Liver structure and function in Yellowtail Kingfish, *Seriola lalandi*, under dietary manipulation

By

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Contributions

Chapters 2, 3, 4, 5 and 6 are presented in stand-alone manuscript format suitable for a journal in the field of aquaculture. Therefore, some unavoidable repetition between chapters is observed, particularly in methods and background information. I wrote each of the chapters, but each chapter has contributions from other people. Mr Benjamin H. Crowe conceptualization, data generation, data analysis, manuscript preparation, thesis preparation, thesis editing, visualization. Associate Professor James O. Harris conceptualization, data generation, data analysis, thesis editing, visualization, project administration. Associate Professor David A.J. Stone conceptualization, data generation, data analysis, thesis editing, visualization, project administration. Dr Todd J. McWhorter data generation, thesis editing. Dr Matthew S. Bansemer conceptualization, data generation, data analysis, thesis editing, visualization, project administration. Dr Fran Stephens data analysis, thesis editing.

Illustrations

All Yellowtail Kingfish, *Seriola lalandi*, illustrations are by Dr René Campbell, copyright 2020. All images are produced with permission of the artist.

Summary

Yellowtail Kingfish, *Seriola lalandi,* is a carnivorous finfish species valued in aquaculture for its fast growth rate and excellent meat quality. The aquaculture industry is reducing fish oil and fish meal content in formulated feeds. Alterations to lipid storage and the effects on liver structure need to be considered when reducing fish oil (FO) (lipid) and fish meal (FM) (protein) content in formulated diets for commercial Yellowtail Kingfish. Changes in either or both protein and lipid level have the potential to alter liver structure, the levels of essential fatty acids, bile acids, amino acids, and other nutrients such as the cholesterol required for maintaining animal health and growth. In this thesis is the first report of gross and somatic indices, liver histology, bile acid and taurine concentrations, liver enzyme concentration and blood biochemistry for wild caught Yellowtail Kingfish from South Australian waters. These data provide a point of reference for the development and further optimisation of formulated feeds for this commercially important species. In this thesis I report on liver structure and energy reserves, concentrations of enzymes involved in cholesterol and bile acid metabolism and blood biochemistry in both wild and cultured Yellowtail Kingfish.

Yellowtail Kingfish may be able to be fed diets with up to 30% crude lipid (CL) and 48% crude protein (CP) without affecting total bile acid synthesis, storage and liver lipid storage. Yellowtail Kingfish fed a 30% wild-derived FM (wd-FM) diet may be fed diets where the FM can be replaced by up to 66.67% FM by-product, 33.3% poultry meal (PM) or 33.3% soy protein concentrate (SPC) without any significant impact on total bile acid synthesis, storage and excretion and liver lipid storage. However, severe bile duct proliferation and periductular fibrosis was observed in the livers of fish fed diets with wd-FM replacement with 10% PM or 10% SPC.

Yellowtail Kingfish may be fed a formulated diet containing either: 1) ~25% dietary lipid level (2.12 g $100g^{-1}$ of ΣLC n-3 PUFA) with up to 100% of the fish oil (FO) component replaced by poultry oil (PO); or 2) ~25% dietary lipid level (2.12 g $100g^{-1}$ of ΣLC n-3 PUFA) with up to 100% of PO with canola oil without an apparent negative impact on any of the parameters measured in this study. Emulsifiers may be required if bile acid production decreases from inclusion of alternative oil sources. The additional of a commercial emulsifier at 40 mg lipid kg^{-1} in diets with different levels of CL (~30 g 100 g^{-1} and ~20 g 100 g^{-1}) presents no benefit for the production of Yellowtail Kingfish.

Yellowtail Kingfish have nutritional plasticity enabling them to grow and maintain enterohepatic function on a variety of diet formulations. Much of this capacity is due to their livers, the organ foremost responsible for cholesterol regulation and synthesis of bile that enables emulsification and uptake of nutrients. Therefore, should an adverse environmental or biological event occur challenging the animals' health further, the impacts this might also have on the liver or whole animal is unknown.

Declaration

I certify that this thesis:

1. does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university

2. and the research within will not be submitted for any other future degree or diploma without the permission of Flinders University; and

3. to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

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Benjamin Harry Crowe

1 November, 2021

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This thesis is dedicated to my Grandpa, Christopher Crowe, whose love and encouragement has spurred me on and who taught me ‘nothing is too much trouble’.

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Thesis extension

Chapter 5:

Crowe, B.H., Harris, J.O., Stone, D.A.J., McWhorter, T.J., Bansemer, M.S. Effect of partial wild derived fish meal replacement on bile acid production and liver structure in Yellowtail Kingfish, *Seriola lalandi*. 18th International Symposium on Fish Nutrition and Feeding. Poster Presentation. 3rd – 7th June 2018. Gran Canaria, Spain. 45 min.

Contributions of research to other reports:

Bansemer, M.S., Stone, D.A.J., Skordas, P., Currie, K.L., Crowe, B., Nankervis, L., Salini, M. (2018). "Reducing dietary wild derived fish meal inclusion levels in production diets for large Yellowtail Kingfish (*Seriola lalandi*)" in Growing a Profitable, Innovative and Collaborative Australian Yellowtail Kingfish Aquaculture Industry: Bringing 'White' Fish to the Market. Stone, D.A.J., and Booth, M.A. (eds). South Australian Research and Development Institute (Aquatic Sciences) (DAWR Grant Agreement RnD4Profit-14-01-027), Adelaide, December 2018.

Chapter 6:

In addition:

Liver histological analyses from each chapter have been compiled and presented.

Crowe, B.H., Harris, J.O., Stone, D.A.J., McWhorter, T.J., Bansemer, M.S., Stephens, F. Histological observations of dietary energy and protein influences on liver structure in Yellowtail Kingfish, *Seriola lalandi*. 5th FRDC Australasian Scientific Conference on Aquatic Animal Health & Biosecurity. Presentation. 8 – 12th July 2019. Cairns, Australia. 15 min.

Chapter 1: General introduction

1.1 Introduction

The global population is expected to increase to 9.6 billion people by 2050, along with the associated task of feeding the increasing population (Dawson and Johnson, 2014; FAO, 2020b). Over the past few decades, an increased global shift towards finfish for human consumption has resulted in increasing growth in the culture of animals within the aquaculture industry to satisfy demand. The shift has also resulted from the majority of world fish stocks becoming unsustainably fished, with the trend driven mostly by limited management and governance capacities in developing countries (FAO, 2020b). The aquaculture industry may be capable of supplying the rapidly increasing global population. Since 2014, supply from cultured species has surpassed that of wild capture fisheries (FAO, 2020a and 2020b). Global aquaculture production is expanding rapidly, increasing from 36.8 million tonnes in 2002 to 73.8 million tonnes in 2014 and reaching a record high at 110.2 million tonnes in 2016 with an estimated value of US$243.5 billion (Mitchell & Lynstad, 2019; FAO, 2020b). The global aquaculture industry is estimated to provide employment to 19.3 million workers (Mitchell & Lynstad, 2019). This employment base is expected to increase as projections by the United Nations suggests by 2030 production growth in aquaculture will be 37 percent great than 2016 (FAO, 2020b).

The growth of aquaculture in Australia has also been rapid with employment in the sector increasing from 3,298 people in 2012-13 to 7,000 people in 2019-20 (ABARES, 2020a and 2022a). Within the combined Australian fishing and aquaculture industry, aquaculture makes up 53% of the total employment (ABARES, 2020a). Australia has seen the proportion of wild stocks fished within biologically sustainable levels increase from 27% in 2004 to almost 85% in 2018 through good stock management (FRDC, 2020a; FAO, 2020a and 2020b). Domestic Australian demand for fish products continues to increase despite the global trend of fisheries

decline. Australian apparent seafood consumption increased by an estimated 1.3% from 2006-07 to 2016-17 (ABARES, 2020b). However, since 2016-17 a 1.83% decrease in apparent seafood consumption has been observed due to consumer behavioural changes influenced by domestic COVID-19 outbreaks (ABARES, 2022b). The value of Australian fishery commodity imports (nominal aquatic animal catches, aquaculture production and imported raw materials) exceeded that of fishery commodity exports by 35.7% in 2019-20 in response to market demand (FAO, 2020a). This demonstrates that aquaculture has a strong and assured future in supplying seafood products in Australia.

While Australian fisheries gross production and gross production value decreased in 2019-20 by2% the aquaculture sector increased by 11% to 106,139 tonnes worth $1.6 billion (ABARES, 2022c). The Australian aquaculture sector now contributes to 38% of the nation's total fisheries and aquaculture gross production value; up from 29% in 1999-2000 (ABARES, 2021 and 2022c). The Australian aquaculture industry is expansive and was dominated by several species in 2019-20 (Table 1.1: ABARES, 2022d).

Table 1.1. Aquaculture production in 2019-20 in Australia (ABARES, 2022d).

Species	Tonnes	Value ($AUD '000)
Salmonids (Atlantic salmon, *Salmo salar*, rainbow trout, *Onchorhynchus mykiss*, and brown trout, *Salmo trutta*)	66,015	890,468
Pacific oyster, *Crassostrea giga*	9,011	114,448
Southern bluefin tuna, *Thunnus maccoyi*	8,345	137,000
Prawns (black tiger brawn, *Penaeus monodon*, banana prawn, *Fenneropenaeus merguiensis*, and karuma prawn, *Penaeus japonicus*)	6,740	134,492
Barramundi, *Lates calcarifer*	3,427	91,083
Blue mussel, *Mytilus galloprovincialis*	2,342	6,043
Abalone (greenlip abalone, *Haliotis laevigata*, blacklip abalone, *Haliotis rubra* and their hybrid)	549	22,002
Silver perch, *Bidyanus bidyanus*	185	2,331

In particular, the growth of Australian aquaculture is driven by the expansion of the salmonid industry which increased production value by 132% from $369 million in 2006-07 to $890 million in 2019-20 with production volume increasing to a peak of 66,015 tonnes (ABARES, 2021 and 2022c). A variety of both freshwater and marine finfish species such as Yellowtail Kingfish, *Seriola lalandi*, black bream, *Acanthopagrus burcheri*, King George whiting, *Sillaginodes punctatus*, snapper, *Pagrus auratus*, mulloway, *Argyrosomus hololepidotu*, and Murray cod, *Macculochella peelii peelii*, have also been trialled and are developing species in aquaculture in Australia (Fotedar and Phillips 2011).

Successful aquaculture ventures require understanding of all aspects of a target species. Specific knowledge of the species' biology, culture methods, farm sites and economics will drive the success or failure of a venture (Lucas and Southgate, 2012). Species must also meet a region's environmental, social and market requirements, including product acceptance, to be successful (Alvarez-Lajonchère and Ibarra-Castro, 2013). Once a species is selected and general choice of aquaculture principles have been established, the refining of culture can commence. Research into genetics, reproduction, feed nutrition, feeding regimes, disease maintenance, and post-harvest technology and processing can be improved to deliver increased growth performance, feed utilisation and animal health, and reduced waste output (Leung et al., 2007; Lucas and Southgate, 2012).

Increased production of commercially formulated feeds for cultured finfish is required to satisfy the increased demand in aquaculture production. As expected, the production of formulated feeds has increased in line with the rapid expansion of aquaculture from 7.6 million tonnes in 1995 to what was predicted to be 70.9 million tonnes required by end 2020 (El-Sayed, 2014). These formulated feeds traditionally contained fish oil and fish meal as the main sources of dietary lipid and protein, respectively. The increasing demand for finfish for human

consumption and competition for fish oil and fish meal from the terrestrial animal production industry are driving up the cost of these raw ingredients (FAO, 2020b). In order to make diets more cost effective and environmentally sustainable, fish oil and fish meal substitution is a high priority area for feed development within the aquaculture industry (Gatlin et al., 2007; Stone and Bellgrove 2013; Stone et al., 2016).

In Australia, nutrition, feeding strategy and health research is assisting the production and profitability of a developing Yellowtail Kingfish, *Seriola lalandi,* industry (Figure 1.1; Stone and Booth, 2018). Fast growth rate and excellent meat quality makes the Yellowtail Kingfish a valuable species. The Yellowtail Kingfish is a carnivorous pelagic finfish species that congregates in schools in temperate and sub-tropical coastal waters throughout the world (Fernandes and Tanner, 2008; Hilton et al., 2008; Booth et al., 2010; Bowyer et al., 2013). Its optimum temperature for growth is 22.8 °C, reaching a harvest size of 3.0 – 3.5 kg in 18 – 24 months (Miegel et al., 2010; Bowyer et al., 2012b). In the wild, Yellowtail Kingfish predominantly feed on prey rich in essential amino acids and long-chain polyunsaturated fatty acids (LC-PUFA) (Fowler et al., 2003). In southern Australia, cultured Yellowtail Kingfish are reared in land-based hatcheries before being transported to sea cages as juveniles for grow out where they are exposed to sea temperatures ranging from 10 °C to 24 °C (Fernandes and Tanner, 2008; Bowyer et al., 2012b). Until recently, the dietary nutrient requirement of cultured Yellowtail Kingfish has been largely based upon formulated feeds for Atlantic salmon and barramundi and closely related *Seriola* spp. cultured throughout Asia and the Americas (Stone and Bowyer, 2013; Stone and Booth, 2018). Similar to other cultured species, Yellowtail Kingfish have been traditionally fed formulated feeds containing fish oil and fish meal as the main sources of dietary lipid and protein, respectively. However, as the cost of raw ingredients increase, the Yellowtail Kingfish industry in Australia is also seeking more cost effective and

environmentally sustainable ingredients for formulated feeds (Stone and Bellgrove, 2013; Stone et al., 2016).

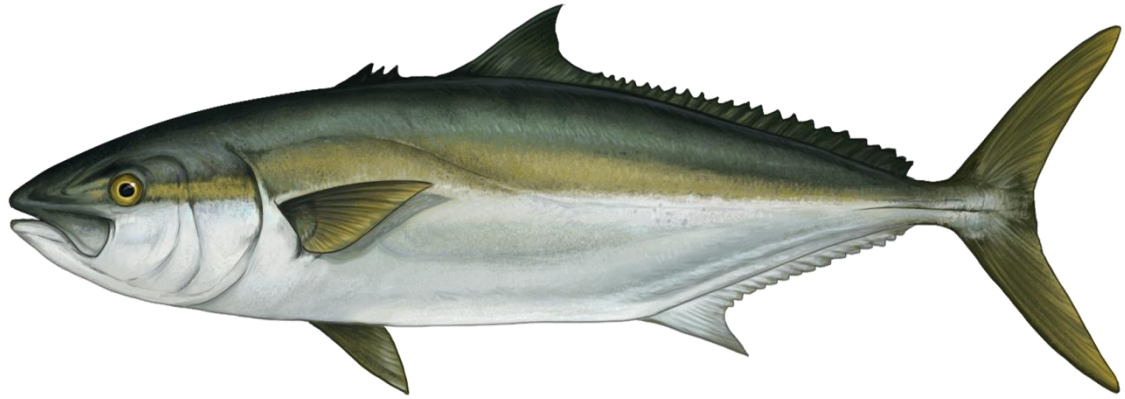

Figure 1.1. The Yellowtail Kingfish, *Seriola lalandi*, illustration René Campbell, copyright 2020.

