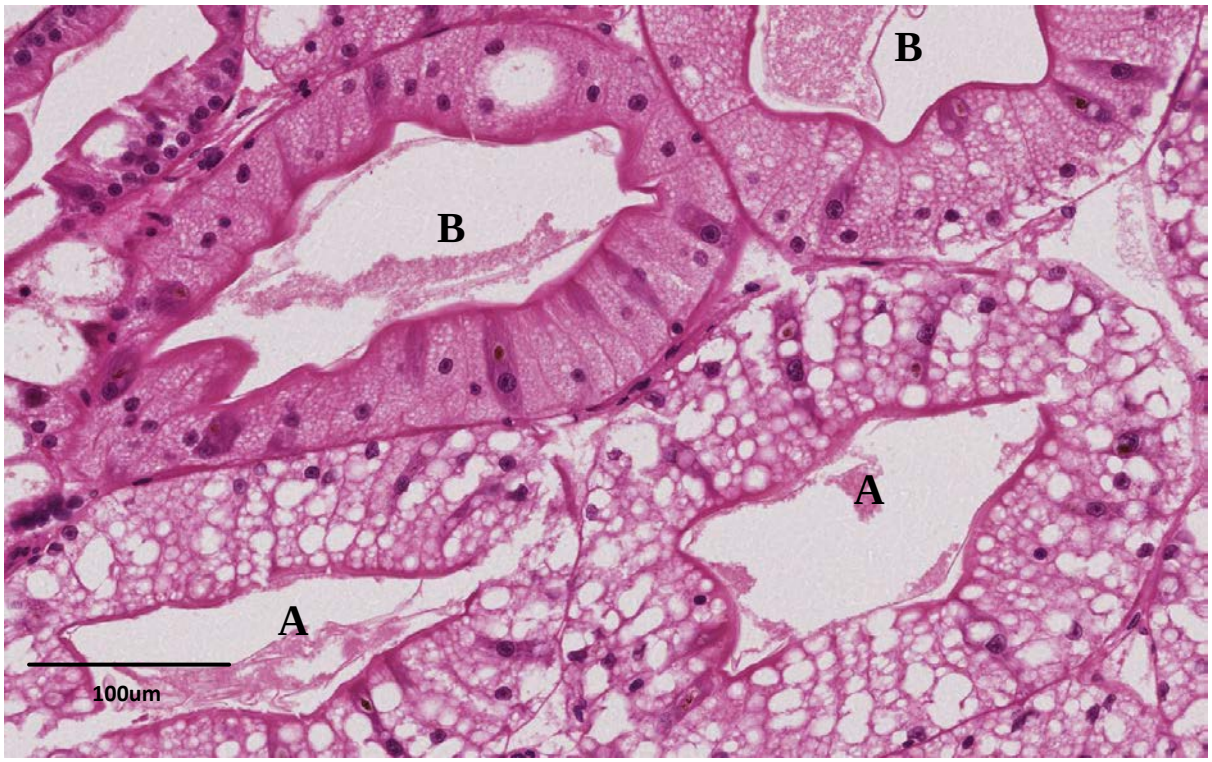


Image 3.2 Tubule lumen with overabundance of lipid storage vacuoles (A) compared to adjacent tubule lumen (B)

Table 3.1 Classification of hepatopancreas lumen structure.

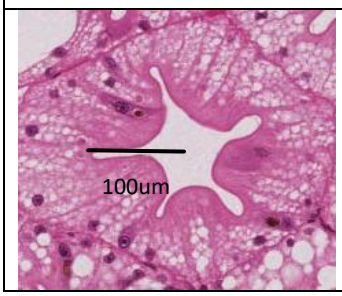
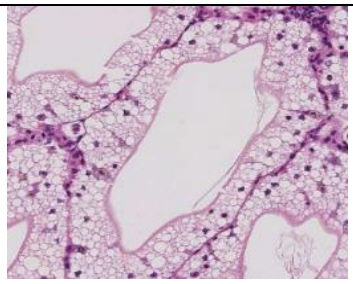
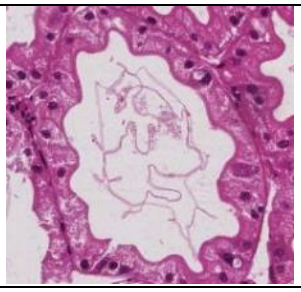
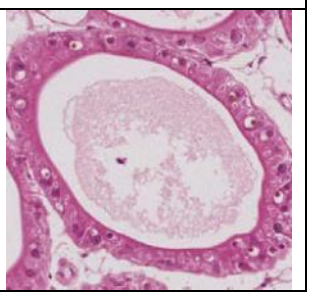
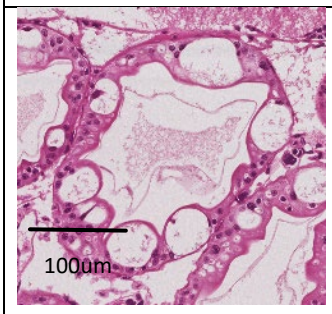
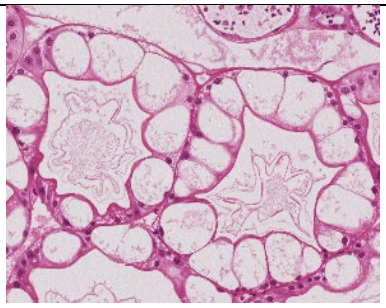
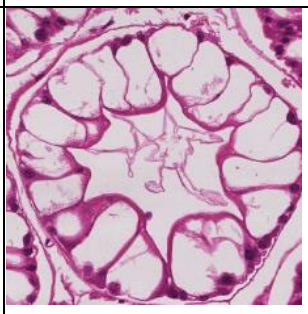
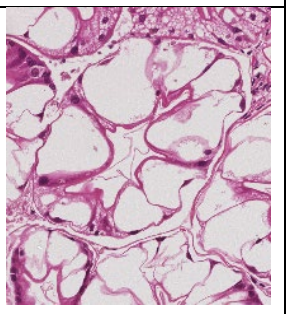
1.	2.	3.	4.
Thick lumen with distinct pointed star shape or inner folds	In between thick/thin lumen with oval/rounded	Thin lumen with folds	Thin lumen with oval/rounded lumen
			

Table 3.2 Classification of hepatopancreas hypertrophic b-cells.

1.	2.	3.	4.
Hypertrophy of b-cells not evident	Number of tubules with hypertrophic b-cells in tubules low, or if present not of concern e.g. lumen/tubule structure is maintained	Medium - high number of tubules with hypertrophic b-cells, b-cells coalescing into one but structure maintained	High number of tubules with hypertrophic b-cells, with structural compromised in lumen due to coalescing b-cells
			

3.2.6. *Histopathological survey – measurements*

The area of the Hep was calculated using the inbuilt measurement tool within the imaging software (Aperio ImageScope). Fifty individual tubules for each sample were measured in the same way to obtain an average tubule size for that hepatopancreatic sample, with this average used to calculate the percentage of cells infected. Total number of granulomas were counted, with the average of 20 random granulomas measured (except in those individuals where there was less than 20 where all were measured). All measurements were converted to mm^2, and reported for each diet treatment as the average ± S.E.

3.2.7. *Performance indices*

Equations and statistical analysis for performance indices (weight gain, survival and FCR) are presented in Chapter 2.

3.2.8. *Statistical Analysis*

Hepatopancreatic measurements and grading scheme scores were averaged for each diet group and subjected to a one-way ANOVA. Homogeneity of variance was determined with Levene's test. Limits for significance were set at $P < 0.05$. Post-hoc analyses of significant terms were performed using Tukey's highly significant difference (HSD) test. Where equal variances were not met and could not be transformed, Tamhane's test was used to test for levels of significance. Results from analysing the raw data for CqBV tubule infection % did not result in any significant differences, although numerical differences were noted. Outliers were removed using a function in GraphPad, and the data was re-run as above with the outliers removed. Statistical analysis was performed using SPSS (Version 25.0).

3.3. Results

3.3.1. *Hepatopancreatic measurements and performance indicies*

Measurements of the whole hepatopancreatic area, number and size of granulomas, and percentage of tubules infected with CqBV and reovirus are presented in Table 3.3. Data is reported as the diet treatment average ± S.E. All redclaw fed the experimental diets were found to be infected with CqBV, with the infection in redclaw fed SBM diet significantly different ($p<0.05$) compared to the initial redclaw, and those fed the FM, DDG and LM diets. There were no significant differences for Hep size, percent of tubules infected with CqReo or for the number of granulomas.

Granulomas were present in each diet treatment with the number in an individual ranging from 1 – 138 and between 4.87 mm^2 to 7.47 mm^2 in size. Granulomas found in redclaw fed the SBM diet (72 ± 67) had an average size of 0.94 ± 0.22 mm^2. In comparison granulomas in the FM diet had an average size of 3.03 ± 3.02 mm^2. Redclaw fed the DDG diet had a weight gain of 8.29% but a Hep size of 53.35 ± 17.35 mm^2, in comparison redclaw fed the FM diet had a Hep size of 73.77 ± 18.89 mm^2, and a % weight gain of 25.6%. However, PBP who had a % weight gain of 12.9% had a comparably lower Hep area of 40.13 ± 13.79 mm^2.

3.3.2. *Grading Scheme*

Grading scheme results are presented in Figure 3.1. There were significant differences ($p<0.05$) for the lumen scores of redclaw fed the LM diet, compared to the initial redclaw, and those fed the FM and SBM diets. ELS scores for redclaw fed the LM diet was significantly different ($P<0.05$) to all dietary treatments, including the initial, but except for redclaw fed PM. ELS scores for redclaw fed the PM was significantly different to the initial redclaw ($P<0.05$), but not to any of the other diet treatments. There were no significant differences in the grading scores for the remaining structural characteristics and pathologies observed.

Table 3.3 Hepatopancreas measurements and infection level in C. quadricarinatus (n = 3 per diet treatment; ± SE)

	Granulomas		**Whole Hepatopancreas**	**Tubules infected with CqBV**	**Tubules infected with Reovirus**
Diet	No.	Size (mm^2)	area (mm^2)	%	%
Initial	45 ± 38	1.09 ± 0.47	57.05 ± 17.45	0.00 ± 0.00[a]	0.05 ± 0.06
PM	38 ± 27	1.40 ± 0.44	49.38 ± 18.04	0.80 ± 0.36[ab]	0.21 ± 0.21
FTM	45 ± 32	3.03 ± 3.02	73.77 ± 18.89	0.21 ± 0.14[a]	0.14 ± 0.12
DDG	30 ± 21	1.83 ± 0.86	53.35 ± 17.35	0.63 ± 0.31[a]	0.08 ±0.04
LM	20 ± 20	0.99 ± 0.30	30.70 ± 8.73	0.09 ± 0.05[a]	0.29 ± 0.35
PBP	41 ± 17	0.85 ± 0.46	40.13 ± 13.79	1.36 ± 0.73[ab]	0
SBM	72 ± 67	0.94 ± 0.22	50.18 ± 12.41	7.82 ± 4.19[b]	0.17 ± 0.14
SM	29 ± 33	1.84 ± 1.15	55.36 ± 35.12	0.71 ± 0.49[ab]	0
Control	39 ± 28	1.60 ± 0.78	62.37 ± 25.84	0.78 ± 0.47[a]	0

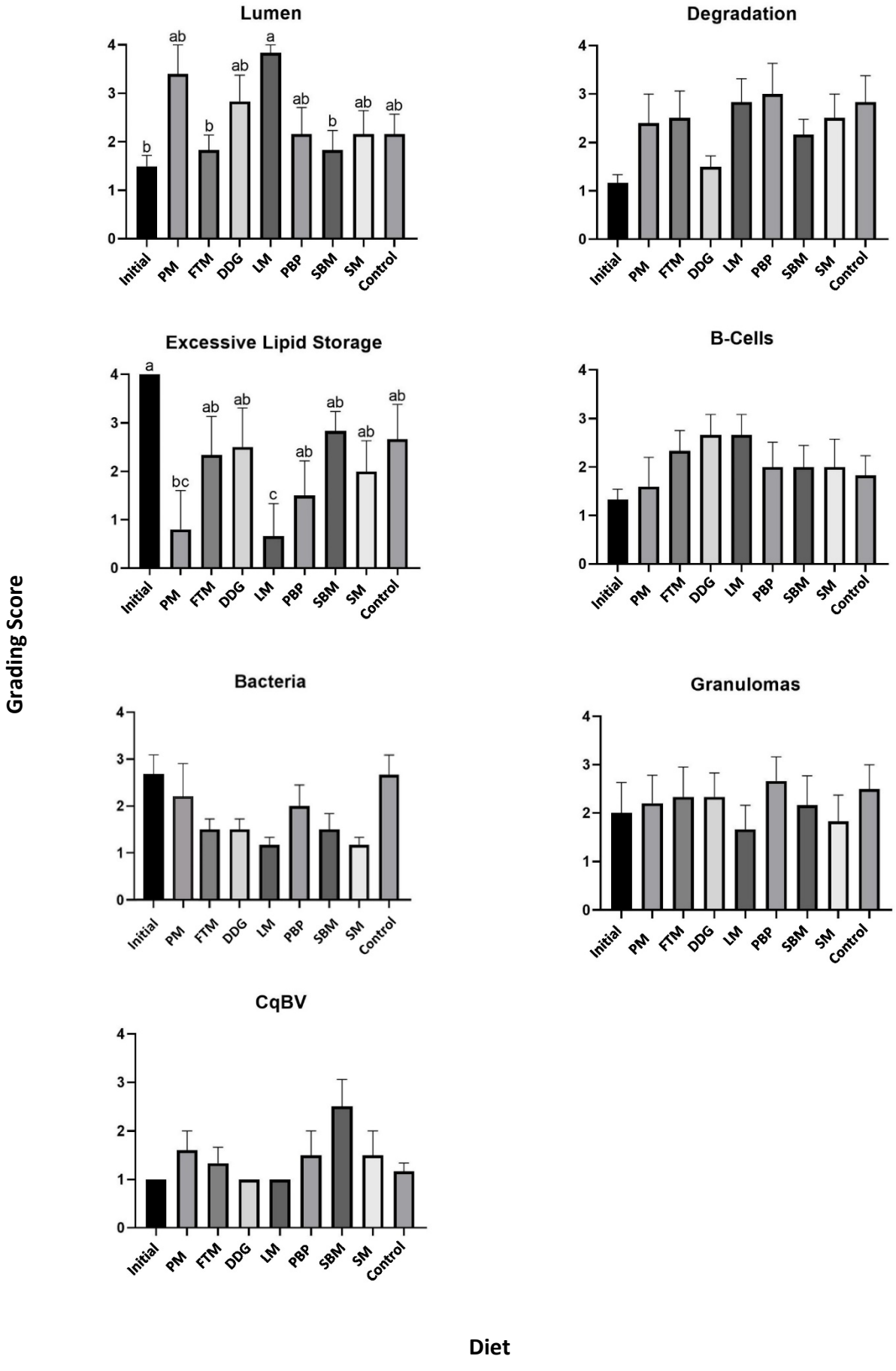


Figure 3.1 Prevalence of different structural characteristics, pathogens and pathologies observed by histopathology in redclaw fed plant-based and animal by-products. Graded score with 1 = low/absent and 4 = severe/high. Values are diet average (SEM)

3.3.2.1. Initial

All initial redclaw sampled showed signs of ELS as indicated by numerous vacuoles. However, structure of the tubule was uniform with thick epithelium and folded inner lumen star-shaped (Image 3.3). B-cells were limited to the distal region; however, tubules with the enlarged B-cells formed a border around the organ. There were no signs of CqBV infections. One individual presented with CqReo. Bacteraemia were present in all individuals, two were at levels of high concern (systematic throughout tissue). Coinfection of CqBV and bacteria was found in one individual. Four presented with granulomas, of those, two had counts ≥60 although they were small in size. Protein precipitation was not observed, while iron deposits were noted in all individuals.