The Australian Yellowtail Kingfish aquaculture industry is operational and expanding. In 2019 the largest Australian Yellowtail Kingfish producer Clean Seas Seafood Ltd, South Australia, produced 3,500 tonnes with reports of increased growth in domestic and international markets and plans to pursue an additional 4,250 tonne capacity lease (CSS, 2020). Likewise, in other parts of Australia the Yellowtail Kingfish aquaculture industry is growing with Indian Ocean Fresh Australia in Western Australia holding an 800-hectare lease. Australia's largest Atlantic salmon producer, Huon, has also announced a new 2200-hectare lease in Western Australia (FRDC, 2020b). The Yellowtail Kingfish biology, culture methods and farm sites, environmental, social and marketplace requirements are well established and understood. The current and most significant challenges the Yellowtail Kingfish industry face are that of declining availability of fish meal and fish oil coupled with increasing cost of these raw ingredients. Market competition to deliver the best performing diets is also a complementary driving force in the research priority area of nutrition.

Fish oil and fish meal contain high levels of essential amino acids and LC-PUFA, particularly eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) (Turchini et al., 2003; Turchini et al., 2009). These essential LC-PUFA are required for optimum growth and health for cultured marine finfish, including Yellowtail Kingfish (Bowyer et al., 2012a; Bowyer et al., 2012b; Castro et al., 2015). Available levels of cholesterol are reduced and phytosterols increased in diets where fish oil and fish meal have been substituted by plant-based ingredients (Morais et al., 2011). The nutritional values of both terrestrial plant and animal oil and meal vary with source and processing thus presenting challenges to diet formulation.

Fish oil and fish meal may only be substituted with some terrestrial sourced lipids and proteins to certain levels before digestive processes are affected (Bowyer et al., 2012a). Previous nutrition studies on Yellowtail Kingfish have focused on fish oil and fish meal substitution with results reported on specific growth rate, feed conversion, digestive enzyme concentration and activity, digestive tract histology, and effects of water temperature and dissolved oxygen on growth and digestive enzyme concentration and activity (Abbink et al., 2011; Bowyer et al., 2012a and 2012b; Booth et al., 2013; Stone and Bowyer, 2013; Stone et al., 2016). Stone and Bellgrove (2013) completed an extensive review of the current knowledge of the dietary nutritional requirements of Yellowtail Kingfish, including: protein, protein to energy ratio, amino acid profiles, taurine, energy, lipids and essential fatty acids, cholesterol, carbohydrates, minerals and vitamins. However, the impact that diet manipulation may have on Yellowtail Kingfish cholesterol synthesis, bile acid synthesis and enterohepatic circulation of bile acid is poorly understood. Currently, the only information available for comparison is from studies with Atlantic salmon and other *Seriola* spp. (Takagi et al., 2005 and 2008; Nguyen et al., 2011; Kortner et al., 2013; Stone et al., 2016).

Teleost fish either acquire cholesterol from the diet or can synthesise it *de novo* in the liver, although in a lesser capacity than mammals (Kortner et al., 2013). Dietary cholesterol is obtained from animal cells with plant cells being void of cholesterol but rich in other sterols: stiosterol, campesterol and stigmasterol (Lecerf and de Logeril, 2011). Cholesterol is the precursor for bile acids that are required for the micellar solubilisation of dietary lipids, esterification, emulsification and digestion of long chain fatty acids (Van Waarde, 1988; Yamamoto et al., 2007; Nguyen et al, 2011; El-Sayed, 2014; Murashita et al., 2014; Zhou and Hylemon, 2014). The amphipathic structure of bile acids facilitates the detergent properties that enable esterification, emulsification and digestion of long chain fatty acids through activation of digestive pancreatic and intestinal lipase (Van Waarde, 1988; Yamamoto et al., 2007; Nguyen et al., 2011; de Aguiar Vallim et al., 2013; Zhou and Hylemon, 2014). In both mammals and teleosts, *de novo* cholesterol synthesis is up-regulated when dietary intake does not meet metabolic demand (Lecerf and de Logeril, 2011).

The liver is the most important organ for cholesterol regulation, influencing plasma lipoprotein levels, bile acid production and elimination of excess cholesterol via faeces, nutrient metabolism, as well as lipid and glycogen storage (Fåhraeus-Van and Spurrell, 2003; Mohapatra and Mishra, 2011; Wang et al., 2012; Holm et al., 2013; Rodrigues et al., 2017). In mature teleosts, the liver is a wedge-shaped organ, and from a left lateral view is located adjacent to the heart and the intestine generally at the base of the visceral cavity (Figure 1.2). The liver can be divided into two main regions: the left lobe making up 30 – 50% of the liver mass and the right lobe making up the other 50 – 70% of liver mass; each region has its own blood supply and bile duct drainage (Wang et al., 2012). Based on vascular and bile duct distribution, these two regions can be segmented further into eight regions (Abdel-Misih and Bloomston, 2010). Of specific interest to the enterohepatic cycle is the porta hepatis (the caudate and medial segments) which is the location of major vascular vessels entering and

exiting the liver carrying nutrient rich blood (Wang et al., 2012). Within this region is the portal vein, which divides into the left and right portal veins when entering the liver, carrying approximately two thirds of the nutrient rich blood with recently absorbed nutrients and one third of the oxygen to the liver (Gadžijev and Ravnik, 1996). The hepatic artery supplies the other third of nutrients and two thirds of the oxygen to the liver (Wang et al., 2012). Despite extensive feed replacement trials for cultured Yellowtail Kingfish the basic liver morphological, enzymatic and biochemical parameters of Yellowtail Kingfish have received little attention to date.

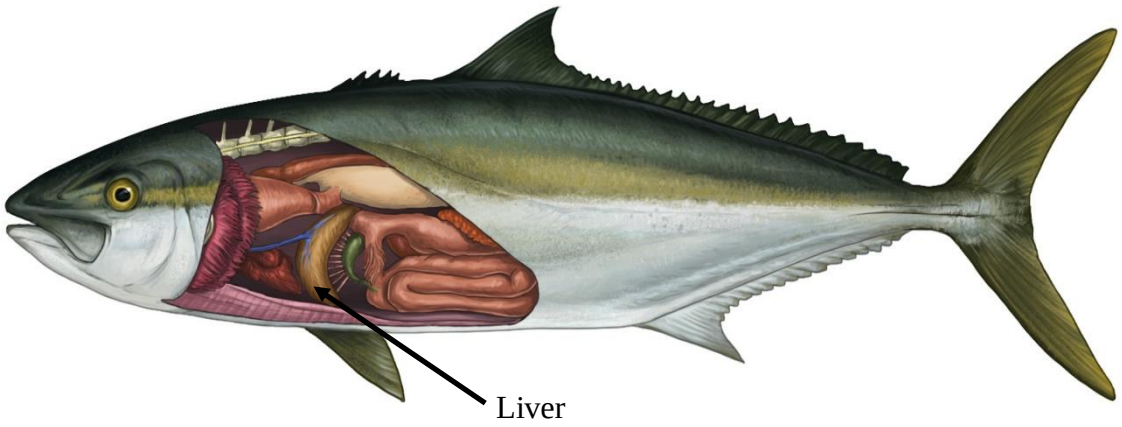

Figure 1.2. The Yellowtail Kingfish, *Seriola lalandi*, internal anatomy, illustration René Campbell, copyright 2020.

1.2 Overall study objectives

Aim

The overall objective of this research was to improve knowledge of Yellowtail Kingfish nutrition. Specifically, the enterohepatic system was investigated by diet manipulation through partial or complete substitution of fish oil and/or fish meal with alternative ingredients.

The aims of this research were to:

1. Obtain structure, energy reserves and enzyme concentration in the liver of wild Yellowtail Kingfish; and,
2. Investigate any changes in cultured fish that diet manipulation of,
    a. lipid level,
    b. protein level,
    c. emulsifier level,
    d. partial fish oil replacement with terrestrial oils, and,
    e. partial fish meal replacement with terrestrial meals,

    may have on:

    i. liver structure and morphology
    ii. liver energy reserves
    iii. blood biochemistry
    iv. liver cholesterol metabolism
    v. liver bile acid metabolism
    vi. total bile acid concentrations of enterohepatic and excretory system tissues.

Five trials were conducted to address these aims. The experimental trials were:

1. Trial 1: Understanding structure, energy reserves and enzyme concentration in the liver of wild Yellowtail Kingfish, *Seriola lalandi*.

    This addressed Aim 1 and is presented in Chapter 2.
2. Trial 2: The effect of diets of differing energy and emulsifier levels on liver and intestinal bile acid concentrations and bile acid and cholesterol metabolism and liver structures in Yellowtail Kingfish, *Seriola lalandi,* at cooler water temperatures.

    This addressed Aims 2.a. and 2.c. and is presented in Chapter 3.

3. Trial 3: The effect of alternative oil diets on liver histological structures and hepatic enzyme concentration in Yellowtail Kingfish, *Seriola lalandi*, at cooler water temperatures. This addressed Aim 2.d. and is presented in Chapter 4.
4. Trial 4: The effect of reduced fish meal diets on liver histological structures and hepatic and enzyme concentration in Yellowtail Kingfish, *Seriola lalandi*, at cooler and warmer water temperatures. This addressed Aims 2.b. and 2.e. and is presented in Chapter 5.
5. Trial 5: The effect of graded dietary protein and lipid levels on liver histological structures and hepatic enzyme concentration in Yellowtail Kingfish, *Seriola lalandi*, at warmer water temperatures. This addressed Aims 2.a. and 2.b. and is presented in Chapter 6.

1.3 Thesis outline

This project was supported by the Australian Government Department of Agriculture and Water Resources as part of its Rural R&D for Profit Programme, the Fisheries Research and Development Corporation (FRDC), the South Australian Research and Development Institute (SARDI), Flinders University, NSW Department of Primary Industries, Clean Seas Seafood, Huon Aquaculture, Ridley Corporation Ltd. and Skretting Australia. Therefore, this research has been conducted and reported with a focus on the commercially relevant information that will be best utilised by the stakeholders of this project. Statistically significant results are included for the scientific community. Observed trends, that may be apparent with further investigation, are discussed as numerical differences as these are relevant to producers and feed manufacturers. The production and performance indices are reported elsewhere by Stone and Booth (2018) but incorporated into each chapter's discussion. This thesis is presented in seven chapters: a general thesis introduction, five data chapters and a general thesis discussion.

Chapter 1 is a general introduction to this thesis that outlines the projected future of global aquaculture, the Australian aquaculture industry and the position of the Yellowtail Kingfish aquaculture industry in Australia. This chapter outlines the need for the alternatives to fish oil and fish meal ingredients in formulated feeds and the limited knowledge that is currently available on the enterohepatic and excretory system.

Chapter 2 presents wild caught Yellowtail Kingfish data on liver structure and morphology, liver energy reserves, blood biochemistry, liver cholesterol and bile acid metabolism and total bile acid concentrations of enterohepatic tissues. This information has not previously been reported in literature and as such gives vital reference for all further work on Yellowtail Kingfish nutrition.

Chapter 3 presents a factorial design experiment where commercial diets of increasing energy levels were provided by fish oil substituted with poultry oil and the addition of a commercially available emulsifier to diets. The chapter presents observed response in liver structure and morphology, liver energy reserves, blood biochemistry, liver cholesterol and bile acid metabolism and total bile acid concentrations of enterohepatic and excretory tissues in sub-adult Yellowtail Kingfish at cooler water temperatures.

Chapter 4 presents an experiment where commercial diets with graded fish oil and diets with fish oil were substituted by graded poultry and canola oil blends. The chapter presents observed responses in liver structure and morphology, liver energy reserves, blood biochemistry, liver cholesterol and bile acid metabolism and total bile acid concentrations of enterohepatic and excretory tissues in sub-adult Yellowtail Kingfish at cooler water temperatures.

Chapter 5 presents an experiment where commercial diets with differing dietary protein levels were provided by the substitution of fish meal with either fish by-product meal, poultry meal or soy protein concentrate. The chapter presents observed responses in liver structure and

morphology, liver energy reserves, blood biochemistry, liver cholesterol and bile acid metabolism and total bile acid concentrations of enterohepatic and excretory tissues in sub-adult Yellowtail Kingfish over an extended period of cooler and warmer water temperatures.

Chapter 6 presents an experiment where commercial diets with increasing dietary energy protein with either a high or low lipid level were used. The chapter presents observed responses in liver structure and morphology, liver energy reserves, blood biochemistry, liver cholesterol and bile acid metabolism and total bile acid concentrations of enterohepatic and excretory tissues in sub-adult Yellowtail Kingfish at warmer water temperatures.

Chapter 7 is the general discussion of this thesis, where the major research outcomes are summarised. This chapter also gives final recommendations to the Yellowtail Kingfish industry and feed manufacturers to improve production. Further research, not investigated in this thesis, is also recommended.

1.4 Experimental animals

The care and use of experimental animals complied with the Australian Code for the Care and use of Animals for Scientific Purposes, 8th edn, 2013. Animal welfare approval was obtained from the Flinders University Animal Welfare Committee (Permit No. E441/16) for the duration of this project.

Chapter 2: Understanding structure, energy reserves and enzyme concentration in the liver of wild Yellowtail Kingfish, *Seriola lalandi*

2.1 Abstract

Knowledge of liver structure and energy reserves in wild Yellowtail Kingfish, *Seriola lalandi,* may facilitate further development of formulated feed for aquaculture. In this study, we investigated liver structure and energy reserves, concentration of enzymes involved in cholesterol and bile acid metabolism and blood biochemistry in wild Yellowtail Kingfish. Wild Yellowtail Kingfish (4 female and 3 male; 6.58 ± 3.12 kg; mean body mass ± SD) were collected by a commercial fishing charter in southern Australia. Hepatosomatic index (HSI) ranged from 0.73 to 1.58% (1.05 ± 0.31 HSI; mean ± SD), visceral somatic index (VSI) ranged from 1.62 to 11.85% (6.36 ± 3.01 VSI; mean ± SD) and visceral fat index (VFI) ranged from 1.51 to 6.28% (3.67 ± 2.07 VFI; mean ± SD). Hepatocytes were devoid of vacuolar fat and had minimal glycogen. Bile ducts demonstrated typical structure and integrity with correctly positioned cuboidal cholangiocytes within bile ducts. Bile duct proliferation with fibrous bridging and periductular fibrosis was observed in some of the fish. The livers had a vacuole volume as a proportion of the liver cell volume (VPLC%) ranging from 1.69 to 6.83 (4.84 ± 2.35 VPLC%; mean ± SE). Total bile acid concentrations of the liver tissue ranged from 13.43 to 21.53 µmoles g^{-1} (18.36 ± 2.7 µmoles g^{-1}; mean ± SD; Table 2.3). Liver concentration of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) ranged from 2.40 to 9.91 µmoles g^{-1} (6.02 ± 3.07 µmoles g^{-1}; mean ± SD). Liver concentration of cholesterol-7α-hydroxylase (CYP7A1) ranged from 1865.6 to 3557.60 µmoles g^{-1} (2521.26 ± 627.00 µmoles g^{-1}; mean ± SD). The blood biochemistry values presented in this study are the first values reported for wild Yellowtail Kingfish.

2.2 Introduction

The Yellowtail Kingfish is a carnivorous pelagic finfish species with a high metabolic rate found in temperate coastal waters throughout the world and valued in aquaculture for its fast growth rate and excellent meat quality (Booth et al., 2010; Bowyer et al., 2013; Fernandes and Tanner, 2008; Hilton et al., 2008). Its optimum temperature for growth is 22.8 °C, reaching a harvest size of 3.0 to 3.5 kg in 18 to 24 months (Bowyer et al., 2012b; Miegel et al., 2010). In southern Australia, cultured Yellowtail Kingfish are reared in land-based hatcheries before being transported to sea cages as sub-adults for grow out, where they are exposed to water temperatures ranging from 10 °C to 24 °C (Bowyer et al., 2012b; Fernandes and Tanner, 2008).

Cultured Yellowtail Kingfish have traditionally been fed formulated feeds containing fish oil and fish meal as the main sources of dietary lipid and protein, respectively. However, increased demand for finfish for human consumption and use in terrestrial animal production systems has led to competition for fish oil and fish meal resulting in the prices of these raw ingredients increasing (FAO, 2020b). In order to make diets more cost effective and environmentally sustainable, fish oil and fish meal substitution with other ingredients is a high priority for feed development within the aquaculture industry generally and specifically for Yellowtail Kingfish diets (Gatlin et al., 2007; Stone and Bellgrove, 2013; Stone et al., 2016).

The fish oil and fish meal included in formulated diets together contain high levels of essential amino acids and long-chain polyunsaturated fatty acids (LC-PUFA) that are essential for optimum growth and health in cultured marine finfish, particularly eicosapentaenoic acid (20:5n-3; EPA) and docosahexaenoic acid (22:6n-3; DHA) (Bowyer et al., 2012a; Bowyer et al., 2012b; Castro et al., 2015; Turchini et al., 2003; Turchini et al., 2009). Substituting fish oil and fish meal with terrestrially sourced lipids and proteins provides a more sustainable approach, but this also has the potential to negatively impact digestive processes, resulting in

reduced growth and health (Bowyer et al., 2012a). Reduced available levels of cholesterol and increased phytosterols are observed in diets where fish oils have been substituted by plant-based oils (Morais et al., 2011). Cholesterol is the precursor for bile acids that are required for the micellar solubilisation of dietary lipids, esterification, emulsification and digestion of long chain fatty acids (El-Sayed, 2014; Nguyen et al., 2011; Murashita et al., 2014; Van Waarde, 1988; Yamamoto et al., 2007; Zhou and Hylemon, 2014).

The liver is the most important organ for cholesterol regulation, influencing plasma lipoprotein levels, bile acid production and elimination via faeces, as well as lipid and glycogen storage (Fåhraeus-Van Ree and Spurrell, 2003; Holm et al., 2013; Mohapatra and Mishra, 2011; Rodrigues et al., 2017; Wang et al., 2012). Mammals and marine teleosts fed high-fat diets that exceed dietary requirements accumulate excessive amounts of intracytoplasmic lipid droplets which may result in liver steatosis or necrosis (Scudamore, 2013; Wang et al., 2015). Histological examination of fish livers is considered one of the means necessary to assess the nutritional impact that formulated feeds may have on cultured species (Aydin et al., 2015). To provide context for this information, it is essential to characterise this in wild animals.

Despite extensive nutrition trials on cultured Yellowtail Kingfish the basic liver morphological, enzymatic and biochemical parameters of wild Yellowtail Kingfish have received little attention to date. At present, the only available histological comparisons for Yellowtail Kingfish liver lipid accumulation are in reports for anchovy, *Engraulis mordax,* sardines, *Sardina pilchardus*, Japanese seabass, *Lateolabrax japonicas*, yellow croaker, *Pseudosciaena crocea*, and Atlantic salmon, *Salmo salar* L. (Hu et al., 2013; Liland et al., 2015; McFadzen et al., 1997; O'Connell, 1976; Wang et al., 2015). The objective of this study was to investigate the liver structure and energy reserves, cholesterol and bile acids metabolism and blood

biochemistry in wild Yellowtail Kingfish to underpin future advances in the development of formulated feeds for this species.

2.3 Methods

2.3.1 Fish source

Seven wild Yellowtail Kingfish, *Seriola lalandi,* were collected by commercial fishing charter during summer of January 2018 off Greenly Island, South Australia. Fish were measured for their body mass (nearest 0.01 g) and fork length (nearest 1 mm; Table 2.1). The condition factor (CF) for each animal was also determined from these measurements as follows (Table 2.1; Bansemer et al., 2018):

$$CF = (\text{fish body mass [g]} / \text{fish length [cm]}^3) \times 100$$

Table 2.1. Gross and somatic parameters of wild Yellowtail Kingfish, *Seriola lalandi*.

Fish / Parameter	1	2	3	4	5	6	7	Mean	SD
Sex	F	F	M	F	F	M	M		
Body mass (kg)	5.80	8.90	2.85	5.40	12.40	5.50	5.19	6.58	3.12
Length (mm)	780.00	920.00	610.00	670.00	1060.00	785.00	760.00	798.00	151.00
Hepatosomatic index (HSI; %)	0.73	1.04	0.74	0.83	1.14	1.58	1.27	1.05	0.31
Visceral index (VSI; %)	1.62	5.72	5.72	11.85	7.31	6.18	6.13	6.36	3.01
Visceral fat index (VFI; %)	6.28	2.61	1.97	5.37	1.51	5.86	2.11	3.67	2.07
Condition factor	1.22	1.14	1.26	1.80	1.04	1.14	1.18	1.25	0.25

2.3.2 Somatic indices

For each fish, the visceral cavity was cut open and the whole viscera removed and weighed, then the liver weighed (nearest 0.01 g) to determine the visceral somatic index (VSI; %) and hepatosomatic index (HSI; %). The visceral fat was removed and weighed (nearest 0.01 g) to determine the visceral fat index (VFI; %). These indices were calculated as follows:

$$VSI\% = (\text{visceral weight [g]} / \text{body mass [g]}) \times 100$$

$$HSI\% = (\text{liver weight [g]} / \text{body mass [g]}) \times 100$$

VFI% = (visceral fat / body mass) ×100

2.3.3 Sample collection

Liver, foregut, midgut, hindgut and gallbladder content were collected, immediately frozen in a – 50 °C blast freezer and stored at – 80 °C until analysis. Two 1 cm^3 sections of the left lobe of the liver were dissected and immediately placed into a histology cassette and fixed with 10% neutral buffered formalin (pH of 7.2) for 24 h for histological evaluation. One of the liver sections from each fish was used for standard paraffin embedding and then sectioned by rotary microtome, while the other was preserved in an 18% phosphate buffered saline (PBS) -sucrose + 0.01% sodium azide (NaN_3) solution with pH 7.1 for cryostat sectioning. Blood samples were obtained from the caudal vasculature using 21 gauge needles and 5 mL syringes.

2.3.4 Liver histology

Once fixed, one liver section from each fish was transferred to 70% (v/v) ethanol and stored at room temperature according to Hu et al. (2013). The sample was dehydrated using standard procedures, embedded in paraffin and sectioned at 5 μm on a rotary microtome. This section of liver from each fish underwent several staining techniques: haematoxylin and eosin (H&E) for a quantitative assessment of the vacuole volume as a proportion of the liver cell volume (VPLC%) and tissue structure as per Crowe et al. (2021) and periodic acid Schiff (PAS) for glycogen deposition. The second liver section samples were sectioned at 10 μm on a cryostat microtome and mounted on polyethylenimine coated slides. This section of liver from each fish underwent several staining techniques: Oil Red O (ORO) for unsaturated neutral lipid deposition and Sudan Black B (SBB) for total lipid deposition. Stained sections were examined at 400-fold magnification using a light microscope (Olympus BF BX50). Three 559,390 μm^2 microphotographs were taken per stained slide using a digital camera (Olympus DP27). Quantitative assessments of the stained deposition volume as a proportion of the liver cell

volume was determined by applying stain sensitive colour thresholds using Fiji ImageJ processing software (National Institutes of Health, Bethesda, Maryland, United States of America).

2.3.5 Biochemical analyses

Samples from individual fish were partially thawed, weighed and homogenised in five volumes of PBS with a pH of 7.1. Homogenisation was performed using a Retch MM 400 ball mill at frequency 30.01 s^{-1} for 4 min with Biospec 2 mm zirconia beads (Catalogue No.11079124zx). The suspensions were centrifuged twice at an acceleration of 10,000 *g* for 10 min at 4 °C (Beckman Coulter Microfuge 16 centrifuge) with the supernatants being extracted to new microfuge tubes between cycles. All assay kits included internal standard solutions and manufacturer instructions were followed for assays. Tissue concentrations are reported per tissue weight (μmoles g^{-1}).

2.3.5.1 Total bile acid concentration

The liver, foregut, midgut, hindgut and gallbladder content from individual fish were partially thawed, weighed and homogenised. The final supernatants were analysed for total bile acid concentrations by use of a commercial fluorometric bile acid assay kit by reading Ex/Em = 560/590 nm after 45 min of dark incubation at room temperature following manufacturer instructions (Catalogue No. MET-5005; Cell Biolabs Inc.®, San Diego, California, United States of America).

2.3.5.2 Taurine concentration

The livers from individual fish were partially thawed, weighed and homogenised. The final supernatants were analysed for taurine by use of a commercial colorimetric taurine assay kit by reading samples at 405 – 415 nm following manufacturer instructions (Catalogue No. MET-5070; Cell Biolabs Inc.®, San Diego, California, United States of America).

2.3.5.3 HMGCR and CYP7A1 enzyme-linked immunosorbent assays

The livers from individual fish were partially thawed, weighed and homogenised. The final supernatants were analysed for HMGCR concentration after reading the absorbance of samples at a wavelength of 450 nm (Catalogue No. CSB-E15772m; CusaBio, Texas, United States of America). The final pooled supernatants were analysed for CYP7A1 concentration after reading the absorbance of samples at a wavelength of 450 nm (Catalogue No. CSB-EL006395FI; CusaBio, Texas, United States of America).

2.3.6 Blood analyses

Two mL of blood was placed into separate Vacuette® or BD vacutainer® tubes (Z serum clot activator or EDTA tubes) and stored indirectly on ice until same-day analysis of blood parameters by IDEXX Laboratories (Unley, South Australia, Australia). Biochemistry parameters were measured but of specific interest were the following parameters: total bile acids, bile salts, cholesterol, triglycerides and total bilirubin.

2.3.7 Descriptive statistics

As a first investigation to establish data for wild Yellowtail Kingfish the range and mean ± SD or SE is displayed with descriptive observations for each parameter investigated.

2.4 Results

2.4.1 Gross observations

A total of seven wild Yellowtail Kingfish (4 female and 3 male) were collected with a body mass range of 2.85 to 12.40 kg (6.58 ± 3.12 kg; mean ± SD) and fork length range of 610 to 1060 mm (798 ± 151 mm; mean ± SD; Table 2.1). The CF of the animals ranged from 1.04 to 1.80 (1.25 ± 0.25 CF; mean ± SD; Table 2.1).

2.4.2 Somatic indices

The wild Yellowtail Kingfish HSI ranged from 0.73 to 1.58% (1.05 ± 0.31 HSI; mean ± SD), VSI ranged from 1.62 to 11.85% (6.36 ± 3.01 VSI; mean ± SD,) and VFI ranged from 1.51 to 6.28% (3.67 ± 2.07 VFI; mean ± SD; Table 2.1).

2.4.3 Liver histology

In general the wild Yellowtail Kingfish hepatocytes were devoid of vacuolar fat and had minimal glycogen (Figure 2.1). Bile ducts demonstrated typical structure and integrity with correctly positioned cuboidal cholangiocytes within bile ducts (Figure 2.2). Bile duct proliferation with fibrous bridging was observed in three of the fish (fish 3, 4 and 5) which also had sloughing within the bile duct lumen (Figure 2.3). Periductular fibrosis characterised by sclerosing fibrous layering around the bile duct giving an “onion-skin” appearance was also observed; no inflammatory cells were present (Figure 2.4). Areas of fatty change and melanomacrophage centres were also observed within the livers of some wild Yellowtail Kingfish (Figure 2.5 and 2.6).