3.3.2.2. Reference Diet (commercial prawn feed)

ELS occurred in all but one of the samples, with epithelium starting to thin and inner lumen becoming round. Degradation of the connective tissue and tubule structure was graded as quite severe in half of the individuals with tubule 'shrinkage' evident. CqBV presented in half of individuals although infection level was low. CqReo was not observed. Bacteraemia were present in all but one individual, with haemolytic enteritis present in one individual. Coinfection of CqBV and bacteria were found in three individuals. Granulomas were also found in all but one individual, with three having ≥60 granulomas. B-cells were limited to the distal regions, although in more than half of the tubules with enlarged B-cells formed a border. Iron deposits were noted in all individuals, and protein precipitation in five.

3.3.2.3. Yellow Pea Meal

Only five individuals were sampled for histology due to the low-survival in one replicate tank. CqBV was present in all individuals with a moderate infection containing multiple lesions. Bacteraemia were present at a high level in two individuals (Image 3.4) with one showing signs of bacteria localised around the blood vessels present in the connective tissue. This was confirmed with a Gram stain. Of note is that the reproductive organs in one individual was compromised with haemoctyic infiltration. Co-infections of CqBV and bacteria were noted in two individuals, with one coinfected with CqBV and CqReo . Granulomas were present in 90% of individuals with counts ≥20 recorded. Overall, tubule structure was poorly organised, the inner lumen wall was thinning and rounding. Tubules with enlarged B-cells formed a border. ELS was only observed in one individual which did not have any bacteraemia present and was

the only one with thick epithelium and a normal level of B-cells. Iron deposits were present in four individuals, with protein precipitation evident in three.

3.3.2.4. Feather Meal

Bacteraemia were present at very low levels in three individuals. CqBV was also present in three individuals but only one was at a moderate level (large number of cells but few lesions). ELS occurred in four individuals. Tubules with enlarged B-cells again formed a border. CqReo was present in two individuals. Co-infection of CqBV, CqReo and bacteria occurred in one individual, no other individuals had co-infections. Granulomas were present in all but two individuals with number of granulomas ≥40. A granuloma the size 7.47 mm was found in one individual. Iron deposits occurred in all but one individual, with protein precipitation in three. In general tubule epithelium was thick, although inner lumen was starting to become ovular.

3.3.2.5. Dried Distillers Grains

CqBV was found in three individuals at low levels, as was CqReo. Bacteraemia were present in half of the individuals but at a level not significant in the Hep. However, one individual had a high-level bacteraemia infection in the reproductive cells. Coinfection of CqBV and bacteria was noted in one individual, with another recorded with CqBV, CqReo and bacteria. Granuloma counts of ≥20 were observed in all but one individual. Lipid storage was excessive in all but two individuals. B-cells were largely located in the distal region forming around the border of the organ. Iron deposits and protein precipitation were recorded in all individuals. Connective tissue and tubule structure was uniform with little degradation noted. Although lumen epithelium was generally thick with folds, there were a number of instances where the lumen was becoming circular.

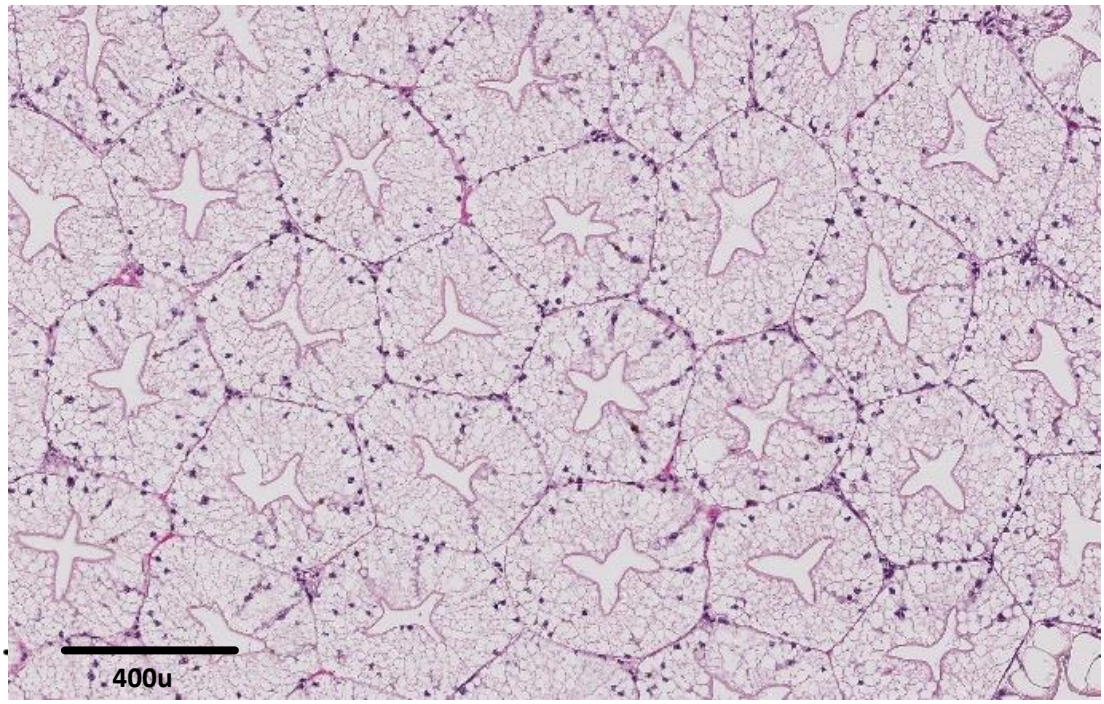


Image 3.3 Hepatopancreas of initial redclaw showing thick epithelium with star-shaped folded inner lumen, with excess lipid storage evident

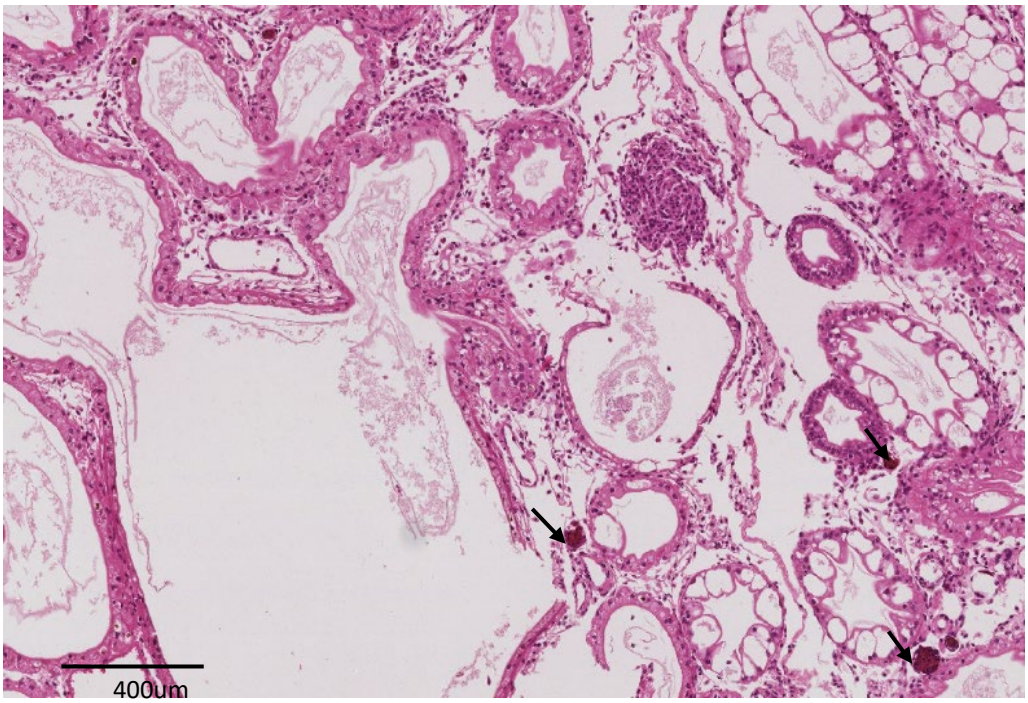


Image 3.4 Hepatopancreas from a redclaw fed the pea meal diet showing high bacterial loads, granulomas (arrows) and disorganised tubules and degradation of overall structure

3.3.2.6. Lupin Meal

All redclaw fed diets containing lupin meal presented with granulomas however not to the same degree as found in the other diet treatments. CqReo was observed in one individual. CqBV was present in three individuals with one with several lesions in a moderate number of tubules. Bacteraemia were present at low levels in two individuals, and one with moderate levels. Three individuals had coinfection of CqBV and bacteria. B-cells were quite prominent with large areas of tubules filled with the enlarged cells. Iron deposits and protein precipitation were observed in all but one individual. Connective tissue was considerably degraded, with tubule structure containing a thin epithelium and rounded lumen, or thick lumen wall but disorganised structure.

3.3.2.7. Poultry-by-Product meal

Low levels of bacteraemia were identified in four individuals, with one also showing moderate levels of bacteria present in reproductive tissue. CqBV was present at low levels in two individuals, with one individual at a moderate to severe level of infection. No CqReo was observed. Coinfection of CqBV and bacteria were noted in three individuals. Granulomas were present in all but one individual and while counts were ≥20, the average size was 0.85 ± 0.46 mm^2). Iron deposits and protein precipitation were noted in all but one individual. B-cells were present in distal areas but were not just limited to the border, increasing into the medial space. Tubule structure and connective tissue was highly degraded in three individuals, although tubule epithelium was thick with some instances where lumen was becoming ovular.

3.3.2.8. Soybean meal

CqBV was found in four individuals, severe lesions and high numbers of cells were infected, with CqBV occurring in adjacent tubules. Granulomas were present in four individuals, with 138 granulomas observed in one individual. Bacteraemia and CqReo was identified at low levels in two individuals. Coinfection of CqBV and CqReo was present in one individual. Iron deposits were noted in all individuals. Protein precipitation was present in all but one. B-cells were prominent around the border, but they were also located in abundance in tubules throughout the organ. Tubule epithelium was thick and folded; however the structure was in some instances disorganised. All samples had a moderate level of ELS.

3.3.2.9. Sorghum

Bacteraemia were present in two individuals at low levels. CqBV was found in half of individuals with one recording high numbers of cells and severe lesions, and in adjoining tubules. CqReo was not observed. Granulomas were present in four individuals, half with ≥40 counts. Iron deposits and protein precipitation were noted in four individuals. B-cells were present in along the border and throughout in all individuals. ELS was evident in all but one individual. Degradation of connective tissue was severe in two individuals. Tubule epithelium was often thick and the lumen trending toward becoming ovular.

3.4. Discussion

Upon gross examination of the Hep a number of structural abnormalities were observed in the redclaw fed the various protein sources including: degradation of the myoepithelial cells, sloughing of cell contents, hypertrophy of B-cells, and thinning of epithelial luminal wall. Additionally, granulomas, bacterial and viral infections (CqBV and CqReo) were also present. No single individual sampled from the digestibility study supported a healthy hepatopancreatic structure and very few were completely pathogen free. This was surprising as the results from Chapter 2 were not indicative of an underlying pathology. The relationship, therefore, between digestibility of feeds and ingredients, and Hep health is not straightforward. There were however, some clear diet effects such as diet treatment mitigating ELS condition. Diet has influenced the histopathology of other crustaceans such as: *P. monodon* fed vitamin deficient diets induced degeneration and detachment of epithelial cells (Naik, 1999); detachment of cells and damage to the basal lamina occurred in *P. muelleri* fed high levels of vitamin E (Fernandez Gimenez et al., 2004); and epithelial cells and compressed R-cells were evident in *P. clarkii* fed low levels of corn starch (Xiao et al., 2014).

There has been a general lack of discussion and attention in redclaw nutrition studies on the role of the Hep as evidenced by a search of the primary literature in which only 22 articles on redclaw nutrition, from 2003 – 2018 included analysis of the Hep. Of those only seven conducted histology, with the other studies conducted different analyses e.g. biochemical, enzymatic and RNA extractions (Appendix A, Table 3). However, the prevalence of viruses in redclaw population is well documented. Surveys by Edgerton & Owens (1999) conducted at northern Queensland farms in 1992 and 1996 showed that CqBV and bacterial infections were quite common in farmed redclaw populations in the region. Groff et al. (1993) and Romero & Jimenez (2002) reported similar detections in redclaw in North America and Ecuador respectively. Recent genetic sequencing indicated that CqReo found in farmed redclaw in

China, originated from Australian stocks exported in the early 1990s (Hayakijkosol et al., 2021). CqReo, which was observed in the present study (although in low numbers), infects the hepatopancreatic tissue, increasing inflammation and is associated with chronic mortalities (Edgerton et al., 2000; Hayakijkosol et al., 2021) and stunting in experimental trials (Hayakijkosol & Owens, 2011). Reovirus was also identified as the causative agent for an epidemic in Mud Crab (*Scylla serrata*) farms in Zhejiang Province, China with mortalities estimated to high as 80% (Chen et al., 2011). CqBV is also associated with mortality and growth suppression in redclaw (Edgerton et al., 1995; Groff et al., 1993; Romero & Jimenez, 2002). Other infections that have impacts on redclaw health and survival include the chequa iflavirus (Sakuna et al., 2017, 2018) which caused mass mortality (20-40%) in redclaw three weeks after a stress event but exhibits no pathognomonic histological lesions, and *Aeromonas hydrophila*, a bacteria that while common in freshwater bodies, caused high mortality in stage 2 larvae (Hayakijkosol et al., 2017). The effects of these infectious agents are clear and their importance cannot be understated but remains largely missing from nutrition studies.

Redclaw in the present study were farm-sourced stock from northern Queensland and had been habituated in a RAS for a number of months prior, with redclaw pooled and randomised for this study. While no structural abnormalities, CqBV or CqReo were observed in the initial redclaw sampled prior to stocking, all initial redclaw sampled had varying levels of bacteria in the Hep. Overall, the level of bacteria was generally lower in the experimental redclaw, in particular in those fed the LM and SM diets when compared to the initial redclaw. However, the severity of the bacteria varied across individuals and diet treatments, in some instances severe bacterial infections were present not only in the Hep but in the surrounding reproductive organs. The provision of particular feed ingredients could act as a food source for bacteria, for example β-glucans, that make up dietary fibre in cereal grains, are fermentable by bacteria (Rurangwa et al., 2009). However, the link between food source and bacterial level is not within the scope of the present study but whether specific ingredients promote bacterial growth would be something to investigate further. The culture of redclaw has not yet been developed commercially for RAS with such systems largely limited to research, and so there is the potential for the culture environment to perpetuate some of the bacterial agents. However, water quality in a RAS is far more controllable than in an earthen pond in which redclaw are currently farmed, further the water in the present study was UV treated and maintained throughout the feeding trial with the culture environment unlikely to be a major contributor to the bacterial levels.

Crustaceans have a partially open circulatory system which transports haemocytes and humoral components thorough the haemolymph (Vazquez et al., 2009). Haemocytes have important roles in phagocytosis, encapsulation, and lysis of foreign cells and are therefore essential for immune function (Smith et al., 2003). Granulomas are a collection of inflammatory cells that form in response to an antigen which can include bacteria (Petersen & Smith, 2013). While not statistically significant, redclaw fed the SBM diet had an abundance of granulomas, although the average size was small, while granulomas of a large size occurred in redclaw fed the FTM diet. The presence of granulomas demonstrated an immune response to an infection. Abundance of granulomas does not necessarily indicate a compromised immune system as in the case of redclaw fed the SBM diet, but larger granulomas do indicate the ability to respond to foreign bodies is diminishing.