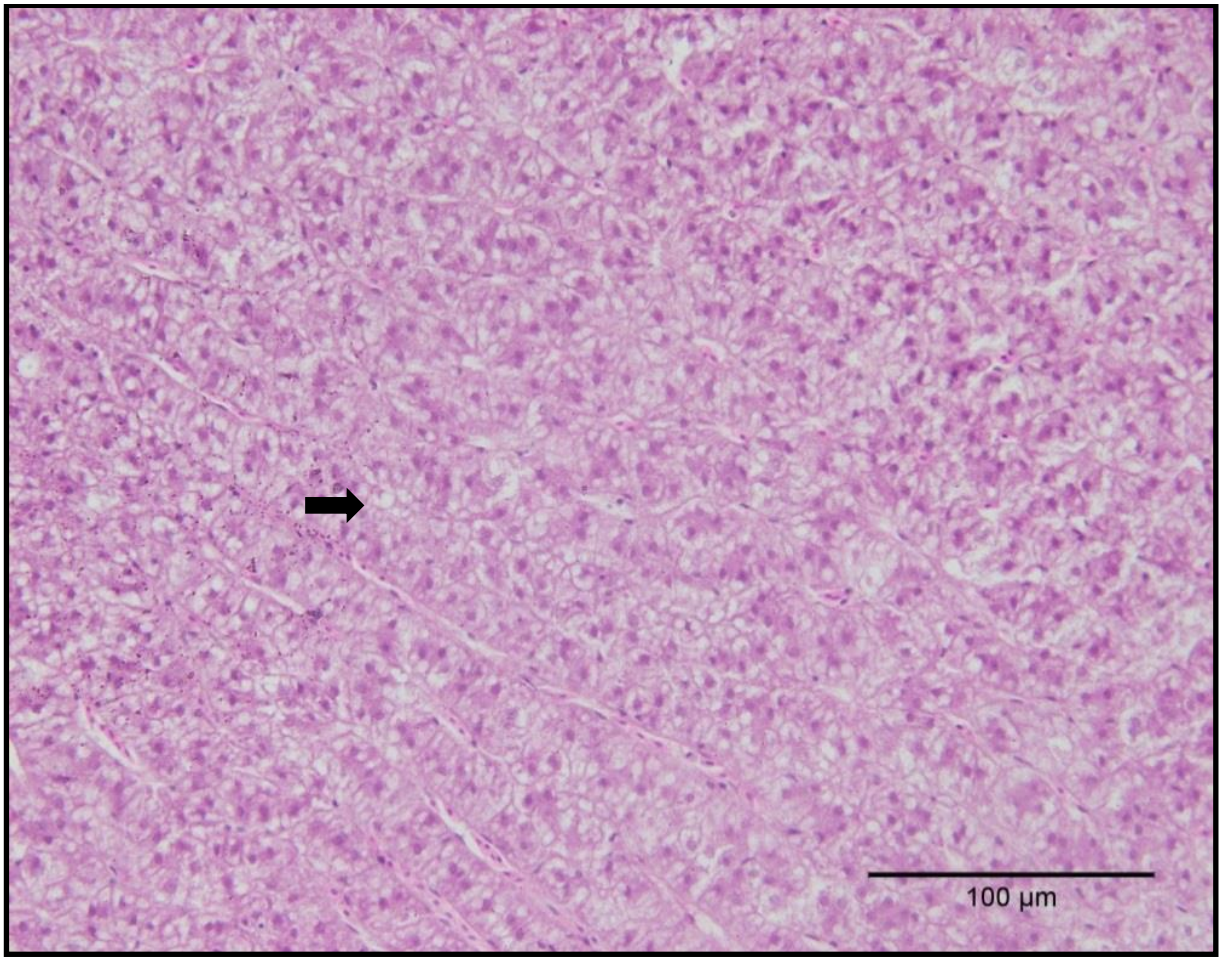

Figure 2.1. Wild Yellowtail Kingfish, *Seriola lalandi*, liver devoid of vacuolar fat within hepatocytes (*arrow*) and minimal glycogen. Haematoxylin and eosin stain. Fish 4; female, 5.8 kg, 780 mm.

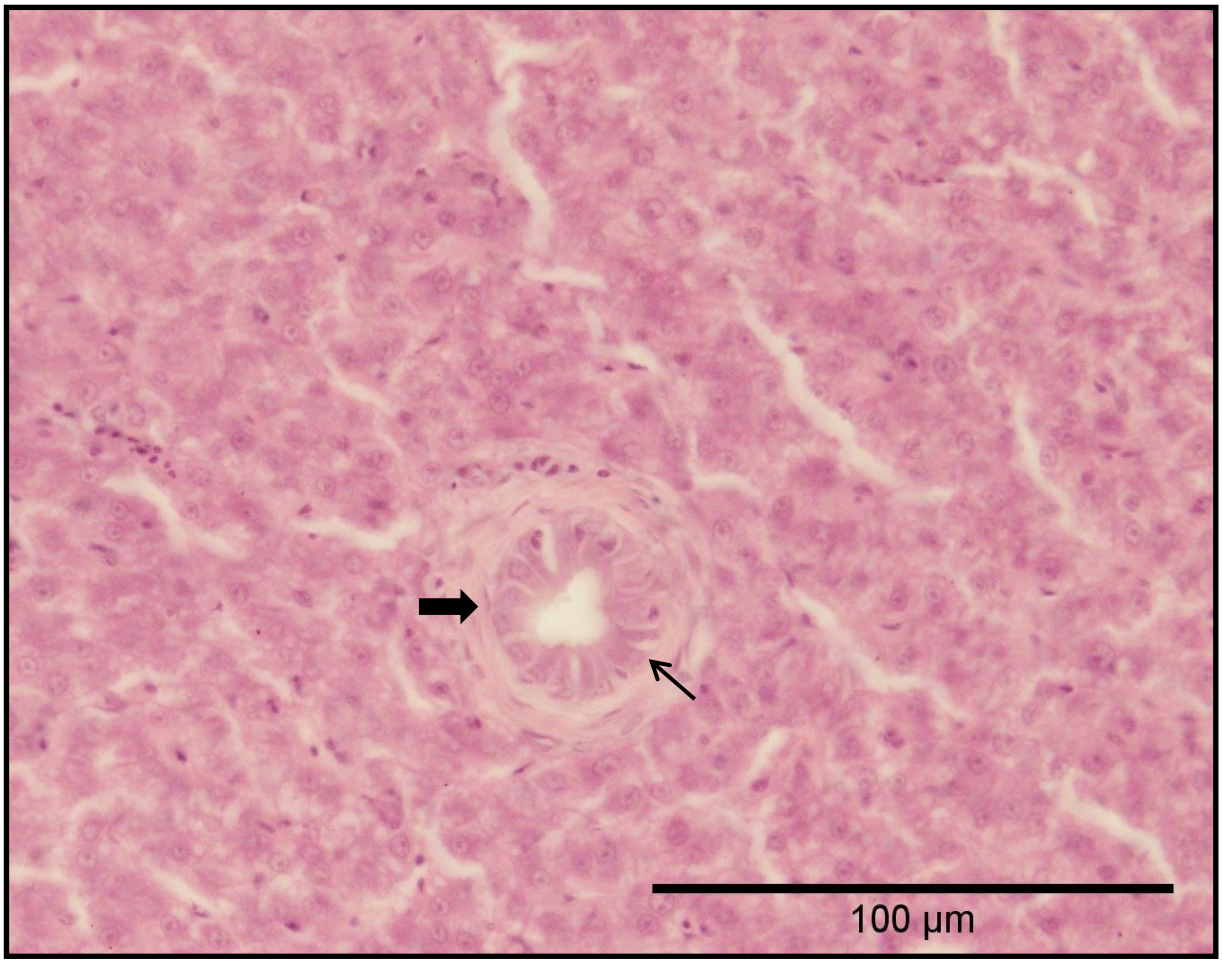

Figure 2.2. Bile duct (*thick arrow*) of wild Yellowtail Kingfish, *Seriola lalandi*, demonstrating typical structure and integrity with correctly positioned cuboidal cholangiocytes within bile ducts (*thin arrow*). Haematoxylin and eosin stain. Fish 4; female; 5.8 kg; 780 mm.

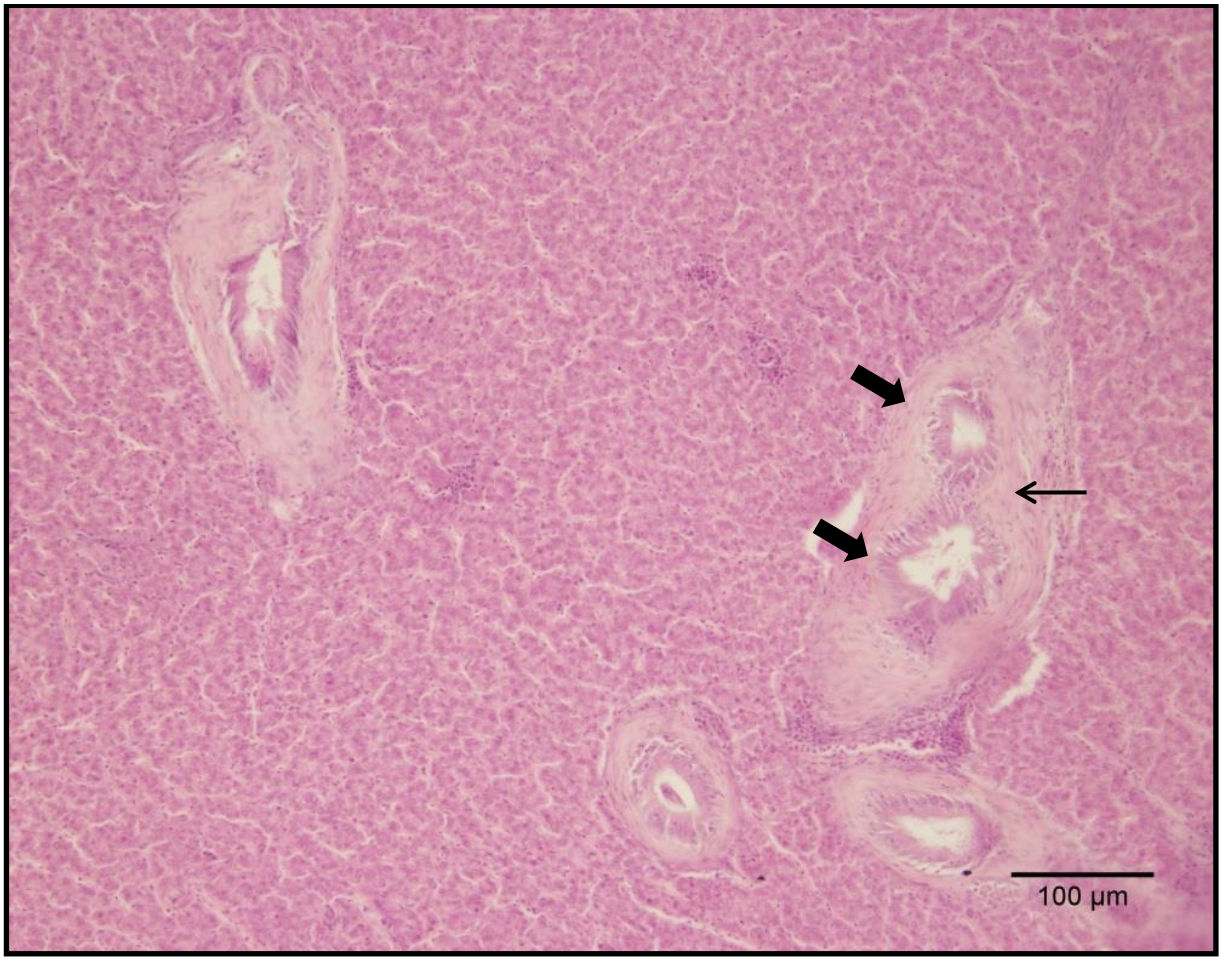

Figure 2.3. Bile duct proliferation (*thick arrow*) with fibrous bridging (*thin arrow*) within liver of wild Yellowtail Kingfish, *Seriola lalandi*. Haematoxylin and eosin stain. Fish 5; female, 12.4 kg, 1060 mm.

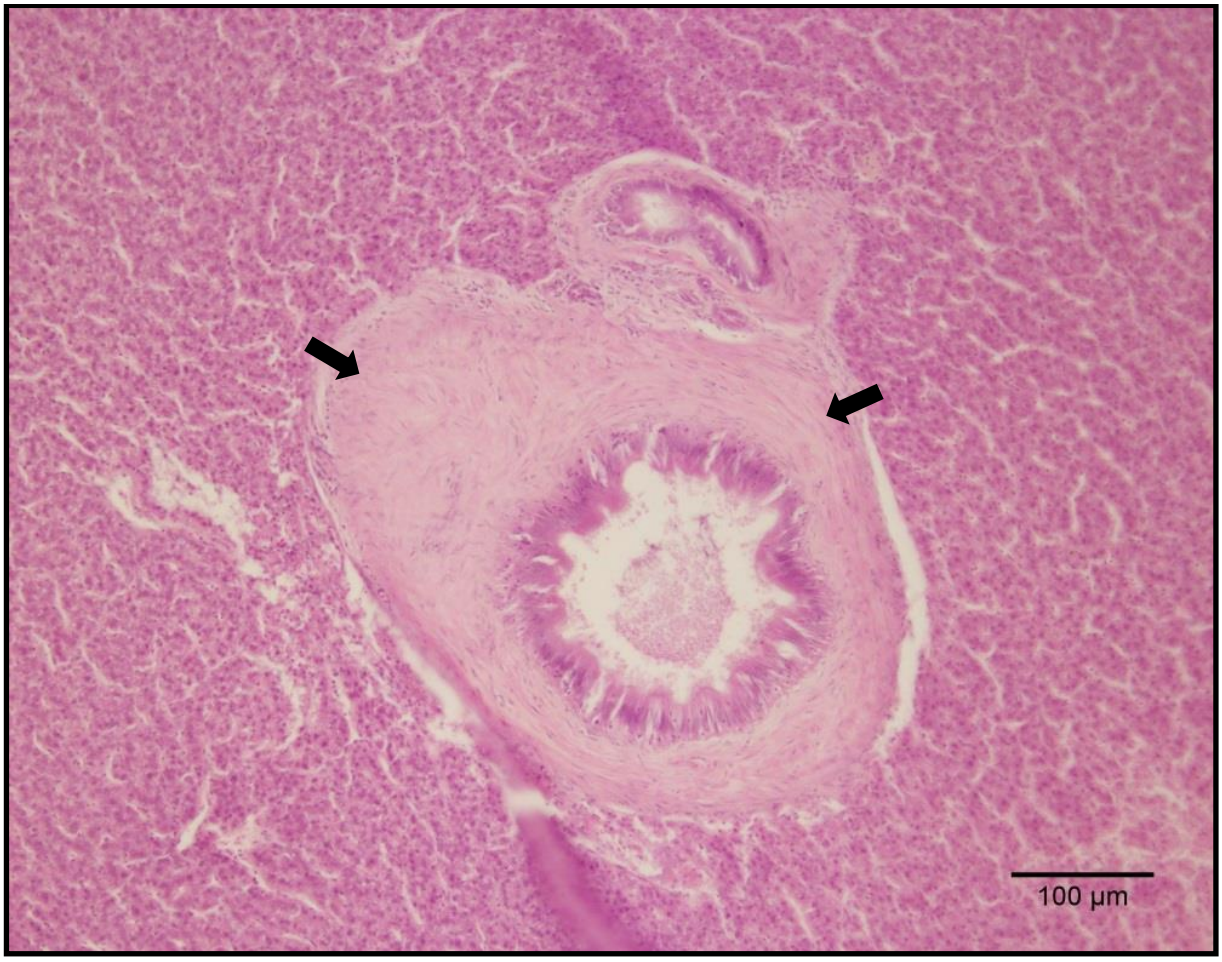

Figure 2.4. Sclerosing fibrous layering around the bile duct giving an "onion-skin" appearance in the liver of wild Yellowtail Kingfish, *Seriola lalandi*. Haematoxylin and eosin stain. Fish 4; female; 5.8 kg; 780 mm.

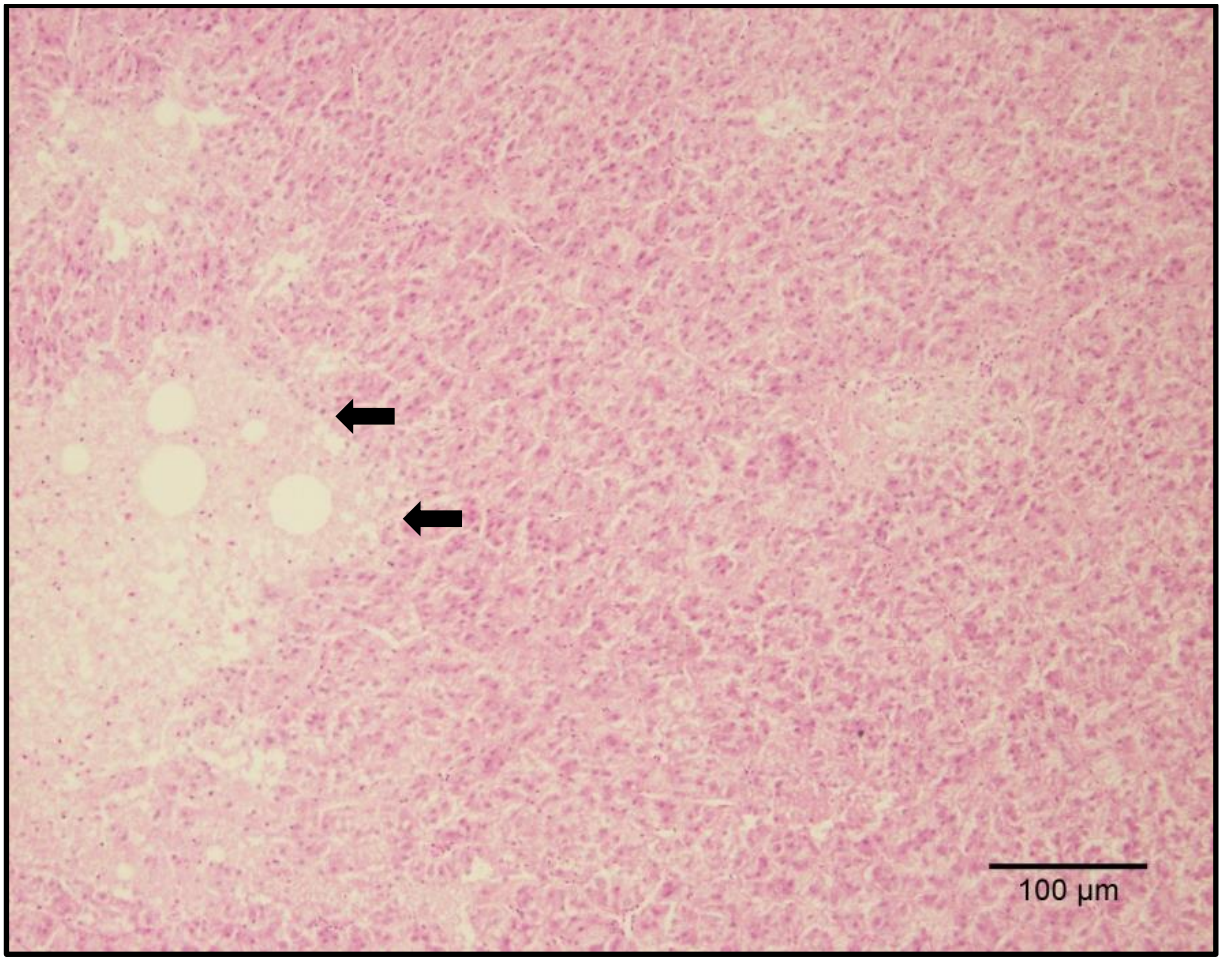

Figure 2.5. Areas of fatty change layer (*thick arrow*) observed within liver of wild Yellowtail Kingfish, *Seriola lalandi*. Haematoxylin and eosin stain. Fish 3; male; 2.85 kg; 610 mm.

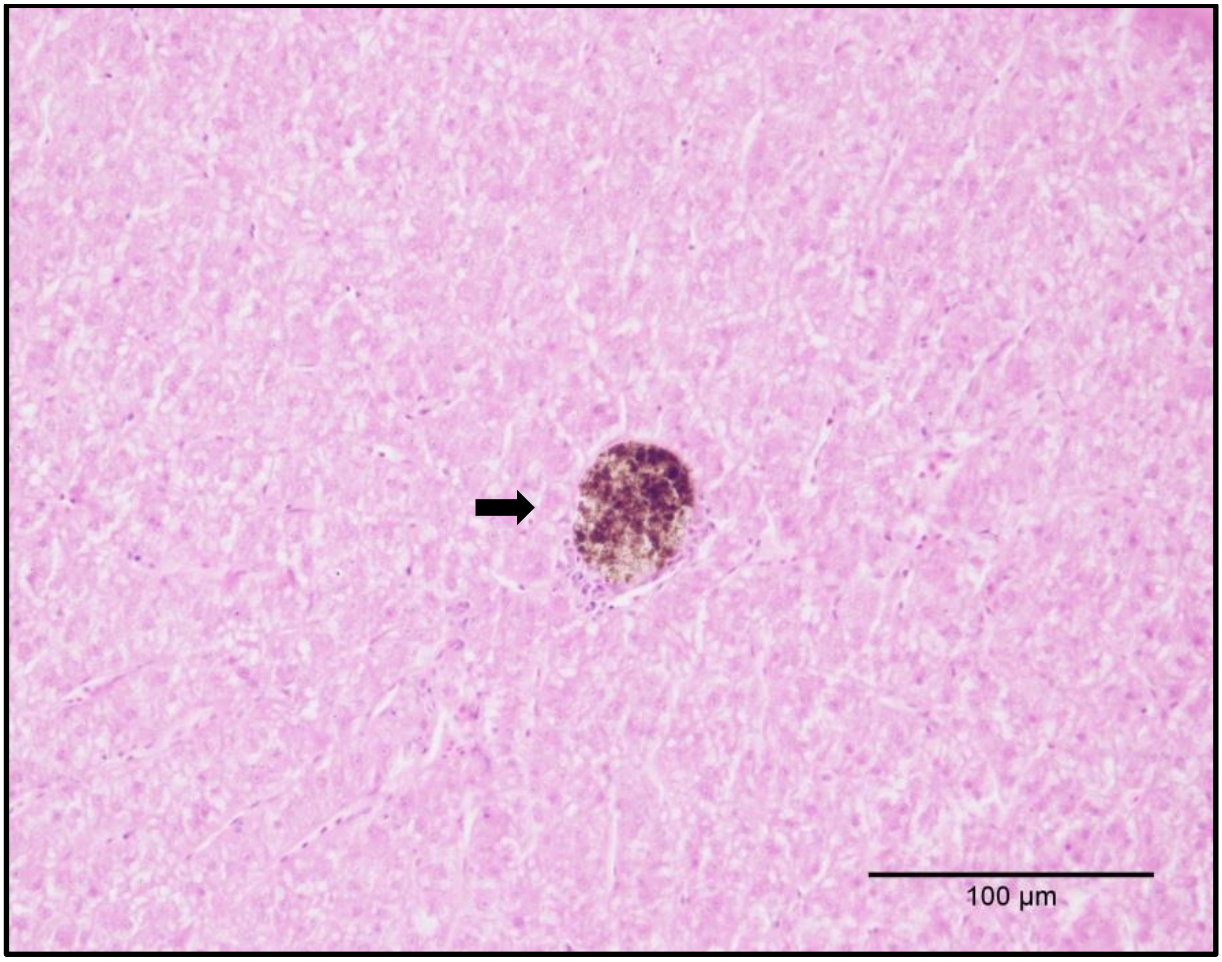

Figure 2.6. Melanomacrophage centre (*thick arrow*) observed within liver of wild Yellowtail Kingfish, *Seriola lalandi*. Haematoxylin and eosin stain. Fish 7; male; 5.19 kg; 760 mm.

The livers of wild Yellowtail Kingfish had a VPLC% ranging from 1.69 to 6.83 (4.84 ± 2.35 VPLC%; mean ± SE; Table 2.2). PAS-positive stained glycogen presented as fine magenta-stained granular material in cytoplasm of hepatocytes. The proportion of glycogen total ranged from 0.05 to 0.30 (0.16 ± 0.22, mean ± SE; Table 2.2). Neutral lipids stained with ORO appeared as red droplets in the cytoplasm of hepatocytes. The proportion of neutral lipids ranged from 0.38 to 5.40 (3.59 ± 0.78, mean ± SE; Table 2.2). Total lipids stained with SBB appeared as black droplets in the cytoplasm of hepatocytes. The proportion of total lipid ranged from 0.04 to 7.21 (2.66 ± 2.31, mean ± SE; Table 2.2).

Table 2.2. Vacuole area and proportion of histology stain on liver sections of wild Yellowtail Kingfish, *Seriola lalandi*[1].

Fish Parameter	1	2	3	4	5	6	7	Mean	SD
VPLC% [2]	6.83±2.07	3.20±2.72	6.10±2.25	1.69±1.11	4.80±2.48	5.33±0.64	5.90±1.03	4.84	2.35
Glycogen	0.07±0.03	0.17±0.10	0.05±0.01	0.32±0.28	0.05±0.03	0.16±0.05	0.30±0.16	0.16	0.10
Neutral lipids	5.24±2.11	0.38±0.27	3.85±1.91	2.87±0.35	2.57±1.60	4.82±2.05	5.40±1.33	3.59	0.78
Total lipids	3.98±1.51	7.21±6.83	0.04±0.02	1.49±1.31	2.17±0.74	1.74±0.70	1.99±0.80	2.66	2.31

[1] Values are mean ± SE; $n = 3$.
[2] Vacuole volume as a proportion of the liver cell volume (Crowe et al. 2021).

2.4.4 Total bile acid concentration

Total bile acid concentrations of the liver tissue ranged from 13.43 to 21.53 µmoles g^{-1} (18.36 ± 2.7 µmoles g^{-1}; mean ± SD; Table 2.3). Compared to the liver, the total bile acid concentrations of the gallbladder content increased in each of the fish to a range of 20.71 to 42.4 µmoles g^{-1} (24.94 ± 7.79 µmoles g^{-1}; mean ± SD; Table 2.3). The total bile acid concentration then decreased in the foregut to a range of 3.99 to 21.04 µmoles g^{-1} (13.29 ± 6.98 µmoles g^{-1}; mean ± SD; Table 2.3). The total bile acid concentrations in the midgut (range 11.00 to 19.45 µmoles g^{-1} [15.57 ± 3.29 µmoles g^{-1}; mean ± SD]) were similar to that observed

in the hindgut (range 10.27 to 24.53 µmoles g^{-1} [15.02 ± 5.59 µmoles g^{-1}; mean ± SD]; Table 2.3).

Table 2.3. Total bile acid concentrations from different sections within the enterohepatic bile acid cycling and excretory system of wild Yellowtail Kingfish, *Seriola lalandi*.

Fish / Parameter	1	2	3	4	5	6	7	Mean	SD
Total bile acid concentration (µmoles g^{-1})									
Liver	16.29	19.21	19.28	20.24	18.52	13.43	21.53	18.36	2.70
Gallbladder content	22.95	20.36	20.71	22.39	22.06	23.68	42.41	24.94	7.79
Foregut	17.97	3.99	5.76	18.48	17.35	8.44	21.04	13.29	6.98
Midgut	19.45	11.66	16.05	16.87	18.95	11.00	14.98	15.57	3.29
Hindgut	10.89	17.51	10.27	24.53	19.52	10.31	12.10	15.02	5.59

2.4.5 Taurine concentration

The liver taurine concentrations ranged from 0.01 to 0.07 µmoles g^{-1} (0.06 ± 0.02 µmoles g^{-1}; mean ± SD) and were similar for each of the fish except for fish 6 which had an 85 – 87% lower liver taurine concentration compared to the other fish (Table 2.4).

2.4.6 HMGCR and CYP7A1 enzymes

The HMGCR enzyme concentration varied in the liver between the different fish with a range of 2.40 to 9.91 µmoles g^{-1} with up to 51% variation from the mean (6.02 ± 3.07 µmoles g^{-1}; mean ± SD; Table 2.4). The CYP7A1 enzyme concentration also varied moderately in the liver between the different fish with a range of 1865.6 to 3557.60 µmoles g^{-1} with up to 25% variation from the mean (2521.26 ± 627.00 µmoles g^{-1}; mean ± SD; Table 2.4).

Table 2.4. Taurine, HMGCR and CYP7A1 concentrations of liver tissue from wild Yellowtail Kingfish, *Seriola lalandi*.