Increased levels of B-cells were observed to some degree in all redclaw fed the diet treatments including those fed the RF. F-cells are specialized for the synthesis and secretion of digestive enzymes during phases of the feeding cycle, differentiating into early stage B-cells, which are the sites for intracellular digestion and assimilation (Al-Mohanna & Nott, 1986; Al-Mohanna et al., 1985). Enlargement and hypertrophy of B-cells were reported in redclaw fed a restricted diet (Calvo et al., 2011) and also in response to diets with different processing of grain legumes (Vasagam et al., 2007). B-cells transition from F-cells following initial digestion in the stomach through the introduction of fragmented proteins/polypeptides into the developing B-cells, which eventually form digestive bodies that merge to form the larger digestive vacuole (Hu & Leung, 2007). B-cells then go through a succession of uptake, intracellular digestion, assimilation and elimination of waste which can occur 12 - 48 hrs post-feeding (Al-Mohanna & Nott, 1986). Xiao et al. (2014) noted an increase of B-cells and corresponding α-amylase in crayfish, concluding crayfish were able to increase digestion of corn starch. However, the appearance of the tubule lumen needs to be taken into consideration when determining whether abundance of B-cells has positive or negative connotations.

A thick lumen wall indicates enough nutrition is being supplied to support the growth of columnar epithelium, while a thinner lumen wall indicates lack of nutrition and a reduced capacity for storage (Manan et al., 2015). Redclaw have a high starvation resistance (Calvo et al., 2018; Stumpf et al., 2010) and it is unlikely that redclaw in Chapter 2 were relying on energy stores when being fed daily. Indeed, complete nutritional depletion of *C. destructor* occurred only after 7 months of starvation, at 10^{o}C (Jones & Obst, 2000). In crustaceans, lipids are a major source of energy and have important roles in reproduction (Sánchez-Paz et al.,

2006). Storage of lipids occurs in the R-cells, which are the most abundant cell type occurring along the length of the tubule (Al-Mohanna & Nott, 1987). Tubules with thick columnar epithelium and abundant R-cells were observed in a number of sampled redclaw in the present study. Increased accumulation of glycogen and lipids in HP were reported for redclaw fed on a cyclic feeding regime of being fed and being food deprived, evidenced by larger vacuoles in the R-cells (Stumpf et al., 2014). Similar observations were recorded by Johnston et al. (2003) when assessing carbohydrate/lipid ratios in *Jasus edwarsdsii*; the authors reported that the increased vacuolation of R-cells corresponded with high density of lipid droplets. Further, the Hep histology images of *J. edwarsdsii* presented in Johnston et al. (2003) are visually similar to those observed in the present study. It would therefore be beneficial to determine whether excessive vacuolisation of R-cells was indeed lipid storage, as it appears that increased lipid storage in the Hep (equivalent to fatty liver) can be detrimental (Cervellione et al., 2017), in some redclaw there were major structural changes in the tubule lumen due to excessive number of vacuoles. In the present study, initial redclaw scored high for ELS, but an improvement was seen in redclaw fed the experimental diets, particularly those fed the YPM and LM diet (Figure 3.1). Redclaw in this study were fixed using Davidson's fixative which does not allow for preservation of lipids. In future, fixing tissue in glutaraldehyde and staining with osmium tetroxide (Cervellione et al., 2017) would be recommended to confirm the presence of lipids under light microscopy and would indicate any beneficial effects of the diets.

The test diets used in the present study comprised 70% commercial prawn feed (Ridley AgriProducts) and 30% of the test ingredient. With the limited published information of the nutritional requirements available for redclaw, and a lack of commercially available feed specifically formulated for redclaw, the prawn diet was considered the best alternative to ensure adequate provision of essential macro and micro nutrients for a crustacean. At the commencement of the experiment the Hep of the initial redclaw had the typical star-shaped inner lumen and honeycomb structure of a healthy Hep, however the tubules did have high lipid storage as evidenced by the vacuolation within R-cells. The initial redclaw had been fed the commercial prawn feed for ~3months. However, nutritional requirements for crustaceans differ between species and the prawn diet over a long period may not have been suitable for redclaw. This is evidenced by the increased levels of bacteraemia, granulomas, tissue degradation and loss of the star-shaped lumen of redclaw fed the RF diet, which was simply the prawn diet re-pelleted with the inert marker included. However, redclaw are often provide non-species specific diets in nutrition studies, with a commercially available tropical fish food,

Tetracolor, surprisingly the most common, used either as a maintenance diet prior to a feeding trial (Chaulet et al., 2012; Stumpf et al., 2014) or as the primary diet (Calvo et al., 2011; Calvo et al., 2013; Calvo et al., 2012; Calvo et al., 2018; Stumpf & Greco, 2015). Calvo et. al., (2011) is one study that used *Tetracolour* as a primary food source and is one of the few published studies on redclaw that clearly show structural changes in relation to early and late feed restrictions. However, it is not clear to what extent the diet itself may have had on the Hep structure. This is an example of the difficulties faced when studying redclaw nutrition.

Results from the present study show it is important to consider performance parameters such as growth and survival, in combination with key health indices such as the Hep and other health parameters such as the presence of L-lactate in the haemolymph, which is an indicator of stress in crustaceans (Bakke & Woll, 2014; Stoner, 2012). While viruses such as the chequa iflavirus require detection by genome sequencing, histology is a relatively simple method to gain an overall health assessment for crustaceans. It is however time consuming to survey whole tissue in detail and grading scheme results are generally qualitative in nature. Berillis et al. (2013) used image processing to study the Hep in lobster species and it was the first-time nutritional condition for crustaceans was discussed using such methodology. Subsequently, Cervellione et al. (2017) developed and validated the use of computer assisted image analysis for morphological factors, including B-cell vacuoles, lipid droplets and total lumen area, for the Hep of *P. vannamei*. While histology is a lethal, using such technology baseline and reference data could be established and used to compile a database of key health indicators and responses to inclusions of different dietary sources for redclaw.

Redclaw have the capacity to utilise a range of feed (Joyce & Pirozzi, 2016), consuming a higher proportion of plant matter as they get older (Loya-Javellana et al., 1993) of which many aquafeed diets are incorporating in greater amounts. Inclusion of plant ingredients for redclaw are supported by the ADCs established in Chapter 2. However, legumes, which include lupins, peas and soybean are known to contain a number of ANFs that interfere with food utilisation and affect the health status of individuals (Francis et al., 2001). Vasagam et al. (2007) recommended legumes be at least heat treated and dehulled before inclusion in diets of prawns following mass mortality of juvenile *P. monodon*. Similarly, a decrease in ANFs and improved proximate composition of chick pea, lentils and field pea followed germination in combination with autoclaving (Kumaraguru Vasagam & Rajkumar, 2011). Ingredients used in this study were milled to <630 ųm**,** extruded and oven dried at 60 ^{o}C for 24hrs. In the present study the tubule lumen wall was significantly ($p<0.05$) thinner in redclaw fed the LM diet which could

possibly an indication of ANFs. Given the extent to which structural abnormalities were observed in the redclaw fed such diets the influence of ANFs on redclaw digestion, and the capacity to reduce their effects by different processing techniques is an area to investigate further.

Diet-related effects were observed in redclaw fed plant and rendered-animal by-products although the ability to determine to what degree particular ingredients contributed is limited as the use of the commercial prawn feed may not be suitable for redclaw over long periods. However, based on the performance results from Chapter 2, and the histopathology results from the present study feather meal can be considered a suitable ingredient to include in formulated diets for redclaw. The SBM diet induced reasonable performance and high ADCs, however it had significantly greater CqBV infections ($p<0.05$) compared to redclaw fed other protein sources. It cannot be discerned in the current study as to whether the SBM diet had a deleterious or beneficial effect on the immune system, and whether this enabled the virus to proliferate, or provide suitable nutrition for immune defence (Zhou et al., 2018). Given the global use of SBM in formulated feeds it would be of great interest to investigate. Lastly, the inclusion of DDG in redclaw diets needs further assessment as growth performance was low, but connective tissue and tubule structure was uniform with little degradation noted in redclaw fed this diet.

Despite instances of severe bacterial infections, structural abnormalities, and cell degradation all the redclaw in the present study where those that had survived to the end of the feeding trial. Indeed, on completion of the feeding trial individuals not sacrificed for histology were returned to holding tanks and continued to thrive and reproduce. When considered together, the ADCs established in Chapter 2 and the current histopathological investigation of those same crayfish, presents a perplexing and somewhat counter intuitive dichotomy. It does reflect however that redclaw are a very robust animal. This doesn't negate the fact however that there needs to be greater awareness in nutrition research in relation to health implications, in particular the relationship between feed ingredients and health, and the impacts of diet in relation to the known viruses and diseases that are present in redclaw populations. Even though studies have identified specific pathogens present in redclaw, in light of the absence of available literature there is a clear disconnection between nutrition and health related research for this species. When redclaw are pathogen-free, improvements in growth and survival occur as a result as evidenced by the increased harvest size of redclaw stocked with pathogen-free eggs (Owens, 2011). Conversely the provision of an optimal diet formulated from ingredients that do not cause structural abnormalities in the Hep, would ensure redclaw are not compromised in their

ability to digest and absorb nutrients, ensuring enough resources are available for the immune system to respond to any foreign bodies. It is clear that co-investigation of diet performance and histology is necessary when assessing nutritional requirements for redclaw, and with improvements in technology this can be easier to achieve.

4. CHAPTER 4: DIETARY LYSINE REQUIREMENT FOR JUVENILE REDCLAW

4.1. Introduction

With the move toward more intensive redclaw aquaculture practices farmers will rely more on compound diets to provide the required nutritional components i.e. fats, carbohydrates, protein and vitamin and minerals. The dietary protein requirement for redclaw is reported to be between 22-35% CP (Cortés-Jacinto et al., 2004; Pavasovic et al., 2007b; Rodríguez-González et al., 2014) using dietary protein sources such as sardine or anchovy meal, and soybean meal. FM is still a commonly used protein in formulated aquafeed because it provides a high quality source of dietary protein and balanced amino acid profile, is a source of vitamins and minerals, has good palatability, digestibility and absorption (Han et al., 2018; Hardy, 2010; Oliva-Teles et al., 2015; Tacon & Metian, 2015). However, it is a finite natural resource, global fisheries are in decline and subsequently the cost for FM has increased as supply is restricted (Tacon & Metian, 2015). This has resulted in a global shift toward alternative sources such as plant-based ingredients including soy, lupin and pea meal (Hansen et al., 2007; Li et al., 2009; Øverland et al., 2009; Pavasovic et al., 2007a). Thompson et al. (2004) reported that FM could be wholly replaced with SBM in diets for juvenile redclaw. Similarly, SBM can replace up to 40% of FM in diets for juvenile Blue Swimmer Crab (*Portunus pelagicus*) (Taher et al., 2017)

Lys is an EAA with a primary function of proteinaceous tissue deposition, and has other biological roles such as being a precursor of carnitine which carries long chain fatty acids into the mitochondria for β-oxidation of lipids. (Tanphaichitr & Broquist 1973). Lys, along with Met, are often the most limiting amino acids in FM replacement diets that contain high proportions of plant protein (Cvie and Eze 2013). Diet formulations containing low levels of FM or animal protein often require a mixture of plant proteins and supplementation with synthetic AA to obtain an optimal AA profile (Fournier et al. 2004). Plant protein sources such as soya do contain relatively high proportions of Lys; however, these may not be as bioavailable as dietary Lys derived from animal proteins (Tome 2013; Berrazaga et al., 2019). Other sources such as wheat gluten and cornmeal are also relatively deficient in Lys (Hansen et al., 2007; Hardy, 2010).

Dietary Lys requirements have been estimated for only a few commercial crustacean species and range from 1.64% for *L. vannamei* (Xie et al. 2012) to 3.2% for *M. japonicas* (Teshima et al. 2002) on a dry diet basis. Understanding the dietary requirement for Lys facilitates the formulation of nutrient appropriate diets to optimise growth as well as increasing the range of

raw ingredients which could be used to manufacture more cost effective, FM replacement diets. There are currently no published studies quantifying any essential EAA requirements of redclaw; therefore, the objective of this study was to determine the dietary requirements of Lys for juvenile redclaw.

4.1. Materials and Methods

4.1.1. *Experimental Diets*

Five isoenergetic (18 MJ gross energy (GE)/kg) and isoproteic (30% CP) diets were formulated to contain one of the following five levels of Lys 0.52, 0.94, 1.40, 1.66 and 2.02/100g diet (herein referred as D1, D2, D3, D4 and D5) (Table4.2). Dietary specifications were based on known requirements for redclaw (Saoud et al., 2012). Wheat gluten and FM were the primary sources of protein, supplemented with crystalline AAs (Table 4.1). There are no published studies quantifying any EAA requirement for redclaw; therefore, the AA mix was formulated assuming appropriate inclusion based on the published data for other crustacean species (Millamena et al., 1998, Palma et al., 2013, Xie et al., 2012). The lowest level of Lys was obtained exclusively from the intact protein sources in the basal diet. The increasing Lys levels were achieved by directly substituting wheat gluten with Lys HCL. The AA composition for each diet is presented in Table 4.2. Diets were created by mixing dry ingredients in a Hobart mixer (A200N, Hobart Manufactures, Brition, UK) for 1 hour prior to the addition of sufficient water to form a dough. The dough was then extruded through a 3 mm die (La Monferrina Dolly II, Italy). Pellets were oven dried at 60 ^{o}C for 24 hrs and stored at -20 °C until required.

Table 4.1 Diet formulation (DM basis)

Diet	1	2	3	4	5
Ingredient (g/kg)					
Fish meal	85	85	85	85	85
Fish oil	42.7	42.7	42.7	42.7	42.7
EAA premix [a]	177	177	177	177	177
Lecithin	10	10	10	10	10
Cholesterol	10	10	10	10	10
Wheat Flour	162.2	162.2	162.2	162.2	162.2
Pregel Starch	318.2	318.2	318.2	318.2	318.2
Wheat Gluten	81.9	73.8	69.7	65.7	61.6
DCP	10	10	10	10	10
Diatomaceous Earth	73	73	73	73	73
Vit. & Min. premix	30	30	30	30	30
Lysine HCL	0	8.1	12.2	16.2	20.3

[a] Essential amino acid mixture (g per 100g diet) arginine, 1.42; histidine, 1.77; isoleucine, 1.42; leucine, 2.66; methionine, 1.42; phenylalanine, 2.12; threonine, 2.30; tyrosine, 2.12; valine, 2.12; tryptophan, 0.35

Table 4.2 Proximate Composition (g/100g) and amino Acid composition of lysine requirement test diets as analysed (%DM)

Diet	1	2	3	4	5
Dry Matter	93.10	94.00	93.10	94.00	94.60
Crude Protein	29.32	29.89	30.08	29.79	30.34
Crude Lipid	5.86	5.87	5.73	5.51	8.12
Ash	9.88	10.32	9.67	9.26	8.88
Energy (MJ Kg^{-1})	18.74	18.81	18.98	19.38	19.16
EAA					
Arginine	1.91	1.79	1.92	1.87	1.87
Histidine	1.32	1.27	1.40	1.28	1.33
Isoleucine	1.76	1.72	1.70	1.66	1.69
Leucine	3.50	3.39	3.47	3.31	3.40
Lysine	0.52	0.94	1.40	1.66	2.02
Methionine	1.70	1.64	1.71	1.64	1.67
Phenylalanine	2.49	2.50	2.41	2.40	2.41
Threonine	2.93	2.67	2.81	2.72	2.80
Valine	2.33	2.22	2.17	2.17	2.21
NEAA					
Alanine	0.61	0.57	0.57	0.57	0.59
Aspartic Acid	0.68	0.63	0.64	0.63	0.64
Cysteine*	0.30	0.28	0.28	0.26	0.29
Glutamic acid	3.85	3.45	3.53	3.45	3.65
Glycine	0.72	0.67	0.68	0.66	0.68
Proline	1.61	1.59	1.54	1.57	1.48
Serine	0.73	0.64	0.67	0.64	0.67
Taurine	0.10	0.10	0.10	0.10	0.11
Tyrosine*	2.45	2.34	2.43	2.34	2.37

*conditionally essential

4.1.2. *Animals and experiment design*

It was apparent from the mortalities that occurring during the feeding trial in Chapter 2 that farm sourced redclaw health may be compromised. To prevent this potential problem from confounding the current study juvenile redclaw used in this study were obtained as craylings (~0.02 g) from a commercial hatchery in North Queensland, Australia and reared at the Marine and Aquaculture Research Unit, James Cook University and on-grown to ~1 g. Approximately 1,000 craylings were kept in 8 x 60 L tanks supplied with mesh and PVC tubes for hides and fed with a commercial prawn feed (Ridley AgriProducts Pty Ltd, Australia) which was ground into a smaller crumble and fed daily, the size of the feed was gradually increased until the craylings were able to grasp the full-sized 3mm pellets. Tanks were integrated within a RAS. Optimal water quality was maintained for the duration of the experiment with tanks provided with filtered, U.V. treated freshwater. Water temperature was maintained at 27.0 ± 1°C, pH between 7 - 8.5, and dissolved oxygen concentration remained above 8 mg/L (Jones, 1990). Photo-period was a 12 h day/night cycle with start of the light cycle at 0700 h each day. The same water quality parameters were applied to the feed experiment.