Fish / Parameter	1	2	3	4	5	6	7	Mean	SD
Taurine (µmoles g^{-1})	0.07	0.07	0.07	0.07	0.06	0.01	0.07	0.06	0.02
HMCGR (µmoles g^{-1})	8.86	7.22	2.40	7.33	9.91	2.41	3.98	6.02	3.07
CYP7A1 (µmoles g^{-1})	3557.60	2997.60	1865.60	2641.60	2205.60	1806.40	2574.40	2521.26	627.00

2.4.7 Blood analyses

All blood biochemistry values are shown in Tables 2.5. Of specific interest to liver function were the blood protein concentrations (48.86 ± 2.85 g L^{-1}; mean ± SD), albumin concentrations (15.00 ± 1.15 g L^{-1}; mean ± SD) and globulin concentrations (33.86 ± 2.12 g L^{-1}; mean ± SD) which were each consistent between fish (Table 2.5). Total bilirubin had a <0.00 mmol L^{-1} concentration for each of the fish and is most likely due to concentrations being under the detection limits of the laboratory test (Table 2.5). Alanine aminotransferase (ALT) ranged from 19.00 to 60.00 IU L^{-1} between each of the fish and up to 47% variation from the mean (35.57 ± 16.56 IU L^{-1}; mean ± SD; Table 2.5). Likewise, alkaline phosphatase (ALP) ranged from 20.00 to 46.00 IU L^{-1} between each of the fish and up to 34% variation from the mean (31.15 ± 10.53 IU L^{-1}; mean ± SD; Table 2.5). Cholesterol concentrations were consistent for each of the fish (9.03 ± 0.59 mmol L^{-1}; mean ± SD; Table 2.5). Triglycerides ranged from 1.92 to 5.47 mmol $L^{-1}$and varied up to 35% from the mean (3.37 ± 1.18 mmol L^{-1}; mean ± SD; Table 2.5). Bile acid concentrations were the most varied with a range from 0.90 to 22.70 mmol L^{-1} and up to 98% variation from the mean (8.01 ± 7.84 mmol L^{-1}; mean ± SD; Table 2.5).

Table 2.5. Blood biochemistry from wild Yellowtail Kingfish, *Seriola lalandi*.

Fish / Parameter	1	2	3	4	5	6	7	Mean	SD
Biochemistry									
Protein (g L^{-1})	47.00	46.00	50.00	50.00	46.00	49.00	54.00	48.86	2.85
Albumin (g L^{-1})	15.00	14.00	15.00	14.00	14.00	16.00	17.00	15.00	1.15
Globulin (g L^{-1})	32.00	32.00	35.00	36.00	32.00	33.00	37.00	33.86	2.12
Total Bilirubin (mmol L^{-1})	0.00†	0.00†	0.00†	0.00†	0.00†	0.00†	0.00†	0.00†	0.00†
ALT (IU L^{-1}) [1]	33.00	22.00	48.00	48.00	19.00	19.00	60.00	35.57	16.56
ALP (IU L^{-1}) [1]	23.00	30.00	44.00	33.00	46.00	20.00	22.00	31.14	10.53
Magnesium (mmol L^{-1})	3.29	4.15	4.47	3.62	3.89	3.50	4.78	3.96	0.54
Cholesterol (mmol L^{-1})	9.40	8.20	9.10	9.30	9.30	8.20	9.70	9.03	0.59
Triglyceride (mmol L^{-1})	3.28	2.55	5.47	3.96	3.85	1.92	2.59	3.37	1.18
Bile Acids (mmol L^{-1})	4.60	4.60	3.80	22.70	15.00	0.90	4.50	8.01	7.84

†denotes value below detection limits of the laboratory test.
[1] ALT, alanine aminotransferase; ALP, alkaline phosphatase.

2.5 Discussion

The condition factor (CF) varied amongst the wild fish. Smaller fish within this study generally had an anterio-posteriorly elongated body shape compared to the larger fish, which displayed anterio-posteriorly compressed body shapes. This resulted in a greater CF as the fish increased in body length. The mean CF range of wild fish from the current study is slightly above but similar to that observed in 120 – 150 mm wild-caught California yellowtail *Seriola dorsalis* (Gill 1863) (0.91 ± 0.07 CF; mean ± SD; Wegner et al., 2018). However, the wild fish from Wegner et al. (2018)'s study were smaller and displayed CFs more similar to the larger (body mass and length) fish in the present study. The mean CF for the wild fish of the current trial was approximately twice the mean CF observed in the closely related species greater amberjack (0.56 ± 0.14 CF; mean ± SD) and highfin amberjack, *Seriola rivoliana* (0.57 ± 0.24 CF; mean ± SD) collected from the East China Sea and Pacific Ocean (Saito, 2012). Cultured Yellowtail Kingfish from a restricted feeding strategy trial had a CF range that was greater (CF; 1.39 to 1.44) than the wild fish of the present study (Bansemer et al., 2018). In comparison to closely related species, the wild fish investigated in the present study were of good condition.

2.5.1 Somatic indices

As far as we are aware this is the first report of HSI, VSI and VFI of wild caught Yellowtail Kingfish from South Australian waters. Although the HSI of the wild fish in the present study varied considerably (up to 30% of the mean) the range is similar to that of cultured juvenile to sub-adult Yellowtail Kingfish (Bowyer et al., 2012b and 2013; Bansemer et al., 2018). When compared to cultured animals the VSI range of wild Yellowtail Kingfish is evidently more variable (up to 47% variation from the mean VSI). The mean VSI and mean VFI of wild Yellowtail Kingfish were also greater in variation than that observed in cultured sub-adult Yellowtail Kingfish (Bansemer et al., 2018; Stone and Booth, 2018; Stone et al., 2020). This

is expected as regular feeding of high lipid diets to cultured Yellowtail Kingfish results in a consistent supply of lipids, leading to excess storage in peripheral tissue and viscera. For example, cultured juvenile Yellowtail Kingfish (initial body mass 22.36 ± 0.05 g; mean ± SD) fed diets containing soy protein concentrate (partially substituting the fish meal component of the diet) had HSI values that ranged from 1.1 to 1.5% and VSI values that ranged from 6.2 to 7.2% (Bowyer et al., 2013). Similarly, in two separate temperature controlled experiments that were conducted at 22.1 ± 0.3 °C (mean ± SD) and 17.6 ± 0.9 °C (mean ± SD), larger cultured juvenile Yellowtail Kingfish (95.6 ± 0.1 g and 101.1 ± 0.1 g; mean body mass ± SE; respectively) had HSI values that ranged from 1.08 to 1.68% and 0.91 to 1.02%, respectively (Bowyer et al., 2012b). In a feed rate and frequency restriction trial larger cultured Yellowtail Kingfish (1.44 ± 0.1 kg; mean body mass ± SE) had HSI and VSI ranges of 0.75 to 1.11% and 4.53 to 5.79%, respectively (Bansemer et al., 2018).

2.5.2 Liver histology

Wild Yellowtail Kingfish hepatocytes were either devoid of or had minimal vacuolar fat (VPLC% <10%). Clear spaces within hepatocytes of cultured Yellowtail Kingfish are caused by the removal of lipids by alcohol treatments during standard H&E histology processing techniques (Fåhraeus-Van Ree and Spurrell, 2003). The optically empty content is identifiable as the previous locations of intracytoplasmic lipid droplets and are particularly prominent in cultured fish fed diets with high energy content (Crowe et al., 2021; McFadzen et al., 1997). Cultured Yellowtail Kingfish (1.87 ± 0.05 kg; initial mean body mass ± SD) fed diets containing low, moderate and high energy displayed VPLC% ranging from 25.68 to 43.33% (Crowe et al., 2021). Sudan Black B stains all available lipids other than steroids (Fåhraeus-Van Ree and Spurrell, 2003). A large range was also observed in the total lipids within the livers which were reduced overall compared to the neutral lipids. Neutral lipids consist of triacylglycerides and

steryl esters that occur in all types of cells. Neutral lipids cluster and form a hydrophobic core of lipid-particles specialised for storage as lipid droplets (Athenstaedt and Daum, 2006). These dramatic difference is most likely due to the infrequent feeding of the wild fish with energy being diverted for metabolic energy and not being stored in hepatocytes (Tocher, 2003), whereas cultured fish experience regular feeding and therefore have more capacity to store excess energy. For example, in adult female and male fish the transfer of lipid content from the liver and other tissues to ovaries during vitellogenesis and gonads during spawning, respectively, results in a decrease in the liver lipid content (Rodríguez-Barreto et al., 2012).

The magnitude of storage of glycogen, total and neutral lipids is dependent on feeding, energy expenditure and other metabolic functions (Tocher, 2003). In aquaculture, carbohydrates are added to formulated feeds for dual roles as binding agents and energy sources. Herbivorous and omnivorous marine and freshwater species more readily digest carbohydrates and store glycogen in the liver and muscle than marine carnivorous species (Hemre et al., 2002). Yellowtail Kingfish rapidly absorb carbohydrates (such as glucose) but due to its slow metabolism it is excreted rather than utilised as an energy source or stored as glycogen (Booth et al., 2013). Booth et al. (2013) suggested that cultured juvenile Yellowtail Kingfish suffered from prolonged hyperglycaemia when fed highly available forms of carbohydrates and an inclusion of >10% of these would compromise fish performance. The diets of wild Yellowtail Kingfish from this study are unknown, but are most likely similar to those caught by Fowler et al. (2003) off Greenly Island and Rocky Islands, South Australia. The dietary carbohydrate component of these wild fish was negligible and presumed to be from indiscriminate feeding on floating objects (Fowler et al., 2003). In this study, hepatocyte glycogen deposits were greatly reduced compared to deposits of total and neutral lipids.

No comparisons are available on the bile duct structure of wild *Seriola* species. The common structure of all vertebrates is that bile ducts have highly polarised cuboidal or columnar cholangiocytes, the size of which correlates with the diameter of the bile duct (Wang et al., 2012). The bile duct structure and integrity with correctly positioned cuboidal cholangiocytes observed within bile ducts in this study was consistent with other wild caught species such as yellowtail flounder, *Limanda ferruginea*, and cultured hybrid sorubim, *Pseudoplatystoma reticulatum* x *P. corruscans* (Fåhraeus-Van Ree and Spurell, 2003; Rodrigues et al., 2017). The majority of bile ducts observed had low levels of the connective tissue sheath that surrounded the bile ducts and veins or arterioles similar to that in wild yellowtail flounder and rainbow trout, *Salmo gairdneri* Richardson (Hampton et al., 1989; Fåhraeus-Van Ree and Spurell, 2003). Bile ducts have longitudinal but spare and discontinuous muscle fibres surrounding them (Wang et al., 2012). Of interest were the larger bile duct fibrous sheath and the fibrous bridging that connected multiple bile ducts. Some bile ducts had significant sclerosing fibrous layering around the bile duct giving an "onion-skin" appearance around the bile duct. Bile ducts are active in the secretion of biliary components and have longitudinal and occasional circular muscle fibres within their structure (Wang et al., 2012). The histology samples from this experiment were taken from the lateral inferior portion of the left hepatic lobe. Muscle fibres surrounding the bile ducts generally increase towards the distal common bile duct making the fibrosis in the lateral inferior region unexpected (Wang et al., 2012). The periductular fibrosis observed in the wild fish is often described as sclerosing cholangitis or cholestatic liver disease (bile flow impairment) in mammals (Nakanuma, 2017). Eventually, bile ducts become replaced by fibro-obliterative lesions that, in some cases, have inflammatory cells but more often inconspicuous inflammation that was not present in the livers of wild fish in the present study (Nakanuma, 2017). This study has provided some understanding to Yellowtail Kingfish liver

structure through contributing to the establishing the normal fibrous layering in wild fish. In the future, additional research on cultured *Seriola* species will be able to use this information.

Myxozoan parasites, *Ceratomyxa seriolae* and *C. buri*, are known to impair the flow of bile in the liver of wild and farmed Yellowtail Kingfish (Hutson et al., 2007). Likewise, anisakid larvae infection in the liver can cause fibrosis and atrophy of the organ (Shamsi et al., 2011). However, no parasites were found in the livers of the wild fish caught for this trial. Bile duct proliferation can also result from bacterial infections, mycotoxins or be a result of the cytotoxic effect of bile acids on the bile ducts (Moschetta et al., 2005; Rhodes et al., 2016; Watanabe et al., 1998). We suggest instead that one of these other forms of chronic hepatic insult that occurred, causing fibrosis around several ducts.

2.5.3 Biochemical analyses

2.5.3.1 Total bile acid concentration

Bile acids are amphipathic molecules synthesized from cholesterol in hepatocytes where conjugation with taurine or glycine occurs before being stored in the gallbladder (Nguyen et al., 2011). Approximately 95% of bile acids are recycled through the enterohepatic circulating system, with the remaining bile acids removed from the body via faecal excretion (Zhou and Hylemon, 2014). In teleosts, cholesterol, along with other lipids such as triglycerides, are hydrolysed by bile salt-activated lipase in the intestine and then get transported via the lymphatic system by lipoproteins, varying in density, to the blood and then carried back to the liver. This completes the enterohepatic circulation of bile acids (Tocher, 2003). Total bile acid concentrations of enterohepatic and digestive tissue from wild Yellowtail Kingfish are not available in literature so a comparison to cultured Yellowtail Kingfish fed fish meal and fish oil diets will be used.

Total bile acid concentrations in the blood of fish from the current trial ranged from 0.9 to 22.7 mmol L^{-1} (8.01 ± 7.84 mmol L^{-1} mean ± SD; Table 2.5). Closely related species, Japanese amberjack *S. quinqueradiata* (mean body mass 200 g) fed a fish meal based diet for 60 days had a blood bile acid concentration of 408 ± 18 mmol L^{-1}, considerably higher than the levels observed in the current study (Maita et al., 2006). Total bile acid concentrations in the blood are elevated postprandially and are a measure of hepatic function (York, 2017). However, when there is disruption to enterohepatic circulation of bile acids via hepatic dysfunction or portosystemic shunting total bile acids in blood increase (Morita et al., 1978). Enterocytes transport bile acids to the basolateral domain for efflux and are transported back to the liver via portal vein circulation (Kullak-Ublick et al., 2004; Murashita et al., 2014).

The total bile acid concentrations of the wild fish gallbladder content ranged from 20.71 to 42.4 µmoles g^{-1} (24.94 ± 7.79 µmoles g^{-1}; mean ± SD). Cultured Japanese amberjack (mean body mass 450 g), fed fish meal and fish oil based formulated feeds have gallbladder content bile acid concentrations of ~320 mmol L^{-1} (Nguyen et al., 2011). In the current study, total bile acid concentrations of the liver tissue ranged from 13.43 to 21.53 µmoles g^{-1} (18.36 ± 2.7 µmoles g^{-1}; mean ± SD). Cultured Japanese amberjack, (107.6 ± 2.6 g; mean body mass ± SD) fed fish meal and fish oil based formulated feeds have liver bile acid concentrations of 2.1 ± 0.3 µmoles g^{-1} (Nguyen et al., 2017).

The total bile acid concentration in the wild fish digestive tract differed between gut sections. The foregut total bile acid concentration ranged from 3.99 to 21.04 µmoles g^{-1} (13.29 ± 6.98 µmoles g^{-1}; mean ± SD). The midgut total bile acid concentration ranged from 11.00 to 19.45 µmoles g^{-1} (15.57 ± 3.29 µmoles g^{-1}; mean ± SD). The hindgut total bile acid concentration ranged from 10.27 to 24.53 µmoles g^{-1} (15.02 ± 5.59 µmoles g^{-1}; mean ± SD). Cultured Japanese amberjack (mean body mass 450 g), fed fish meal and fish oil based formulated feed have bile acid concentrations of ~45 µmoles g^{-1} in the anterior intestine and ~23 µmoles g^{-1} in

the posterior intestine (Nguyen et al., 2011). When fed diets with soybean proteins replacing the fish meal component of the diet the bile acid levels were reduced to between ~30-40 µmoles g^{-1} in the anterior intestine and ~18 – 23 µmoles g^{-1} in the posterior intestine (Nguyen et al., 2011). The reduction observed was considered to be due to soybean meals containing undigested high molecular fractions that bind to bile acids and increase their excretion from the body pool (Nguyen et al., 2011). Increased pooled bile acid levels in the cultured fish compared to wild caught fish could be due to the constant availability of feed and ability to synthesise bile acids. However, it is not possible to have known the exact feeding habits that could have influenced the bile acid pool of these wild caught fish.

2.5.3.2 Taurine concentration

To the best of our knowledge taurine liver levels for wild *Seriola* spp. are not present in literature for comparison. The taurine level in the livers of the wild fish caught in this study ranged from 0.01 to 0.07 µmoles g^{-1} (0.06 ± 0.02 µmoles g^{-1}; mean ± SD). Taurine is required for osmoregulation, membrane stabilisation and conjugation with bile acid before the latter is stored in the gallbladder (Nguyen et al., 2011; Takagi et al., 2005). In fish the ability to synthesise taurine differs among species and the ability to do so is particularly lower, or negligible, in marine carnivorous species due to a reduced level or absence of cysteine sulfinate decarboxylase compared with freshwater species (El-Sayed, 2014; Takagi et al., 2011). In marine fish, taurine is required and obtained through dietary intake. Taurine levels are high in wild-fish derived fish meals (approximately 4,000 to 12,000 mg kg^{-1}) and vary depending on the protein source and processing (El-Sayed, 2014; Spitze et al., 2003; Salze and Davis, 2015). In cultured Yellowtail Kingfish metabolism of lipids is reduced by a lack of available dietary taurine for bile acid conjugation and lipid emulsification and absorption, resulting in poor growth performance (Bowyer et al., 2012b: Stone and booth, 2018). Although taurine has not

traditionally been considered an essential amino acid for cultured marine finfish, studies in recent years have revealed significant improvements to health and growth when it is included in formulated feeds. Khaoian et al. (2014) considered taurine an essential supplement for yellowtail, even when fish meal was in the diet. The liver taurine levels of the current trial assists in the further understanding of Yellowtail Kingfish nutrition by establishing these concentrations.

2.5.3.3 HMGCR and CYP7A1 enzymes

Cholesterol is essential for bile acid production as well as for disease resistance and vital to cell membrane growth and function (Liland et al., 2013; Norambuena et al., 2013). Fish either acquire cholesterol from the diet or synthesise it *de novo* in the liver, although in a lesser capacity than mammals (Kortner et al., 2013). Formulated feeds containing fish oil and fish meal are high in available cholesterol, which is known to inhibit the synthesis of cholesterol *de novo* in fish (Norambuena et al., 2013). When formulated feeds have the fish oil and fish meal component replaced by certain terrestrially-sourced lipids and proteins, dietary cholesterol is reduced (Bowyer et al., 2012b; Liland et al., 2013). This has the potential to impact production of bile acids, the amphipathic molecules synthesized from cholesterol in hepatocytes (Nguyen et al., 2011). Mevalonate synthesis is the irreversible committed step of cholesterol synthesis regulated by HMGCR in the liver which is up regulated if *de novo* cholesterol is required (Kortner et al., 2013; Maita et al., 2006). The HMGCR concentration in the livers of wild fish from the present study ranged between 2.40 and 9.91 µmoles g^{-1} (6.02 ± 3.07 µmoles g^{-1}; mean ± SD). Likewise, the CYP7A1 enzyme concentration in the liver of different fish ranged between 1 865.6 and 3 557.60 µmoles g^{-1} (2 521.26 ± 627.00 µmoles g^{-1}; mean ± SD; Table 2.4). Cultured Atlantic salmon increased liver HMGCR gene expression is associated with decreased dietary cholesterol (Morais et al., 2011). Cholesterol catabolic pathway is stimulated

when increased levels of cholesterol are in the diet. The exact diet and feeding habits of these fish prior to sampling is unknown. However, Fowler et al. (2003) established that wild Yellowtail Kingfish feed on high energy fish species which would have high concentrations of cholesterol. The HMGCR and CYP7A1 enzyme concentration of the current study contributes to the understanding of *de novo* cholesterol synthesis within Yellowtail Kingfish.

2.5.4 Blood analyses

Blood biochemistry values are reported in Tables 2.5 and contribute to the establishment of a baseline understanding of the parameters observed in wild Yellowtail Kingfish. Certain body dysfunctions and diseases can be characteristically diagnosed by the accumulation of proteins not usually present in blood plasma (Hoboken, 2008). Here we discuss the specific parameters of interest to liver function. Blood protein concentration represents many proteins with specific functions, including but not limited to immune response enzymes, clotting factors and transporter proteins (Higgins, 2013). Albumin is the most abundant protein in plasma and is synthesised in the liver where its function is to transport water insoluble proteins and unconjugated bilirubin in blood plasma and maintain blood plasma volume (Higgins, 2013). When examined together, bilirubin and albumin concentrations are a relative indicator of liver synthetic function and overall nutritional status of the subject (Lee et al., 2009). Bilirubin is a derivative of heme during the phagocytosis of aged red blood cells and is commonly used as an indicator of liver function, with elevated levels indicating biliary tract disease, cholestasis or liver dysfunctions (Higgins, 2013; Lee et al., 2009; Wang et al., 2012). Albumin concentration was consistent amongst the fish (15.00 ± 1.15 g L^{-1}; mean ± SD) in the current study whilst the total bilirubin levels were zero. By comparison cultured Yellowtail Kingfish fed graded levels of dietary fish oil, using poultry oil as the replacement, presented albumin levels between 19.44 ± 0.97 and 23.44 ± 3.02 g L^{-1} (Stone et al., 2020). The human plasma

albumin reference range is 35 – 50 g L^{-1} and bilirubin reference range is <21 µmol L^{-1} (Higgins, 2013). Closely related cultured Japanese yellowtail, fed a fish meal-based diet, had albumin levels of 11.90 ± 1.00 g L^{-1} (mean ± SD) after a 36 week feeding trial (Khaoian et al., 2014). In a satiation trial, Kawanago et al. (2014) reported cultured Japanese yellowtail, fed a commercial diet with 52.2% crude protein and 14.9% crude fat, to have albumin concentrations up to 20.00 ± 2.00 g L^{-1} (mean ± SD) and bilirubin concentration of 4.00 ± 1.00 g L^{-1} (mean ± SD). Albumin and bilirubin concentrations in fish of the present trial were both lower than these values and the mammalian reference ranges, although albumin concentrations were within ranges seen in other closely related cultured species. Bilirubin concentrations, most likely under the range of the laboratory test's limit of detection, were negligible compared to closely related cultured species.

Both ALT and ALP levels had considerable variation between the fish; 35.57 ± 16.56 and 31.15 ± 10.53 IU L^{-1} (mean ± SD) respectively (Table 2.5). Alkaline phosphatase is required in liver and biliary tract cells for normal function but has no apparent function in blood. Increased blood levels are the result of increased energy processing by the liver with excess leakage into the blood (Kawanago et al., 2014). Thus, elevated ALP can also be used as a biochemical indicator of cell injury or necrosis and can be used as a measurer for cholangitis (Higgins, 2013; Nakanuma, 2017). Hepatic ALT is considered the most important aminotransferase in fish livers and is regulated by hormonal and nutritional status with increased activity seen when fish are fed high protein-low carbohydrate diets (Metón et al., 2015). By comparison, cultured Yellowtail Kingfish have an ALT range between 9.67 ± 0.88 and 16.33 ± 4.30 IU L^{-1} and ALP between 10.44 ± 2.26 and 13.33 ± 1.35 IU L^{-1} (Stone et al., 2020). Normal human ALT reference ranges are 10 – 40 IU L^{-1} and ALP reference ranges are 30 – 150 IU L^{-1} (Higgins, 2013). In a satiation trial, cultured Japanese yellowtail had ALT concentrations of 6.7 ± 1.5 to 9.0 ± 2.8 IU L^{-1} (mean ± SD) and ALP concentrations of 28.2 ± 4.3 to 41.6 ± 6.3 IU L^{-1} (mean

± SD; Kawanago et al., 2014). Kawanago et al. (2014) showed that both ALT and ALP activity were related to feeding rations and refeeding time with suggestion that these should be carefully considered when used as nutritional indicators. The ALP levels were elevated in two of the seven fish (Fish 3 and 5) and ALT were elevated in three of the seven fish (Fish 3, 4 and 7) suggesting that they may have more recently fed or were experiencing some form of hepatic stress, although no other liver function tests support the latter suggestion. The ALT concentrations of fish from the current study (19 – 60 IU L^{-1}) were greater than cultured Yellowtail Kingfish, the mammalian reference range and closely related species. The ALP concentrations of fish from the current trial (20 – 46 IU L^{-1}) were greater compared to cultured Yellowtail Kingfish, but slightly under and towards the lower end of the mammalian reference range and but similar to that observed in closely related species.

Plasma cholesterol concentrations are a combination of dietary cholesterol and *de novo* cholesterol synthesised in the liver, with the latter triggered by a decrease in dietary cholesterol (Maita et al., 2006). The cholesterol concentrations of the wild fish were consistent with one another (9.03 ± 0.59 mmol L^{-1}; mean ± SD; Table 2.5). Decreased blood cholesterol concentrations are the result of cholesterol utilisation for bile acid synthesis and for potential removal of cholesterol from the body (Kortner et al., 2013). Triglyceride levels ranged from 1.92 to 5.47 mmol L^{-1} (3.37 ± 1.18 mmol L^{-1}; mean ± SD; Table 2.5). Mammalian species are considered to have hyperlipidaemia if plasma cholesterol levels are >5.0 mmol L^{-1} (hypercholesterolemia) and if plasma triglyceride levels are >1.8 mmol L^{-1} (hypertriglyceridemia; Higgins, 2013). According to mammalian measures, some fish examined here had hyperlipidaemia, highlighting the care that must be taken when interpreting potential hypo- and hyper- cholesterolemic effects. Specific knowledge of cholesterol metabolisms for cultured species is largely based on mammalian studies and when reported for fish is fragmented at best. The levels of plasma cholesterol recorded in the current trial are

greater than that observed in a closely related cultured species juvenile greater amberjack and juvenile Yellowtail Kingfish (Bowyer et al., 2012b; Dawood et al., 2015). We recognise that whilst fasting is not necessary for total cholesterol, triglyceride concentrations should ideally be measured after a 12 – 24 hour period of overnight fasting due to the rise in levels observed after feeding (Higgins, 2013). This is however not possible for wild caught fish as the time of feeding is unknown and unable to be controlled.

The diet of wild fish caught off Greenly Island and Rocky Islands, South Australia by Fowler et al. (2003) were most similar to the animals within this study. The lipid composition of the whole body and specific organs and tissues can be influenced by the nutritional balance of diet and feeding regimes which can change according to stage of development for both wild and cultured fish (Rodríguez-Barreto et al., 2012). Size, sex, reproductive status, geographical location and environmental conditions experienced by wild fish and water quality control of cultured fish can also contribute to varying body compositions (Alasalvar et al., 2002; Rodríguez-Barreto et al., 2012). Feeding in particular is known to significantly influence the quality and nutrition profile of cultured fish. Wild caught South African Yellowtail Kingfish are reported to have a superior fatty acid composition that is likely to have greater nutritional benefits to the consumer than cultured Yellowtail Kingfish (O'Neill et al., 2015). A recent study by Stone et al. (2020) showed that the whole fish fatty acid profile of cultured Yellowtail Kingfish mirrored the fatty acid profile of the diets. In particular, EPA, docosapentaenoic acid (DPA), DHA and long chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) significantly increased with increasing dietary provision of EPA, DPA, DHA and LC n-3 PUFA (Stone et al., 2020).

Wild greater amberjack, *Seriola dumerili*, diet consists of smaller finfish such as Atlantic horse mackerel, *Trachurus trachurus*, and Atlantic sea bream, *Boops boops*, both containing high levels of polyunsaturated fatty acids (Rodríguez-Barreto et al., 2012). Similarly, in Australia,

Yellowtail Kingfish predominantly feed on smaller fish species: Fowler et al. (2003) collected 77 fish from the northern part of the Spencer Gulf in South Australia and 36 fish were collected from the southern end of the Eyre Peninsula off Greenly Island and Rocky Islands, South Australia. In their study, 33.8% of the northern fish were found to have stomach contents that consisted of predominantly fish species, some crustaceans and several molluscs. Several fish stomachs contained plant material and grain, indicating indiscriminate feeding on floating objects (Fowler et al., 2003). However, within this gulf several Yellowtail Kingfish sea-cage farms are established and the likelihood of several of the fish originating from the farms was supported by stomach content results and body morphometry (Fowler et al., 2003). The authors reported the stomach contents of 91.7% (33/36) of the Yellowtail Kingfish were predominantly of fish content, some crustacean and cephalopods and salps. Identifiable fish in stomach content were red bait, *Emmelichthys nitidus,* blue mackerel, *Scomber australasicus,* and Australian herring, *Arripis georgianus* (Fowler et al., 2003). A greater collection of fish over different seasons and geographical locations would have improved the comparison and analyses of fish collected within this study.

2.6 Conclusion

In the current study, as far as we are aware, we provide data that is the first report of gross and somatic indices, liver histology, bile acid and taurine concentrations, liver enzyme concentration and blood biochemistry for wild caught Yellowtail Kingfish from South Australian waters. A greater collection of fish over different seasons and geographical locations would have improved the comparison and analyses of fish collected within this study. We have contributed towards the establishment of baseline data that will assist in the future advance and development of formulated feeds for this commercially important species.