Juvenile redclaw (1.68 ± 0.26 g) were randomly assigned to each of the five diet treatments. For each diet treatment there were three replicates 60L tanks, with seven redclaw per replicate. Redclaw were housed individually in clear plastic containers (17.4 cm x 12.7 cm x 7 cm) within each tank to prevent cannibalism occurring, particularly after moulting. Containers were perforated with 3mm holes to facilitate water flow. Redclaw were hand-fed twice daily at 0700 and 1500 hours *ad libitum* for 84 days. Uneaten pellets were individually counted and removed after 30 minutes. Pellets were manufactured to a uniform size, with the average pellet weights (0.01 g) (n = 200) for each diet pre-determined. Uneaten and partially consumed pellets were noticeable and were accounted for and feed intake data adjusted accordingly.

On conclusion of the feeding trial redclaw were fasted for 24 hrs, weighed, euthanized in an ice-water slurry and frozen for future proximate composition analysis.

The dietary Lys and nutrient data in this study are presented on a crude basis; it was beyond the scope of this study to determine Lys digestibility due to the size of the redclaw and the time that would be required to collect sufficient faecal material for chemical analysis.

4.1.3. *Histology*

Chapter 3 highlighted the necessity of co-investigating the health condition of redclaw when conducting nutrition studies. Samples were collected from juvenile redclaw prior to stocking (n = 4). Hep samples were collected, processed and analysed using the same methodology in Chapter 3. Due to amount of sample dry matter required for proximate analysis and the small size of redclaw only one individual from each diet treatment was sampled at the conclusion of the Lys requirement feeding trial. However, no grading scheme or statistical analysis was applied as the primary purpose was to simply identify whether there were any health or dietary concerns, not investigate diet effects.

4.1.4. *Chemical Analysis*

Whole redclaw were homogenised using a food processor (Kenwood FP580 Multi-Pro Food Processor, Australia). A small sub-sample (~1 g) of homogenate was taken to determine whole carcass dry matter content. The remaining homogenised material placed into -80°C freezer prior to being freeze-dried for 24 h (Virtis benchtop 2 K,VWR, Australia). Moisture content (diets and whole carcass) was determined gravimetrically by oven-drying to a constant weight at 105°C. Dried samples were milled to a fine powder consistency and were analysed for crude protein, crude fat and ash using standard laboratory methods (AOAC no. 950.46B, JAOAC 33, 749 (1950), JAOAC 36, 279 (1953) respectively). Gross energy was calculated using the energy conversion equivalents for protein (23.65 kj/g), lipid (39.55 kj/g) and carbohydrates (17.16 kj/g) (NRC, 2011). Amino acid content was determined by HPLC method (Chemcentre, Bentley, WA).

4.1.5. *Performance Indices*

Performance indices were calculated as follows:

Weight Gain (g)	Wf – Wi
Percent Weight Gain (WG, %)	100 x (Wf – Wi) / Wi
Specific growth rate (SGR, % day)	[(Ln Wf – Ln Wi) / t] x 100
Feed Conversion Ratio (FCR)	Total feed intake / total weight gain
Protein efficiency ratio (PER)	WG% / (feed intake x %CP)
Survival	(Final no. crayfish / Initial no. crayfish) x 100

Where W_f is the final body weight (g), W_i is the initial body weight (g), WG

4.1.6. *Statistical Analysis*

Results are expressed as mean ± S.E. Analysis of covariance (ANCOVA) was performed to test the effect of diets on performance indices with initial weight as the covariate. Analysis of variance (ANOVA) was performed to test the effect diet on final whole body proximate and amino acid composition. Normality of the data was tested using the Shapiro-Wilk test. Where data was not normally distributed a Kruskil-Wallis test was applied. Where there were significant differences between treatments, Tukey's multiple comparison test was used to identify the source of significance. Statistical analyses was performed using NCSS and SPSS. Robust segmented linear regression was applied to analyse the relationship between weight gain and dietary Lys level to determine optimal dietary Lys specification (% diet). Regression analyses were performed using GraphPad 8.1.0.

4.2. Results

4.2.1. *Survival.*

After 84 days there was 100% survival across all treatments with the exception of one individual mortality occurring in the D4 treatment.

4.2.2. *Growth Performance*

Bodyweight of redclaw more than doubled across all diet treatments at the conclusion of the feeding trial. Results of growth performance of redclaw fed different levels of Lys are presented in Table 4.3. Growth (g) of juvenile redclaw was significantly greater ($P<0.05$) for D4 (2.00 ± 0.08 g) compared to those fed both D1 (1.59 ± 0.07 g; $p<0.01$), D2 (1.71 ± 0.01; $p<0.01$) and D5 (1.57 ± 0.13 g, $p<0.01$). SGR was significantly different among treatments ($P<0.05$), with redclaw fed D4 a SGR of 1.02 ± 0.24 % day, while redclaw fed D5 had the lowest SGR of 0.83 ± 0.13 % day. Growth tended to increase with dietary Lys intake up to D4 after which there was a significant decrease in relative growth for redclaw consuming D5.

4.2.3. *Feed utilization*

Feed consumed was highest for redclaw fed D2 which was significantly different ($p<0.01$ to redclaw fed D1, D2 & D3. Similarly, redclaw fed D3 consumed significantly more than redclaw fed D6 ($p<0.01$). FCR differed significantly ($p<0.01$) for D2 which had the highest FCR value of 2.60 compared to other redclaw fed D1, D4, D5 with FCR ranging from 1.83 –

1.96. While not statistically significant the lowest FCR occurred for redclaw fed D4 with 1.83±0.09. Protein efficiency retention was not significantly different. The Lys intake (g/day/crayfish) was significant (P<0.05) for D1 against all other dietary treatments (p=<0.01); and between D2 and D5 (p=0.01). Robust segmented linear regression determined the optimal dietary Lys level to be 1.72% equivalent to 5.76% dietary protein (Figure 1).

4.2.4. *Proximate and amino acid whole body composition*

Proximate and amino acid whole body composition are presented in Table 4.4. Ash, protein, fat and energy were not affected by dietary treatments (P>0.05). Body composition for juvenile redclaw for all diets averaged 30.39 ± 0.87% protein, 18.02 ± 0.64% Ash, 7.79 ± 0.77% Fat and 12.11 ± 0.45 MJ Kg^{-1} gross energy. Moisture level ranged 77% - 83% with significant differences (P<0.05) for redclaw fed D3 compared to redclaw fed D1 and D2. Whole-body EAA content was not significantly different between redclaw fed any of the diet treatments. AA Arg and glycine for redclaw fed D1 (1.63 ± 0.13 and 1.25 ± 0.09 respectively) were significantly different than redclaw fed D3 (1.99 ± 0.09 and 1.58 ± 0.08 respectively). No significant differences were found among redclaw fed the diet treatments when considering all other AAs (Table 4.4). Lys composition averaged 1.64 ± 0.08, Arginine 1.82 ± 0.08 and Met 0.46 ± 0.02 g/kg.

4.2.1. *Hepatopancreas analysis*

Hep of the initial crayfish showed some abnormalities in terms of structural disorganisation of tubules, thinning epithelium and disorganised haemal sinuses, hypertrophy and abundance of b-cells (Image 4.1). The structural changes were not as severe as observed in Chapter 3 and the juveniles are comparably in good health. Juvenile crayfish fed the Lys diets showed a marked improvement structurally with tubules organised and tightly arranged, reduced abundance of b-cells, greater tubule lumen and connected haemal sinuses (Image 4.2). Further, there were no bacterial or viral pathologies present in any of the juvenile redclaw sampled.

Table 4.3 Growth performance of C. quadricarinatus fed diets containing different lysine levels (mean ± S.E.)

Diet	1	2	3	4	5	Pooled SEM
Initial BW (g crayfish^{-1})	1.68	1.67	1.67	1.67	1.68	0.43
Final BW (g crayfish^{-1})	3.27^{a}	3.39^{ab}	3.51^{bc}	3.67^{c}	3.25^{a}	0.46
Weight Gain (g crayfish^{-1})	1.58^{a}	1.71^{ab}	1.84^{bc}	2.00^{c}	1.57^{a}	0.06
Weight gain (%)	110.26^{a}	114.38^{a}	122.09^{ab}	142.95^{b}	101.63^{a}	27.60
SGR (%day^{-1})	0.86^{b}	0.89^{bc}	0.93^{bc}	1.02^{c}	0.83^{ab}	0.15
Feed Intake (g crayfish^{-1})	2.33^{a}	3.10^{b}	2.49^{a}	2.18^{a}	2.33^{a}	0.12
FCR	1.47^{a}	1.81^{b}	1.35^{a}	1.09^{a}	1.48^{a}	0.09
PRE	0.02	0.02	0.02	0.03	0.02	0.00
Survival (%)	100	100	100	95.24	100	0.95
Lys Intake (g/crayfish)	0.01^{a}	0.03^{b}	0.04^{bc}	0.04^{bc}	0.06^{c}	0.00

Values in the same row with different superscripts are significantly different (P<0.05)

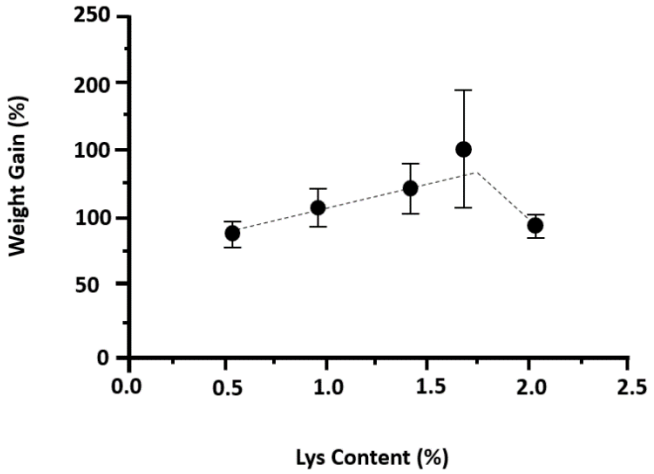


Figure 4.1 Weight gain response of redclaw (n=3; ±SE) fed increasing levels of dietary Lys. Robust segmented linear regression indicating optimal dietary Lys inclusion at 1.72%

Table 4.4 Whole body proximate and amino acid composition of juvenile redclaw fed experimental diets (mean ± S.E.) DM basis

Diet	1	2	3	4	5	Pooled SEM
EAA (g/kg)						
Arginine	1.25	1.46	1.55	1.34	1.35	0.08
Histidine	0.51	0.60	0.63	0.55	0.56	0.02
Isoleucine	0.85	0.94	1.02	0.86	0.94	0.05
Leucine	1.61	1.84	2.02	1.71	1.75	0.08
Lysine	1.45	1.71	1.83	1.58	1.62	0.07
Methionine	0.42	0.48	0.51	0.44	0.46	0.02
Phenylalanine	0.94^{a}	1.07^{ab}	1.16^{b}	0.96 ^{ab}	1.00^{ab}	0.04
Threonine	0.97	1.10	1.16	1.02	1.05	0.04
Valine	0.94^{a}	1.07^{ab}	1.16^{b}	0.96^{ab}	1.03^{ab}	0.04
NEAA (g/kg)						
Alanine	1.63^{a}	1.89 ^{ab}	1.99^{b}	1.77^{ab}	1.81^{ab}	0.05
Aspartic Acid	2.22	2.55	2.71	2.30	2.40	0.11
Glutamic acid	3.19	3.72	3.99	3.40	3.53	0.16
Glycine*	1.25^{a}	1.51^{ab}	1.58^{b}	1.37^{ab}	1.40^{ab}	0.05
Proline*	0.89^{a}	1.05^{ab}	1.08^{b}	0.94^{ab}	0.97^{ab}	0.03
Serine	1.00	1.12	1.19	1.02	1.05	0.04
Taurine	0.16	0.16	0.16	0.14	0.15	0.02
Tyrosine*	0.77^{a}	0.89^{ab}	0.97^{b}	0.78^{ab}	0.86^{ab}	0.04
Proximate Composition (g/100g)						
Ash	17.56	16.73	19.72	18.48	17.65	1.07
Protein	29.04	29.11	32.73	29.77	31.38	1.14
Fat	8.86	5.91	7.11	9.20	7.89	0.94
Calorific Energy MJ Kg^{-1}	11.38	11.55	12.86	13.16	11.60	0.39
Moisture	76.59^{ac}	76.80^{c}	83.07^{bc}	80.33^{c}	80.79^{c}	0.01

* Conditionally essential

Values in the same row with different superscripts are significantly different (P<0.05)

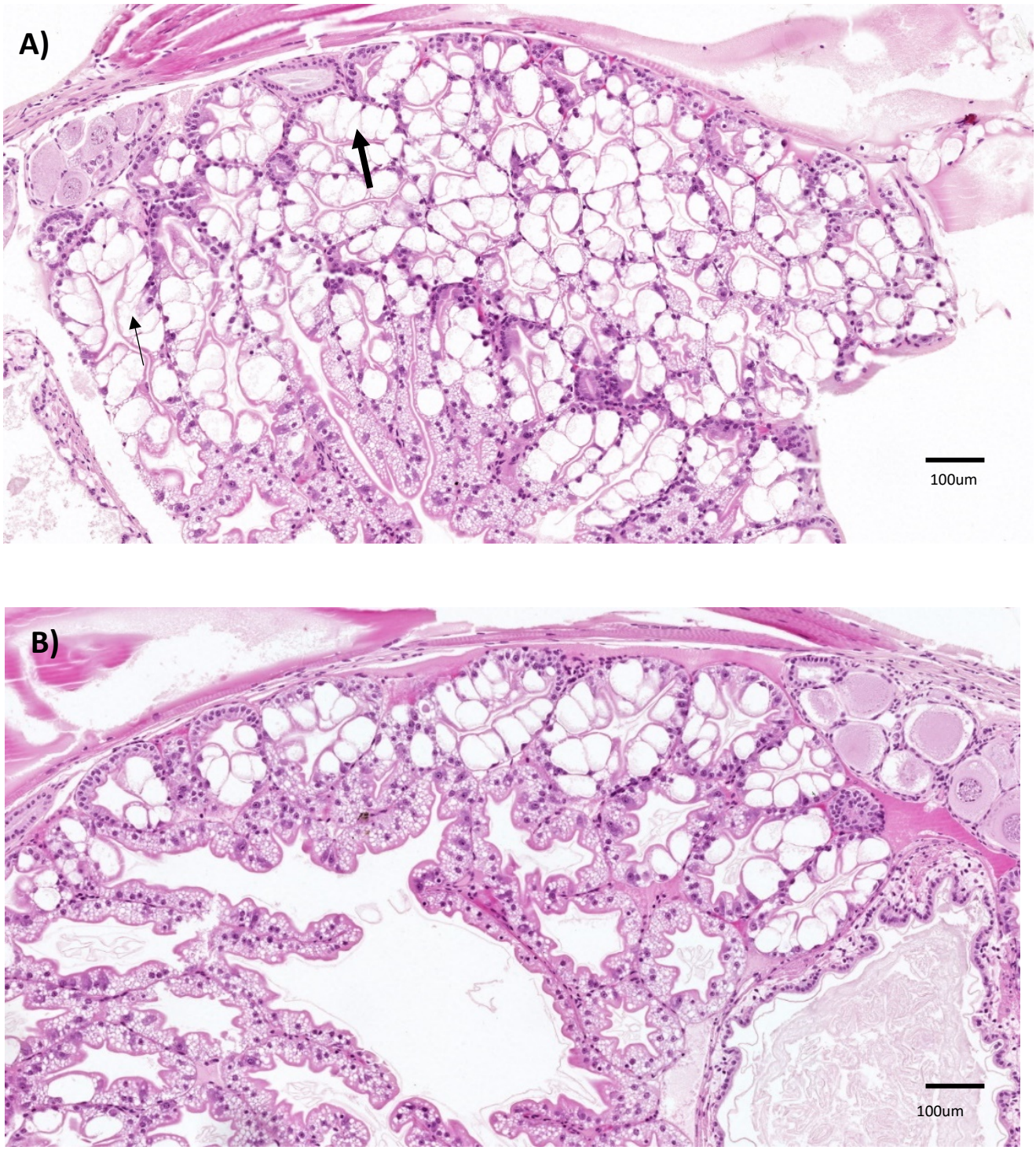


Image 4.1 Hepatopancreas of juvenile redclaw sampled prior to stocking showing a) hypertrophic b-cells (arrow) and b) slight structural abnormalities including structural disorganisation of tubules and haemal sinuses

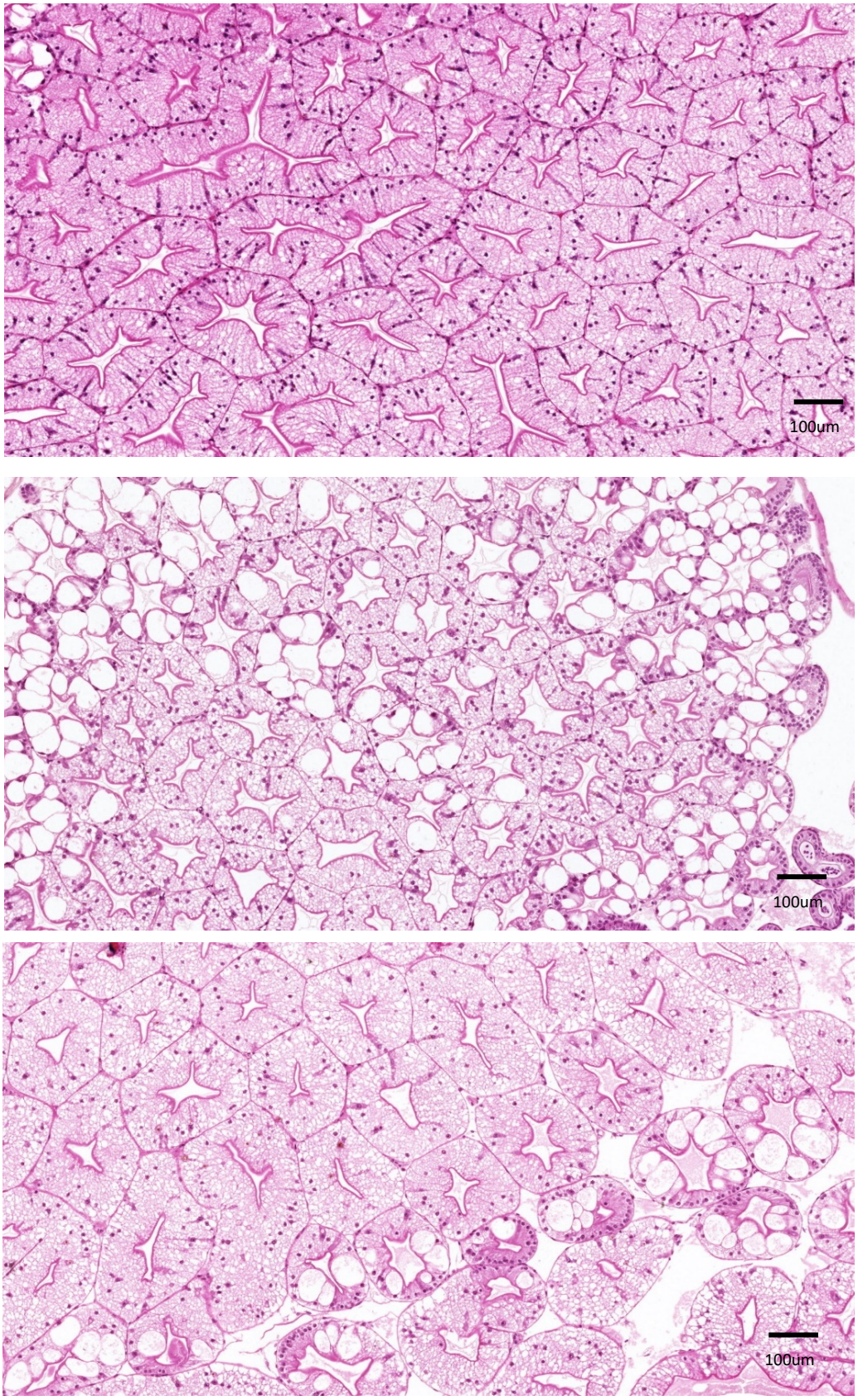


Image 4.2 Hepatopancreas of three juvenile redclaw (each image is a different individual) fed the lysine diets showing organised structure with typical star-shaped lumen

4.3. Discussion

In the present study the optimal dietary Lys inclusion for juvenile redclaw was found to be 1.72%, equivalent to 5.76% of dietary protein. These results are similar to published values for other crustacean species including *L. vannamei* at 1.64% (40% dietary CP) (Xie et al., 2012), Japanese Blue Crab (*Portunus trituberculatus*) at 2.17% (49.5% dietary CP) (Jin et al., 2013), Atlantic ditch shrimp (*Palaemonetes varians*) at 2.42% (45% dietary CP) (Palma et al., 2013) and *P. monodon* at 2.08% (35-45% dietary CP) (Millamena et al., 1998).

Feed consumed was significantly greater for redclaw fed D2, however this intake was not reflected in increased growth performance. Rather, the greatest growth occurred in redclaw fed D4 with average increase of 143%. Moreover, juvenile redclaw fed D5, which had the highest Lys level of 2.0%, grew significantly less compared to redclaw fed D4 (142.95%) ($p>0.05$), and had the least amount of growth overall (101.63%) including those fed the basal diet which had only 0.5% Lys (110.26%). Lys deficiency has been shown to impair protein synthesis, and therefore growth (Green & Hardy, 2008; Peres & Oliva-Teles, 2008) which corresponds with the lower growth in redclaw fed diets other than D4, however the decline in growth for redclaw fed D5 indicates possible deleterious effects or an imbalance in amino acid profile. Millamena et al. (1998) reported a decline in growth rates for *P. monodon* beyond the optimum requirement levels for Lys and Arg with the authors suggesting possible toxic effects of excessive dietary AAs. High concentrations of AAs may affect the rate of absorption of other AAs, with imbalances occurring due to malabsorption (Berge et al., 2004). Zhou et al. (2011) reported decreased growth and feed utilisation for black sea bream when disproportionate levels of Arg and Lys were provided. However, no competitive Lys-Arg effects was reported for Barramundi (*Lates calcarifer*) (Murillo-Gurrea et al., 2001) and Japanese flounder (*Paralichthys olivaceus*) (Alam et al., 2002). Berge et al. (1999) concluded that Lys should be presented at a higher concentration than Arg in Atlantic salmon with results showing that Lys has a both a stimulatory and inhibitive effect on the uptake of Arg, while increased arginine reduced uptake of Lys. In the present study the ratio of Arg to Lys was approximately 1:1 in redclaw fed the best performing diet (D4). A similar ratio for Arg to Lys was reported by Dong et al. (2018) for *M. rosenbergii* with the amino acids supplied at 1.4:1 arginine to Lys respectively, and for *L. vannamei* a ratio of Lys and Arg of between 1:0.88 and 1:1.05 was reported to be optimal (Feng et al., 2013). Information on the optimal ratio for Arg to Lys required in crustacean diets in general is scant, and non-existent for redclaw. Understanding of the relationships of AA interactions in general is also extremely limited for redclaw. The

optimal ratio of Arg:Lys cannot be concluded from the present study and is an area that requires further investigation.

While performance indices showed the highest %WG and SGR occurred in D4, growth was comparatively lower than that reported for juvenile redclaw used in other studies. However, the FCR values in the present study were quite good for this species ranging from 1.83 (D4) – 2.60 (D2). FCR's are similar to Garza de Yta et al. (2012) (2.1 – 2.7) and less than those reported by Pavasovic et al (2007) (5.84 – 9.31) and Thompson et al. (2005) (3.03-5.73). Comparatively lower growth rates in the current study are unlikely to be a result of starvation, as juveniles were fed twice daily, and can be contributed to the relatively low feed intake. For example, feed intake of redclaw (stocked at 0.1 g) in the study of Garza de Yta et al. (2012) was ~9.8 g feed which is triple that of the current study when converting to as fed basis, and subsequently growth of redclaw was also fourfold greater. The relatively reduced intake in the current study may have been an artefact of holding redclaw individually to prevent cannibalism, altering feeding behaviour and/or the reduced palatability of the diets as a result of formulating with relatively high proportion of starch and synthetic AAs which was necessitated to achieve the target nutrient specifications.

The Lys treatments in the current study were chosen to illicit a dose response evidenced predominantly by growth and feed efficiency. Survival may have been impacted should the Lys content have been formulated to be more deficient and/or redclaw have been exposed to malnutrition over a greater period of time. Figure 4.1 indicates an appropriate experiment design with respect to dietary Lys concentration and experiment timeframe of 12 weeks to demonstrate a dose response. Further, 100% survival of redclaw combined with different growth responses on the different diets indicates that other essential nutrients were likely to be at adequate inclusion levels and weren't limiting. Synthesis and deposition of protein is most efficient when all EAAs are supplied in adequate proportion (Bureau & Hua, 2010; Green & Hardy, 2008; Wu, 2009). As EAA requirements for redclaw are unknown a premix was used in the present study, based on the specifications on other crustacean species *P. monodon (*Millamena et al.,1998), *P. varians* (Palma et al., 2013) and *L. vannamei* (Xie et al., 2012). The fact that growth responses increased with dietary Lys level indicate that the remaining AA met or exceeded requirements. The current study notwithstanding, the absence of empirical data on the EAA requirements of redclaw, and *Cherax spp.* in general, is a major concern. Initially proposed by Wilson (1991) and Brown (1995), the ideal protein concept (IPC) has been widely applied to poultry, pigs and dairy cattle nutrition ((Boyd & Haydon, 2018; van

Milgen & Dourmad, 2015) and uses Lys as a reference amino acid to target proportionate concentrations for each of the other essential amino acids (NRC, 2011). While not conducted in the present study, it is recommended that the IPC be employed to provide baseline information on EAAs to aid in the appropriate formulation for redclaw.

It is important to consider the process of how proteins, and therefore AA's are consumed and digested. In crustaceans digestion occurs in the Hep which occupies much of the cephalothoracic cavity. In a review by Wang et al. (2009) the authors surmised the role of AA on gut integrity and function. For example, Arg stimulates intestinal fluid secretion, while glycine and Lys are reported to have protective effects on gut mucosa. Both Lys and Arg are assumed to share the same carrier system for transportation and uptake in the brush border membrane of the intestine (Berge et al., 1999). Degradation of the myoepithelial cells, sloughing of cell contents, hypertrophy of b-cells, and thinning of epithelial lumen were dietary effects observed in the redclaw fed different protein sources in Chapter 3. Further, bacterial and viral diseases were recorded as a result of the redclaw sourced were from pond raised stock. To control for these factors, redclaw for the present study were raised from craylings, sourced from a hatchery in Northern Queensland. The results from Chapter 2 & 3 highlighted the relevance of coinvestigation in relation to nutrition studies and it is important to clarify that the health status of the juveniles used in the AA study were considered. Further, the DM needed for proximate analysis, necessary to determine Lys requirement, restricted the ability to sacrifice redclaw for histology purposes. It was noted in Chapter 3 that the prawn diet may not be suitable for redclaw over long periods, with the prawn feed likely to be too high in fat content. The craylings used in this study had been raised on this feed until they reached the sizes required for stocking purposes and the Hep histology of the initial redclaw samples support this hypothesis. While there is no evidence that the health of the juveniles was compromised or impacted in any way due to the Lys diets used the low replication reduces the confidence in such conclusions. However, it is unlikely that the six individuals randomly sampled were by chance the only healthy individuals in the system.

To conclude, the results from the present study estimate the dietary Lys specification for juvenile redclaw to be 1.72%, equivalent to 5.76% of dietary protein. Establishing the requirement for Lys is a necessary step towards optimising formulated feeds for redclaw. As plant-based proteins are increasingly being used as alternatives to fish meal, it is necessary to understand the availability and role of limiting AAs like Lys to accurately formulate nutritionally complete diets. Investigations on other important EAAs such as Met are needed,

as too is a better understanding of the interactions between AAs such as Lys and Arg and the potential effects on the growth of redclaw.

5. GENERAL DISCUSSION

5.1. Overview

The continued development of the redclaw industry requires the transition from semi-intensive to more intensive culture which necessitates a greater reliance on high quality feeds. While redclaw have been farmed since the 1980s a commercially available feed formulated based on their specific nutritional requirements is still yet to be developed. This is in part due to limited published information on nutritional requirements of redclaw. The research presented in this thesis has contributed towards the knowledge base required to formulate an optimal diet. Specifically, it determined the dietary Lys requirement, assessed the protein and AA digestibly of five plant based, and two animal-rendered protein sources, and investigated diet effects on the overall health status of redclaw through a histological examination of the main digestive organ, the Hep.

Below, Chapter's 2-4 are summarised and recommendations made on the application of the research outcomes, and suggestions for future research are made. Further, future directions related to redclaw nutrition studies are also discussed.

5.2. Protein and amino acid digestibility of selected feed ingredients

With the reduction of FM in formulated feed, a combination of protein sources are required to meet the same or similar amino acid profile found in FM. Increasingly, these are plant based proteins whose AA profile often contain lower levels of EAA compared to animal products (refer Table 1.3 in Chapter 1). When assessing quality of ingredients, several key factors should be considered: chemical composition of ingredient, ingredient digestibility and palatability, nutrient utilisation, and ingredient functionality (Glencross et al. 2007). It is therefore necessary to understand the digestibility of ingredients to accurately formulate a nutritionally complete diet that meets the desired profile (Allen et al., 2003). In Chapter 2 the digestibility of seven protein sources (YPM, DDG, LM SMB, SM, PBP and FTM) were determined. The results demonstrated that both plant and animal-rendered ingredients are readily digested by redclaw. Digestible protein was similar among the ingredients (86.9% to 104.4%), as was DM (81.5% to 97.4%). ADCs for AAs have not previously been reported for redclaw, with results for all ingredients showing AA digestibility to be high (>80%). Overall, the digestibility of plant-based and animal rendered ingredients are quite good which ultimately provides greater flexibility for feed formulation.

Source and accessibility of ingredients are important when considering suitability in feeds, therefore locally produced ingredients in Australia such as canola, corn and safflower (ABARES, 2020) would be beneficial ingredients to assess. Canola meal has been shown to replace up to 20% of FM in diets for *M. japonicus* (Bulbul et al., 2013), while in *L. stylirostris* up to 30% of soybean meal, fish meal and wheat was replaced (Cruz-Suarez et al., 2001) with neither study reporting negative effects. Safflower is a crop produced in Australia, mainly for oil extraction, however meal is a by-product and is an emerging protein alternative elsewhere. A 20% inclusion of safflower meal in *O. mykiss* diets had no negative effects on growth performance, nutrient digestibility or body composition (Ustaoglu Tiril & Kerim, 2015). Similar results were reported for *L. vannamei* fed diets with high-protein safflower meal as total replacement of soybean meal, or partial replacement of FM, although authors noted further research was required in regard to differences between sources and the potential ANFs present and their effects (Galicia-González et al., 2010). Other novel ingredients of interest would be insect meals and seaweed (Hua et al., 2019). Black solider fly larvae was shown to be suitable replacement of FM in diets for *L. vannamei* (Cummins Jr et al., 2017); while seaweed additive such as *Ulva lactuca*, improved growth and carotenoid content in *L. vannamei* (Elizondo-González et al., 2018).

The protein ADC values for LM, PBP and SBM in Chapter 2 are comparable to Pavasovic et al., (2007), and the results for SM are similar to Campaña-Torres et al., (2005). Comparison across independent studies on redclaw provides confidence in terms of both the reliability of the results presented in this thesis and, by inference, then using the data to formulate a feed based on the ADC values. This is of particular relevance in light of the histological results from Chapter 3.

5.3. Histopathological surveys of redclaw fed a variety of dietary protein sources

In Chapter 3 the complexity in studying redclaw nutrition was made evident upon the histological examination of the Hep from the farm sourced redclaw from Chapter 2. The results showed severely compromised animals with a number of structural abnormalities observed, along with viral and bacterial infections, with very few individuals completely pathogen free. The two viral infections identified in this study, CqBV and CqReo, can cause depressed growth, increase inflammation, and lead to higher mortality rates in redclaw (Edgerton et al., 1995; Groff et al., 1993; Hayakijkosol & Owens, 2011; Romero & Jimenez, 2002). Histological analysis in Chapter 3 revealed clear diet related effects on the structure of the Hep and therefore

on the overall health status of redclaw. Of note is that without histopathological assessment, it would not have been evident that the redclaw were compromised to the extent that was seen. There were no indications based on the ADC values, feed intake or performance data from Chapter 2. Redclaw have been distributed worldwide, with detections of CqBV in North America and Ecuador (Groff et al., 1993, Romero & Jimenez 2002), and are susceptible to other infections endemic to the country located e.g. yellow head virus in Thailand (Soowannayan et al., 2015) and overseas populations are unlikely to be completely pathogen-free. As has been highlighted previously, there is little discussion or research on the health status of redclaw used in nutrition studies, and it raises questions on how to interpret already published studies, how the industry can respond, and to what extent latent diseases are normal for redclaw. Despite the presentation of significant histopathology, the overall performance of redclaw indicates that they are a hardy species.

The abnormalities observed in the redclaw from Chapter 2 raises some questions, but it also offers an opportunity to investigate what potential benefits redclaw can receive from dietary immunostimulants. Major limitations still exist for providing a nutritionally complete diet for redclaw as there is a lack of information concerning requirements for vitamins and minerals, EAAs and the effect of ANFs on redclaw digestion and nutrient absorption. Information on vitamin and mineral requirements is severely lacking for redclaw, but based on studies on other commercially important crustacean species such as *L. vannamei* (Niu et al., 2009); *P. monodon* (D'Abramo et al., 1994), and *P. leniusculus* (Celada et al., 2013), ascorbic acid (Vitamin C) would be of interest as it is frequently reported as an essential nutrient for growth, survival, stress resistance and successful moulting. Similarly, determining Vitamin E requirement would be highly recommended as it was established it as necessary to maintain normal hepatopancreatic structure in *P. muelleri* (Fernández Gimenez et al., 2004). Additionally, the role of dietary carotenoids may be of interest, as they have been shown to improve crustacean immunity (Babin et al., 2010) and increase growth and survival (Wade et al., 2017). Similar is the use of immunostimulants to enhance innate defence mechanisms (Song et al., 2014). In crustaceans, defence mechanisms such as the prophenoloxidase (proPO) system is triggered into action by the presence of peptidoglycans and β-1,3-glucan (Vazquez et al., 2009). Glucans are believed to modulate the immune function by binding to macrophages (Meena et al., 2013). β-glucans are β-D-glucose polysaccharides occur in the cell walls of plants such as wheat, barley and oats (Murthy et al., 2009), and are also present in the plant sources used in this study. Van Hai & Fotedar (2009) reported an increase in the surface structure of prawn

intestines and better nutrient absorption when fed Bio-Mos®, a commercially available prebiotic, and β-1,3-D-glucans. The presence of such compounds may explain why some diets, such as DDG, improved the Hep structure. Providing ingredients that have the potential to perform both a nutritional and immunostimulant role is interesting, particularly with increase used of plant-based proteins in aquafeed, and the potential for improvements in production efficiency.

Chapter 3 highlights the importance of co-investigation of health (or disease) in nutrition studies and the benefits to using histopathology as an investigative tool. Histology is a relatively simple and effective method to determine the health status of redclaw, but has only been used in a handful of published studies on redclaw nutrition (refer Table 2 in Appendix A). Research on redclaw nutrition should include a health focus, forming part of the experimental design. Where possible, studies should utilise hatchery raised redclaw, or craylings in which the exposure to infected individuals or viral vectors is non-existent, e.g. inflavirus and bunyaviruses (RNA viruses) have insect vectors found in pond environments (Sakuna et al., 2018). Currently there is insufficient data on the Hep in relation to nutrition and specific dietary effects of redclaw. Establishing baseline data for Hep assessment utilizing computer imaging to automate processes as per Berillis et al. (2013) and Cervellione et al. (2016) would facilitate relevant comparisons across different studies.

5.4. Dietary lysine requirement for juvenile redclaw

Prior to the research undertaken in this thesis there were no published data available on AA requirements for redclaw. In Chapter 4 the dietary Lys requirement for redclaw was determined to be 1.72%, equivalent to 5.76% of dietary protein, and is a major outcome for this thesis. This is a major contribution to the development of a nutritionally balanced diet for redclaw and provides the first insights into the AA requirements for this species. Further work on the full range of AA requirements, will add to this understanding.

Lys requirement can be used as the reference AA to estimate proportionate concentrations for each of the other essential amino acids (NRC, 2011). The ideal protein concept (IPC) is a method based on the concept that whole body amino acid profile is indicative of dietary requirements, and while requirements are affected by genetic, environmental and dietary factors, the ratio of EAAs will remain relatively constant (Akiyama et al., 1997; Furuya et al., 2015) Availability of similar sized animals, and logistical issues meant it was not possible to apply the IPC to redclaw in this thesis. While the IPC provides an estimate only of AA

requirement, it does provide a useful starting point to formulate diets in the absence of empirical data.

As discussed in Chapter 4, a better understanding of the interactions between AA would be of relevance due to the potential Arg/Lys interactions that have resulted in decreased growth and feed utilisation for other species, such as *P. monodon* (Millamena et al., 1998). If plant-based protein sources are being incorporated into diets, consideration on how AAs are utilised is also important. Taurine is found in very low to absent levels in plants (Lunger et al., 2007) and while it can be synthesised, taurine deficiency has shown to result in poor growth performance in teleost fish (Salze & Davis, 2015), and is responsible for green liver syndrome in Japanese amberjack (*Seriola quinqueradiata)* (Takagi et al., 2005), and Red Sea Bream (*Pagrus major*) (Takagi et al., 2011). Studies on taurine requirement for crustaceans is limited, however a study by Dong et. al., (2018) showed improvements in growth and immune function in *E. sinesis* when optimal taurine requirements were met. Further, *P. monodon* has the ability to synthesise taurine, but the ability to do so is affected by the level cystine (Richard et al., 2011). Similarly, Met has been shown to spare the requirement for taurine in yellowtail kingfish (Candebat et. al. 2020). The interaction of sulfur amino acids with respect to requirement in redclaw is currently unknown and is an important area of further research particularly as Met is often one of the first limiting amino acids when using plant derived dietary proteins.

5.5. Future Directions

The information in this thesis highlights the need for closer investigation on the effect of diet and health of redclaw. The next logical step in redclaw nutrition is understanding the role of microbiota on health, growth and survival. An emerging area of nutrition research, microbiota relates to the commensal, symbiotic and pathogenic microorganisms (Peterson et al., 2009) that make up a hosts microbiome. The microbiome plays important roles in modulating immune response, nutrient absorption, and metabolic process, with some of these beneficial bacteria able to supress or eliminate pathogenetic compounds (Yukgehnaish et al., 2020; Zoqratt et al., 2018). Function and abundance of microbiota can be influenced by feed intake, hormone secretion, feed intake, stress and environmental conditions (Bikel et al., 2015). Importantly, bacteria or viruses can compete with host microbiota for resources and antimicrobial compounds (Brestoff & Artis, 2013; Cornejo-Granados et al., 2017). Microbial imbalances could offer an explanation for the lowered immune function and severe infections that were observed in some individuals sampled in Chapter 3. The use of probiotic diets have been shown to improve growth, metabolic activity and reduced pathogen abundance in Marron (*Cherax*

cainii) (Foysal et al., 2019) and would be a useful areas of research to reduce the impact of diet related effects.

Understanding the genetic potential that currently exists in the current population will also help to understand the mechanisms surrounding growth, and nutrient uptake in redclaw. A breeding program for redclaw was conducted by RIRDC and the NQCFA from 2007 – 2012 which aimed to reduce inbreeding depression while selecting for faster growth and to produce pathogen free stock (Stevenson et al., 2013). However, results from this thesis indicate that these are still issues that exist and the genetic potential is largely untapped. Crossbreeding of three groups from different regions in Mexico enhanced commercially important traits (Hernández-Gurrola et al., 2020) but it is not apparent that any follow-up genetic studies have been conducted for the Australian populations. The assessment of the genetic integrity of a population is important as an improvement in feed formulations will provide only limited benefits to genetically compromised stock.

5.6. Overall conclusion

The results from this thesis provide the baseline information required to enable diets to be formulated based on the Lys requirement for redclaw, and with an understanding of ingredients digestibility. However, there needs to be greater consideration on the health status of redclaw, and the interactions between diet and health both positive and negative. Histopathological techniques should be implemented more frequently as a diagnostic tool in nutrition studies on redclaw. Providing a formulated feed based on the nutritional requirements of redclaw but containing ingredients that improve immune function and growth would be ideal, but this objective requires further research into AA and vitamin and mineral requirements. The redclaw industry has great potential for improvement if baseline information on nutritional requirements continues to be established, and the relationship between diet and health is made a primary focus for future studies.

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APPENDIX A - MATERIAL

TABLES

Table 1. Articles that have investigated redclaw digestibility from Scopus search

Authors	Title	Year	Source title	DOI
Rachmawati, D., Prihanto, A.A., Setyobudi, R.H., Anne, O.	Effect of papain enzyme in feed on digestibility of feed, growth performance, and survival rate in post larvaes of freshwater lobster [cherax quadricarinatus (Von martens, 1868)]	2018	Proceedings of the Pakistan Academy of Sciences: Part B	
Sacristán, H.J., Fernández-Gimenez, A.V., Chaulet, A., Franco Tadic, L.M., Fenucci, J., López Greco, L.S.	Effect of different diets on digestive enzyme activities, in vitro digestibility, and midgut gland structure in juvenile crayfish, Cherax quadricarinatus	2016	Acta Zoologica	10.1111/azo.12134
Campaña-Torres, A., Martinez-Cordova, L.R., Villarreal-Colmenares, H., Civera-Cerecedo, R.	Carbohydrate and lipid digestibility of animal and vegetal ingredients and diets for the pre-adult redclaw crayfish, Cherax quadricarinatus (von Martens)	2008	Aquaculture Research	10.1111/j.1365-2109.2008.01980.x
Pavasovic, A., Anderson, A.J., Mather, P.B., Richardson, N.A.	Effect of a variety of animal, plant and single cell-based feed ingredients on diet digestibility and digestive enzyme activity in redclaw crayfish, Cherax quadricarinatus (Von Martens 1868)	2007	Aquaculture	10.1016/j.aquaculture.2007.08.027
Campaña-Torres, A., Martínez-Córdova, L.R., Villarreal-Colmenares, H., Civera-Cerecedo, R.	Carbohydrate and lipid digestibility of animal and vegetal ingredients and diets for juvenile Australian redclaw crayfish, Cherax quadricarinatus	2006	Aquaculture Nutrition	10.1111/j.1365-2095.2006.00388.x
Pavasovic, A., Richardson, N.A., Mather, P.B., Anderson, A.J.	Influence of insoluble dietary cellulose on digestive enzyme activity, feed digestibility and survival in the red claw crayfish, Cherax quadricarinatus (von Martens)	2006	Aquaculture Research	10.1111/j.1365-2109.2005.01389.x
Campaña-Torres, A., Martinez-Cordova, L.R., Villarreal-Colmenares, H., Civera-Cerecedo, R.	In vivo dry matter and protein digestibility of three plant-derived and four animal-derived feedstuffs and diets for juvenile Australian redclaw, Cherax quadricarinatus	2005	Aquaculture	10.1016/j.aquaculture.2005.02.058

Table 2. SCOPUS query string and resulting number of research articles

SCOPUS query string		No. research articles
1	TITLE-ABS ("Cherax Quadricarinatus") OR TITLE-ABS ("redclaw") OR TITLE-ABS ("C. quadricarinatus")	501
2	TITLE-ABS ("Cherax Quadricarinatus") OR TITLE-ABS ("redclaw") OR TITLE-ABS ("C. quadricarinatus") AND TITLE-ABS ("nutrition") OR TITLE-ABS ("nutritional") OR TITLE-ABS ("feed") OR TITLE-ABS ("ingredient") OR TITLE-ABS ("requirement") OR TITLE-ABS ("diet") OR TITLE-ABS ("food")	128
3	TITLE-ABS ("Cherax Quadricarinatus") OR TITLE-ABS ("redclaw") OR TITLE-ABS ("C. quadricarinatus") AND TITLE-ABS ("nutrition") OR TITLE-ABS ("nutritional") OR TITLE-ABS ("feed") OR TITLE-ABS ("ingredient") OR TITLE-ABS ("requirement") OR TITLE-ABS ("diet") OR TITLE-ABS ("food") AND TITLE-ABS ("hepatopancreas") OR TITLE-ABS ("histology") OR TITLE-ABS ("histopathology")	22
Scopus (https://www-scopus-com) on 20 February 2020		

Table 3. List of research articles from SCOPUS query string 3

Hepatopancreas Treatment	**Analysis**	**Diet**	**Reference**
Frozen in liquid nitrogen	RNA extraction	Formulation provided	Wu et al., 2018
Bouin and Baker solution	Histology	Tetracolour	Calvo et al., 2018
Bouin solution	Histology	Experimental diets from (Gutiérrez & Rodríguez 2010)	Castillo et al., 2017
Homogenation	RNA extraction	N/A	Tan et al., 2016
Bounin solution	Histology	Tetracolour	Stumpf et al., 2015
Frozen in liquid nitrogen	RNA extraction	Not specified	Dammannagoda et al., 2015
Bounin solution, frozen	Histology, biochemical and enzymatic analysis	Broodstock fed Tetracolour, and *Elodea sp*. Juveniles from those were fed experimental diets a) tetracolour and b) Gutiérrez and Rodríguez (2010)	Stumpf et al., 2014
Oven dried, frozen -80	Dry weight, biochemical analysis	Tetracolour	Calvo et al., 2013
Dissection, frozen	GSI, HIS and Biochemical composition	Formulation based on Rodríguez-González et al. (2009).	Rodríguez-González et al., 2013
Dissection, frozen -80	GSI, HIS and Biochemical composition	Formulation provided	Wang et al., 2013
Tissue sample	RNA extraction	N/A	Li et al., 2013
Homogenation	Biochemical analysis	Tetracolour and Elodea sp. then formulation specified	Chaulet et al., 2012

Histological method from López Greco et al., 2007.	Histology	Tetracolour, *Elodea sp.*	Calvo et al., 2012
Homogenation	Biochemical composition	Formulation provided	Rodríguez-González et al., 2011
Histological method from López Greco et al., 2007.	Histology	Tetracolour	Calvo et al., 2011
Dissection, frozen	GSI and biochemical composition	Formulation provided	Li et al., 2011
Frozen -80	Biochemical composition	Formulation provided	Gutierrez & Rodríguez (2010)
Frozen -80	Biochemical composition	Commercial pellets (Hongma Feed Company, Shanghai, China) with 35% crude protein and 8% lipid, which is close to the optimum for the development of the gonads (Rodríguez-González et al. 2006)	Li et al., 2010
Dissection, Homogenation	GSI, HSI and biochemical composition	diets formulated according to Cortés-Jacinto et al. (2003).	RodrÍguez-González et al., 2009
Homogenation	Digestive enzyme assay	prepared in accordance to Civera & Guillaume (1989); supplemented with different protein sources	López-López et. al. 2005
Davidson's fixative	Histology	Commercial shrimp pellets (32% crude protein, 8% lipid)	GarcÍa-guerrero et al., 2003

Tetracolour is a commercially available feed provided to tropical fish under the TETRA brand.
GSI = gonadosomatic index, HSI = hepatosomatic index

APPENDIX B – MATERIAL

TABLES

IMAGES

Table 1. Grading scheme used to assess the health of C. quadricarinatus fed alternative protein sources

	Degradation
Score	Histological Finding
1	Structural degradation is not evident
2	Degradation occurring in low to moderate number of tubules/regions of hepatopancreas
3	Moderate number of tubules/regions of hepatopancreas is degraded
4	Severe structural degradation throughout hepatopancreas
	Structure
Score	Histological Findings
1	Connected and uniform; cell walls are touching and there is uniformity in tubule shape/size e.g. honeycomb
2	Connected but not uniform; cells walls are touching but the structure is not uniform
3	Not connected and uniform; cell walls are not connected but are still uniform
4	Not connected and not uniform; cells walls are not connected and not uniform
	Tubule Lumen
Score	Histological Findings
1	Thick epithelium with a distinct star shaped or folded lumen
2	In between thick/thin epithelium with oval/rounded lumen
3	Thin epithelium with folded lumen
4	Thin epithelium with oval/rounded lumen
	Abundance of B-cells
Score	Histological Findings
1	Expected level or b-cells (b-cells are not in every tubule and limited to medial and distal parts)
2	Tubules with b-cells are form a border around tissue
3	Tubules with b-cells forming a multi-layer border
4	All or majority of tubules contain b-cells i.e. 3+ layers in border not restricted to medial and distal parts
	Hypertrophy of B-cells
Score	Histological Findings
1	No hypertrophy of b-cells in tubules
2	Low to medium level of hypertrophy of b-cells, with few collapsing into larger ones
3	Medium level of b-cell hypertrophy in tubules, with mstructural collapses
4	All or majority of tubules have hypertrophy of b-cells, with
	Granulomas
Score	Histological Findings
1	Few if any granulomas <10
2	Low to moderate numbers of granulomas <30
3	Medium to high number of granulomas <50
4	Severe/systemic number of granulomas i.e. 50+
	Bacteria
Score	Histological Findings
1	Bacteria not evident or at levels not a concern
2	Low levels of bacteria in localised area
3	Medium level of bacteria in tissue
4	High/systemic level of bacteria in tissue/region
	CqBV

Table 1. Grading scheme used to assess the health of C. quadricarinatus fed alternative protein sources continued

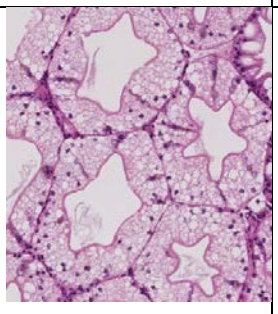
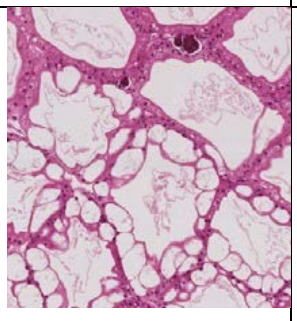
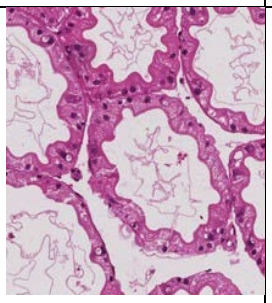
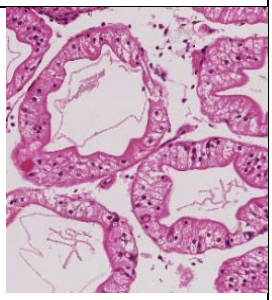
Score	Histological Findings
1	infection/lesions are not evident or at levels not significant
2	low to moderate number of pathogen/lesions, or high number of lesions but in few tubules
3	Moderate numbers of pathogens/lesions, or low no. lesions but in multiple tubules
4	High numbers/severe lesions in multiple tubules

	Lipid Storage
Score	Histological Finding
1	Lipid vacuoles normal/no evidence excessive lipid
2	Increase in vacuoles, but no structural changes in lumen
3	Increase in vacuoles, some structural changes in lumen changes
4	Increased vacuoles, with major structural changes in lumen (coalescing into larger)

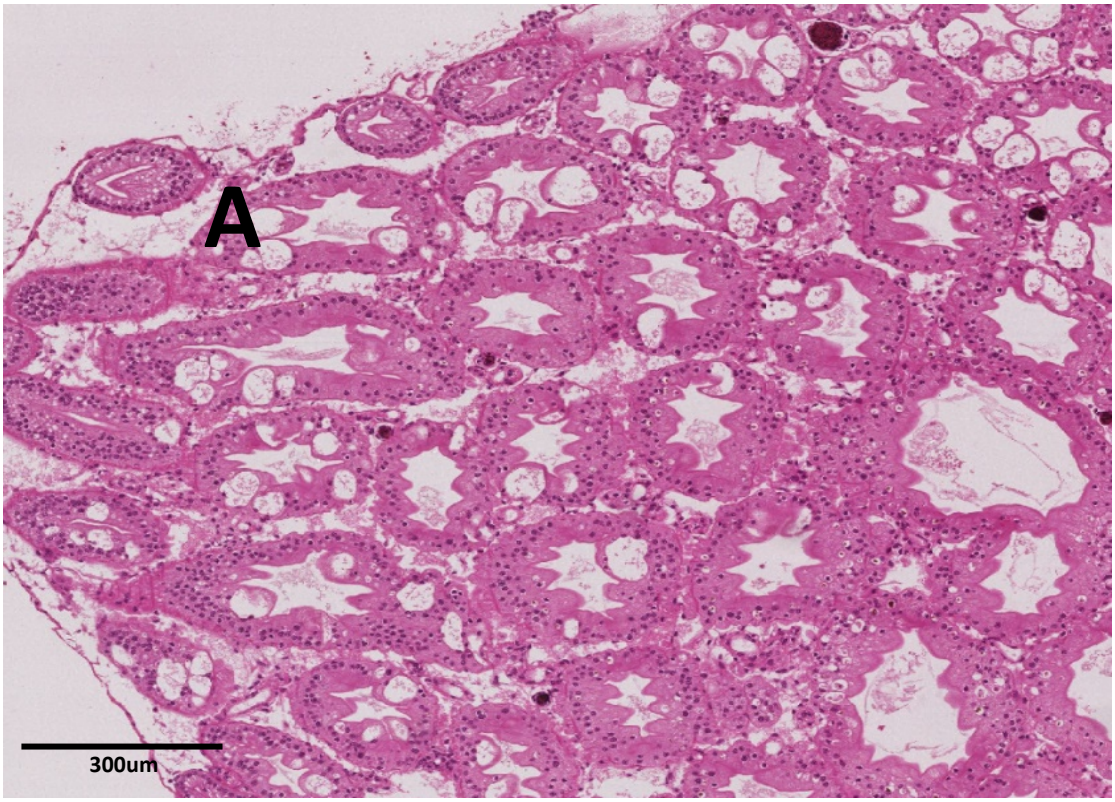
Table 2. Number of hepatopancreas tubules infected with CqBV when comparing the transect method or whole tissue analysis

Slide #	Quadrant	Pea Meal (PM)		Feather Meal (FM)		Dried Distillers Grains (DDG)	
		Transect	Whole	Transect	Whole	Transect	Whole
1	1	2	4	0	0	0	0
	2	0	3	0	4	0	1
	3	0	7	0	0	1	2
	4	3	5	0	1	0	0
2	1	1	9	0	2	0	4
	2	1	10	0	1	0	0
	3	3	7	0	2	0	0
	4	1	5	1	2	0	0

Table 3. Classification of overall hepatopancreas structure relevant to identifying health status of redclaw

1. Connected and uniform = the cell walls are touching and there is uniformity in shape e.g. honeycomb	2. Connected but not uniform = cell walls are touching but the structure is not uniform in shape	3. Not connected and uniform = cell walls are not connected but are still uniform in shape	4. Not connected and not uniform = cell walls are not connected and not uniform in shape
			

Images taken using Aperio ImageScope. Magnification is **300um**



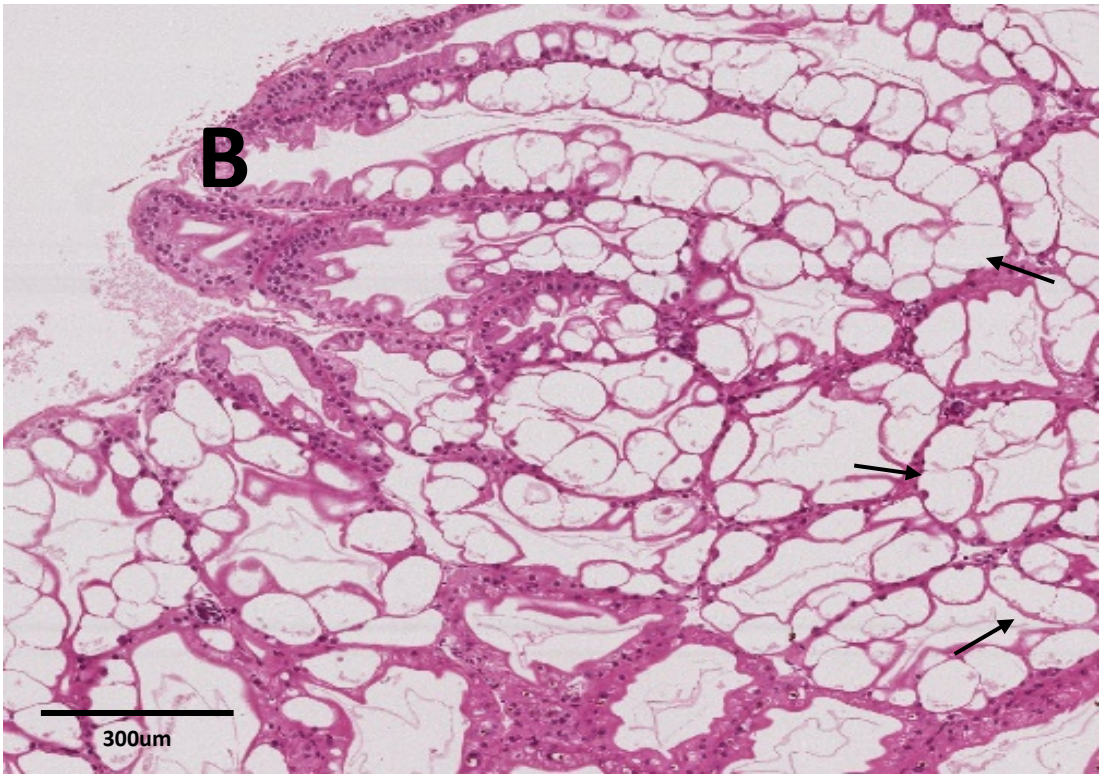


Image 1. A) Normal level of b-cells. B) increased abundance of tubules with enlarged b-cells, arrows show b-cells that have coalesced into larger ones

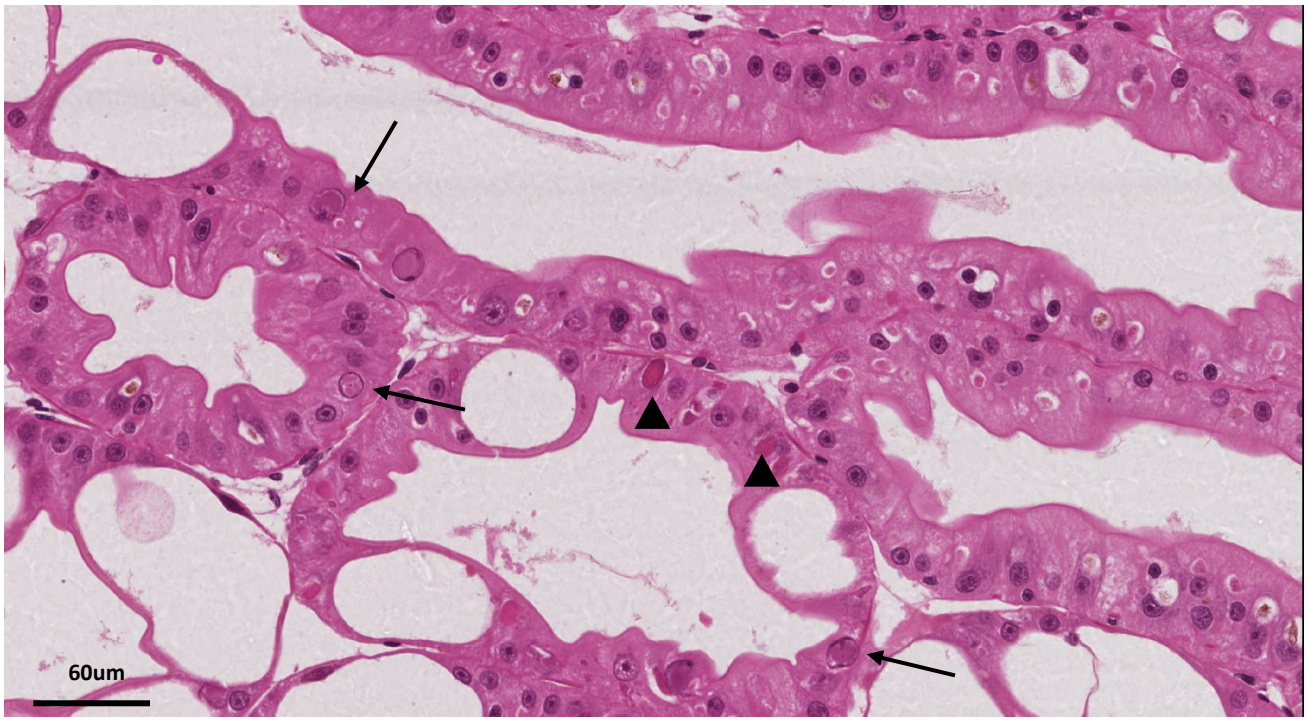


Image 2. Coinfection of Cherax quadricarinatus bacilliform virus (CqBV)(arrows) and Reovirus (triangle). Note the displaced nucleoli with marginated chromatin and infection in adjacent tubules. From redclaw fed the soybean diet.

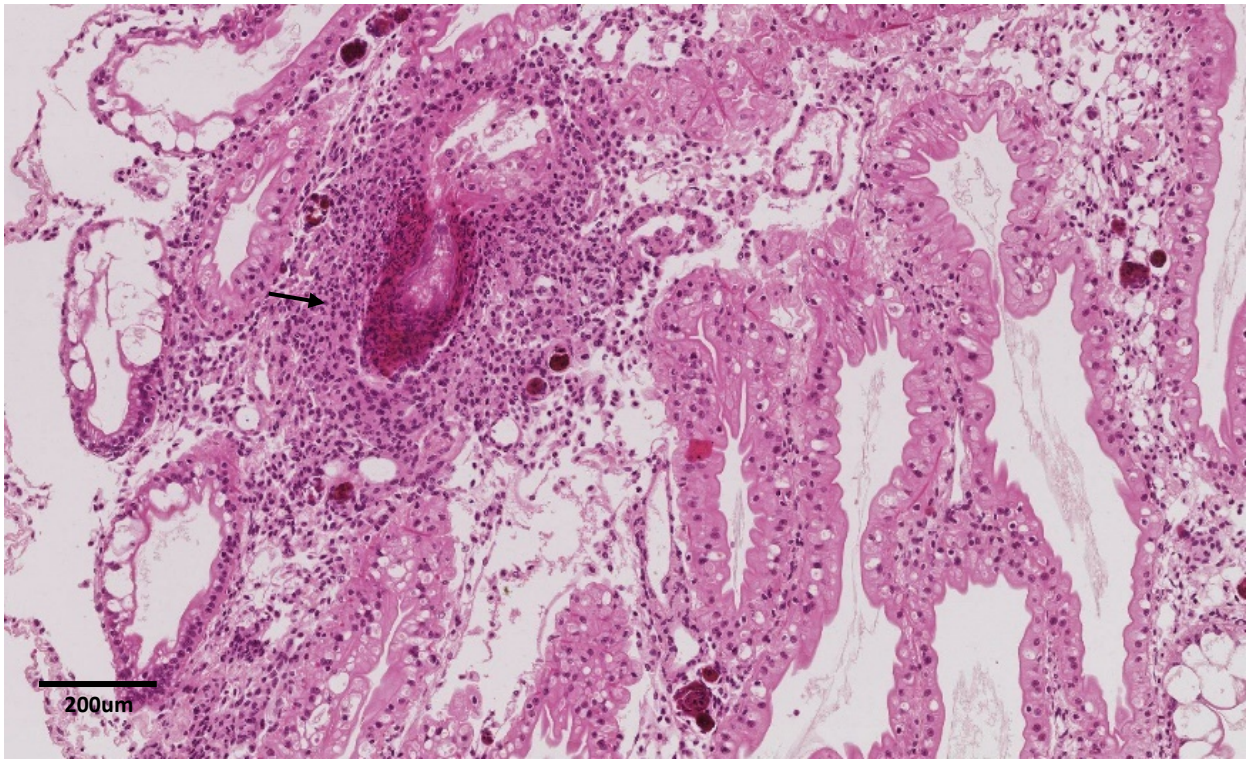


Image 3. Bacteria in hepatopancreatic tubules

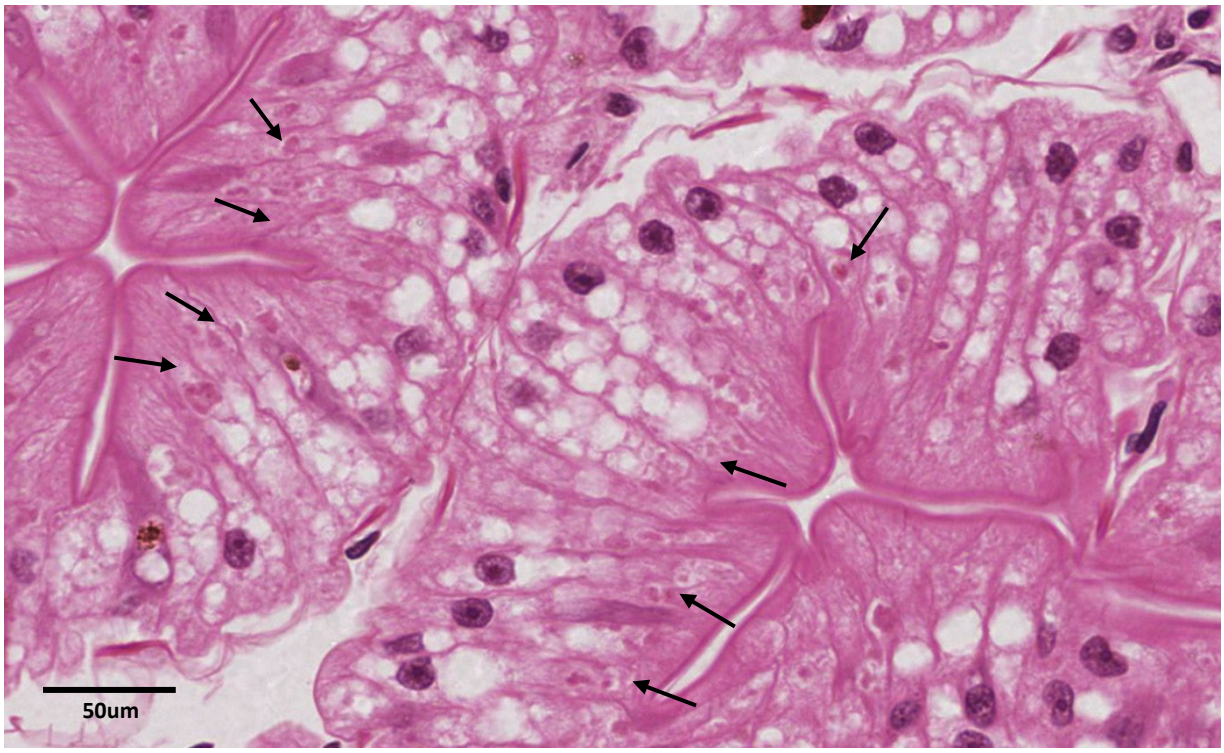


Image 4. Arrows indicate abnormality in lumen epithelium believed to be protein precipitation

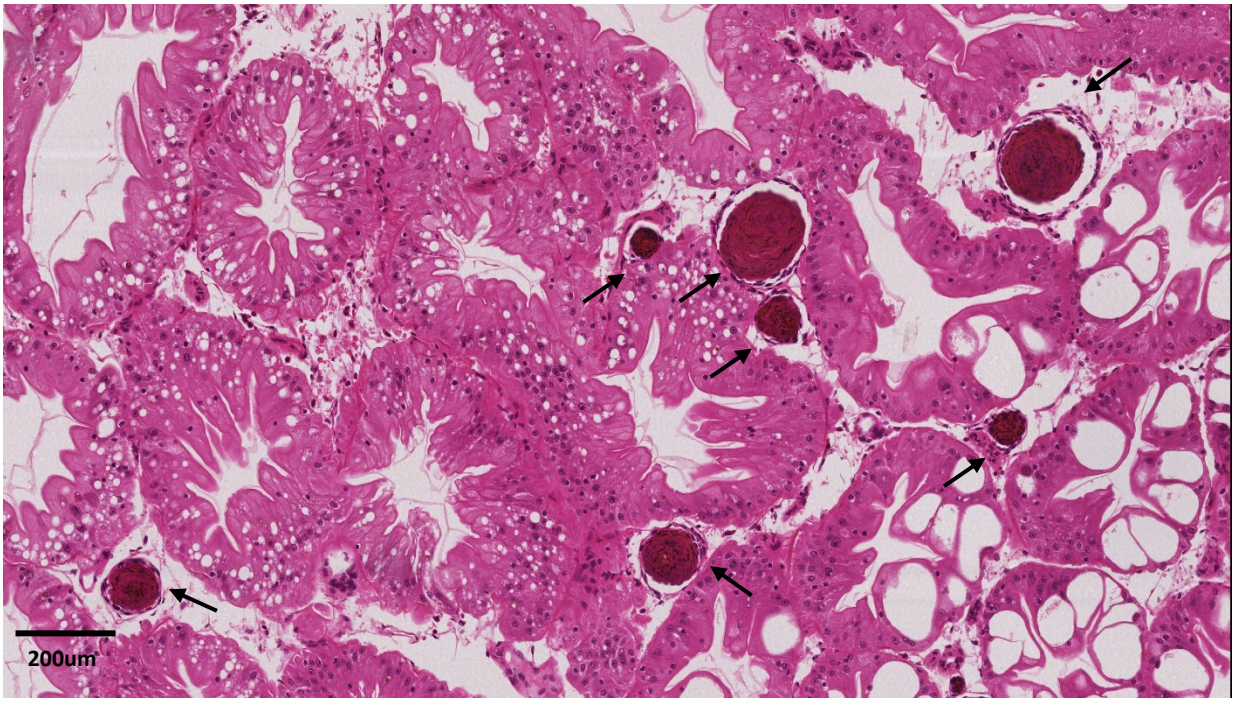


Image 5. Large systemic granulomas (arrows) in hepatopancreas. From redclaw fed the feather meal diet.

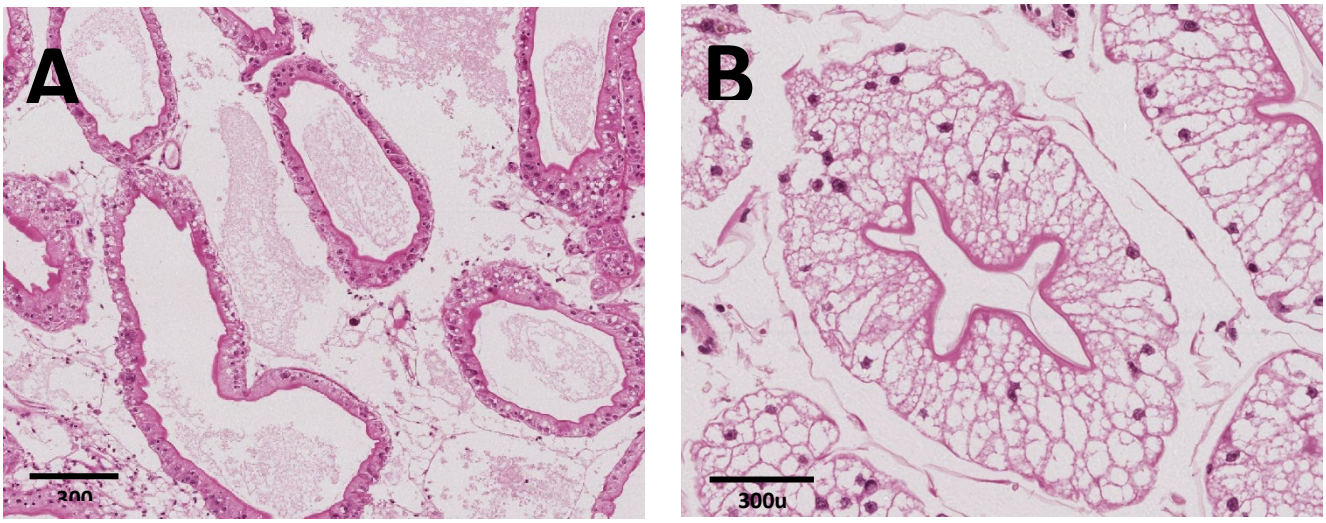


Image 6. A) degradation of the myoepithelial cells and sloughing of cell contents B) inner lumen shrinking away from basal lamina

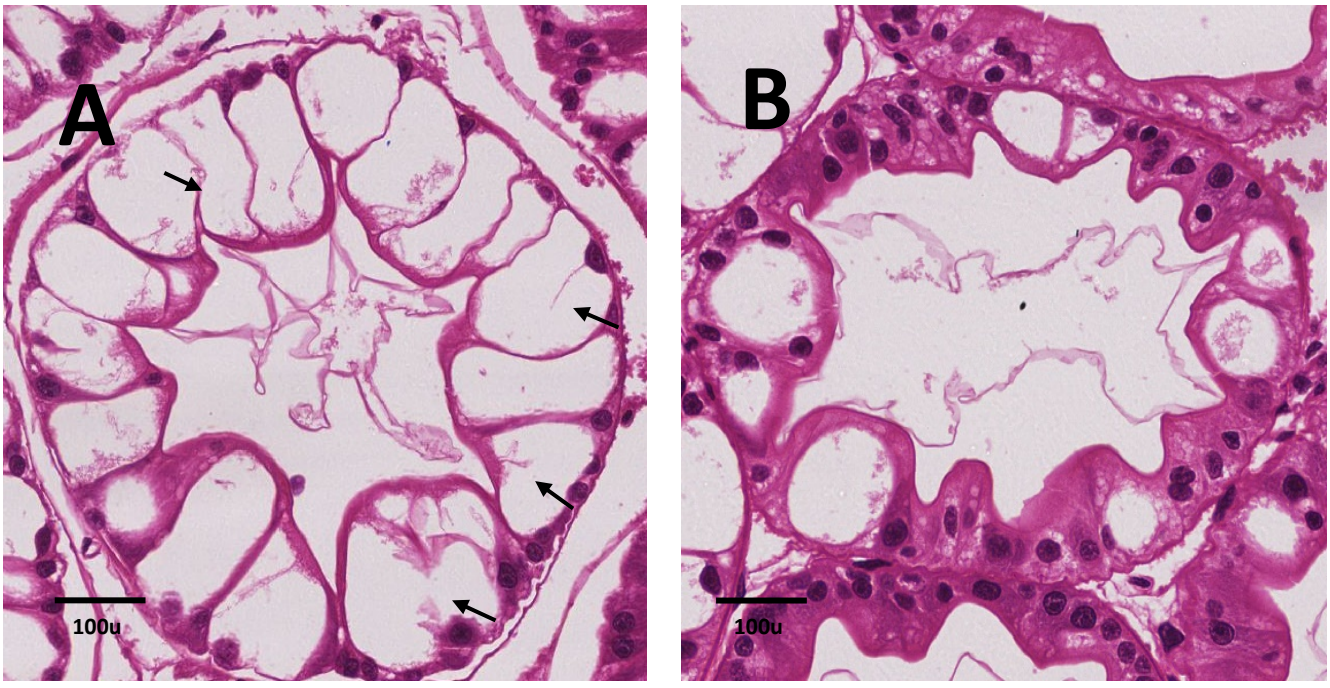


Image 7. A) hypertrophy of b-cells. Arrows indicate b-cells that have coalesced into larger ones B) tubule with normal b-cells