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Chapter 3: Investigating liver structure and function in Yellowtail Kingfish, *Seriola lalandi,* in response to diet energy and emulsifier level manipulation

3.1 Abstract

Supplementation of emulsifying agents in formulated feeds may be required if bile acid production decreases as a consequence of inclusion of alternative oil sources. In this study the effect that diets with different levels of crude lipid (CL, ~30 g 100 g^{-1} [Diets 1 and 2] and CL, ~20 g 100 g^{-1} [Diets 3 and 4] with or without the addition of a commercial emulsifier [LYSOFORTE® Liquid; 40 mg lipid kg^{-1}]) have on liver structure and energy reserves, concentration of enzymes involved in cholesterol and bile acid metabolism and blood biochemistry were investigated in cultured Yellowtail Kingfish, *Seriola lalandi*. The addition of the emulsifier had no significant effect on the hepatosomatic index (HSI) or visceral somatic index (VSI) but these were significantly greater ($P < 0.05$) in fish fed 30% CL diets when compared to the fish fed 20% CL diets. All treatments led to extensive large vacuolisation within hepatocytes, although fish fed 20% CL diets presented overall more homogenous hepatocyte distribution and consistently positioned cuboidal cholangiocytes compared with the livers of fish fed 30% CL diets. Mild bile duct proliferation was observed in livers of all fish and increased in the livers of fish fed 30% CL diets. Added emulsifier did not significantly affect total bile acid concentrations of the foregut, liver or gallbladder content in fish fed any of the diets. Crude lipid level did not significantly affect total bile acid concentrations in the liver or gallbladder but was significantly greater in the foregut ($P < 0.05$) of fish fed the 20% CL diets when compared to foregut tissue of fish fed the 30% CL diets. Neither taurine nor 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) concentrations of liver tissue were significantly affected by either dietary lipid level or the addition of the emulsifier. Liver tissue cholesterol-7α-hydroxylase (CYP7A1) concentrations of fish fed the 20% CL diets were numerically reduced compared to that of fish fed the 30% CL diets. The addition of the emulsifier had no significant effect on blood biochemistry parameters measured. Blood total bile acid and triglyceride levels were significantly greater ($P < 0.05$) in fish fed the 30% CL

diets when compared to the fish fed the 20% CL diets. Neither cholesterol nor total bilirubin concentrations were significantly different between any of the treatments. The additional of this commercial emulsifier at 40 mg lipid kg^{-1} in these diets did not impact parameters measured. Emulsifying agents at different concentrations or of different sources may have desired effects and their uses should be investigated.

3.2 Introduction

The Yellowtail Kingfish, *Seriola lalandi,* is a carnivorous pelagic finfish species with a high metabolic rate that congregates in schools in temperate coastal waters throughout the world. It is favoured in aquaculture because of its fast growth rate and excellent meat quality (Fernandes and Tanner, 2008; Bowyer et al., 2013). Cultured Yellowtail Kingfish were traditionally fed formulated feeds containing fish oil (FO) and fish meal (FM) as the main sources of dietary lipid and protein, respectively. However, increased demand of finfish for human consumption and FO and FM to be used in diet formulation for the terrestrial production animal industry has led to competition and increases in the prices of these raw ingredients (FAO, 2016a and 2016b). In order to make diets more cost effective and environmentally sustainable, FO and FM substitution is a high priority area for Yellowtail Kingfish feed development (Gatlin et al., 2007; Stone and Bowyer., 2013; Stone et al., 2016).

When FO is replaced by some terrestrially-sourced lipids, particularly plant-based sources, dietary cholesterol is also reduced (Bowyer et al., 2012b; Liland et al., 2013). Cholesterol is essential for bile acid production as well as for disease resistance and is vital to cell membrane growth and function (Liland et al., 2013; Norambuena et al., 2013). Teleost fish can either acquire cholesterol from the diet or can synthesise it *de novo* in the liver, although in a lesser capacity than mammals (Kortner et al., 2013). The *de novo* synthesis of cholesterol involves a series of enzymatic processes regulated by the rate limiting step of mevalonate synthesis by 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR). Juvenile European sea bass, *Dicentrarchus labrax,* fed diets void of FO and containing soybean oils as the primary lipid source showed significant up regulation of the HMGCR gene compared to fish fed diets with FO as the primary lipid source indicating a compensatory mechanism to produce cholesterol (Torrecillas et al., 2018). However, fish fed the diet void of FO had significantly decreased

final weight, length and SGR compared to fish fed diets containing FO (Torrecillas et al., 2018). Furthermore, the vegetable oil diet contained defatted FM achieved through chloroform extraction which may have only been partly efficient in removing lipids, leaving a remnant FO component in the experimental diet. Contrary to this, Atlantic salmon, *Salmo salar*, fed diets with either FO as the primary lipid source or a graded blend of FO, camelina oil and poultry oil as the primary lipid source saw no significant alterations to HMGCR regulation across treatment groups (Hixon et al., 2017). Furthermore, the final weight, length and SGR were not significantly different between fish fed the different treatments (Hixon et al., 2017). The level at which dietary FO can be replaced by terrestrially-sourced lipids, particularly plant-based sources, is species dependent. If reduction in dietary cholesterol occurs, *de novo* cholesterol synthesis may not compensate for the reduction and could thus lead to reduced bile acid synthesis and nutrient uptake.

Bile acids are steroids synthesised from cholesterol in liver hepatocytes (Mohapatra and Mishra, 2011; Nguyen et al, 2011; Holm et al., 2013). Bile acids are synthesised via the neutral or classical pathway which is regulated by cholesterol-7α-hydroxylase (CYP7A1), resulting in the formation of primary bile acids, cholic acid and chenodeoxycholic acid (Kullak-Ublick et al., 2004; de Aguiar Vallim et al., 2013). To a lesser extent an alternative pathway, regulated by sterol 27-hydroxylase (CYP27A1), also produces chenodeoxycholic acid in mammals (de Aguiar Vallim et al., 2013; Moghimipour et al., 2015). The alternative pathway results in the formation of hyocholic acid in pigs and muricholic acid in rodents (Holm et al., 2013). In fish, CYP7A1 is considered the main rate-limiting enzyme contributing to bile acid synthesis, by regulating the conversion of cholesterol to 7α-hydroxycholesterol (Crosignani et al., 2011; Deng et al., 2014; Guerra-Olvera and Viana, 2015). Together 7α- and 27- hydroxylation of cholesterol contribute over 95% of bile acid synthesis through the classical neutral pathway or alternative pathway, respectively (Crosignani et al., 2011; de Aguiar Vallim et al., 2013;

Moghimipour et al., 2015). Once synthesised, bile acids are secreted into the canaliculi, drained into bile ducts and finally after being conjugated with taurine or glycine are stored in the gallbladder as bile salts (Mohapatra and Mishra, 2011; Nguyen et al, 2011; Holm et al., 2013; El-Sayed, 2014; Guerra-Olvera and Viana, 2015). After feeding, the gallbladder contracts and the contents are secreted into the small intestine to assist in the micellar solubilisation of dietary lipids, esterification, emulsification and digestion of long chain fatty acids (Van Waarde, 1988; Yamamoto et al., 2007; Nguyen et al, 2011; El-Sayed, 2014; Murashita et al., 2014; Zhou and Hylemon, 2014). The term bile acids is used hereafter to describe the total of conjugated and unconjugated bile acids.

Any reduction in available cholesterol for bile acid synthesis ultimately affects the digestion of nutrients, resulting in reduced emulsification efficiency of dietary lipids. Supplementation of emulsifying agents in formulated feeds may be required if bile acid production decreases as a consequence of inclusion of alternative oil sources. Sunflower, soybean, canola and other oil seed lecithin have been used as dietary emulsifiers in food production, pharmaceutical and dietetics (Guiotto et al., 2015). In rainbow trout, *Oncorhynchus mykiss*, (235 – 375 g) a dietary additive of soy lecithin increased energy digestibility (Hung et al., 1997). Likewise, increased growth was observed in Atlantic salmon fry (1.5 g) when fed soy lecithin at a dietary inclusion rate of 30 g kg^{-1} compared to fry fed a diet void of the soy lecithin additive (Hung et al., 1997).

The aim of this study was to understand the cholesterol and bile acid metabolism and liver structure in sub-adult Yellowtail Kingfish fed different commercial diets with: 1) different dietary crude lipid levels; and 2) the addition of a commercial emulsifier. This was achieved by measuring the concentration of rate limiting enzymes for cholesterol and bile acid synthesis and total bile acid concentrations. Liver histological samples were observed for potential

structural changes. Routine liver function indicators were observed for any biochemical changes that may have been induced by the different treatments.

3.3 Methods

3.3.1 Experimental description

In this study, a factorial design was utilised, with two lipid levels and two emulsifier levels, resulting in four treatment combinations (Table 3.1). The biochemical composition of the four diets is displayed in Table 3.1. The experiment was conducted over an 84-day period at the South Australian Research and Development Institute (SARDI) South Australian Aquatic Sciences Centre (SAASC) at West Beach, Adelaide, Australia. At the commencement of the experiment, individual fish were weighed (kg) and measured for fork length (mm) after the fish had been anaesthetised in seawater using AQUI-S® (AQUI-S® New Zealand Ltd.) at a concentration of 0.02 mL L^{-1}. A total of 276 fish (body mass 1.12 ± 0.11 kg; fork length 426 ± 13 mm; mean ± SD) were randomly distributed between twelve 5,000 L fibreglass tanks with 23 fish per tank. Each of the four treatments was randomly assigned to three replicate tanks. Fish were fed to satiation once daily 09:00 h (Stone and Booth, 2018).

Table 3.1. The nutrient composition of experimental diets fed to Yellowtail Kingfish, *Seriola lalandi*, for 84 days.[1]

Item (as fed) [1]	Diet 1	Diet 2	Diet 3	Diet 4
Nominal dietary lipid level (%)	30	30	20	20
Dietary emulsifier (mg lipid kg^{-1})	0	40	0	40
Analysed proximate composition (g $100g^{-1}$)				
Moisture	4.8	7.7	6.6	7.5
Crude protein	41.9	41.0	47.4	47.1
Crude lipid	30.0	28.6	19.6	19.0
Ash	9.7	9.7	11.0	10.9
Carbohydrate [2]	13.6	13.0	15.4	15.5
Gross energy (MJ kg^{-1})	20.5	19.8	17.9	17.7
Analysed fatty acids (mg 100 g^{-1})				
Palmitic C16:0	6370	6010	4030	3920
Linoleic C18:2n-6	3270	2850	1940	1910
Docosapentaenoic C22:5n-6 (DPA)	46	61	43	48
Eicosapentanaeoic C20:5n-3 (EPA)	1050	1450	1090	1040
Docosahexaenoic C22:6n-3 (DHA)	870	1110	930	900
EPA + DPA + DHA	2090	2780	2200	2110
Other (mg $100g^{-1}$)				
Cholesterol	293	297	277	275

Diet 1: 30% lipid, no emulsifier; Diet 2: 30% lipid, with emulsifier; Diet 3: 20% lipid, no emulsifier; Diet 4: 20% lipid, with emulsifier.
[1] Pellet kernel, fish oil and poultry oil to manufacture diets were supplied by Skretting Australia (Cambridge, Tas., Australia).
[2] Carbohydrate = 100 - (moisture + lipid + protein + ash).

3.3.2 Experimental system

The recirculating aquaculture system (RAS) was kept undercover in ambient conditions with supplemental fluorescent lighting provided during the natural light period. Ambient temperature sea water was treated by settlement and sand filtration and circulated through the RAS. The sea water was then returned through filter-screen baffle-boards, pumped through a drum filter with 70 μm^2 filter pores (HDF1603 Hydrotech, Saint-Maurice, France), returned to

the biofilter, and finally treated with UV light disinfection (D-32051 Wedeco, Herford, Germany) at a rate of 35,000 L h^{-1} by two electric centrifugal pumps (Grundfos, Regency Park, Australia) before being returned to the tanks. One hundred percent of the water volume of each 5,000 L tank was replaced daily.

3.3.3 Fish source and acclimation

Sub-adult Yellowtail Kingfish, *Seriola lalandi*, were obtained from Clean Seas Pty. Ltd. sea cage facilities at Port Lincoln, Australia. The fish were acclimated for 8 weeks in 5,000L fibreglass tanks supplied by the RAS at SARDI SAASC and fed a commercial diet (Ridley Corporation Ltd. Pelagica diet; gross energy 19.3 MJ kg^{-1}, crude lipid [CL] 24%, crude protein [CP] 46%) before the commencement of the experiment.

3.3.4 Experimental diets

In this study, four treatments referred to as Diet 1, Diet, 2, Diet 3 and Diet 4 contained two different nominal levels of CL (20% CL and 30% CL) with or without the addition of a commercial emulsifier (Table 3.1). The diets were measured and determined to be formulated to contain CL at 29.3 ± 0.7 g 100 g^{-1} (Diets 1 and 2) and CL at 19.3 ± 0.3 g 100 g^{-1} (Diets 3 and 4; Table 3.1). Diets 2 and 4 contained a nominal 40 mg lipid kg^{-1} dietary emulsifier (Table 3.1). All diets were formulated and manufactured by Skretting Australia Pty Ltd (Cambridge, TAS, Australia) using cooking extrusion technology (9 mm pellets). The pellet kernel was formulated to contain 30% fish meal, and contained ~10% crude lipid. The pellet kernels were top coated at Aquafeeds Australia (Mount Barker, South Australia, Australia) with a blend of fish oil and poultry oil to achieve a total dietary lipid level of 20% or 30%. The experimental diets were analysed for biochemical composition according to methods in the British Pharmacopoeia Commission (2004) or German Institute for Standardization (DIN) (2000) by

SARDI. A one kg sample of each diet was collected, ground and analysed for proximate composition (moisture, protein, fat, ash, total carbohydrate and energy) and fatty acid profile.

The four experimental diet ingredient formulations were commercially confidential; however, the corresponding proximate diet composition, fatty acid profiles and proximate amino acid profiles are shown in Table 3.1.

3.3.5 Water quality

Water quality parameters were monitored daily, unless otherwise indicated. Temperature (°C, mean ± SD) and dissolved oxygen (% saturation, mean ± SD) were monitored using an OxyGuard Handy Polaris temperature and dissolved oxygen probe (OxyGuard International A/S, Farum, Denmark). The pH (mean ± SD) was monitored using a Eutech pH Testr 30 multiparameter handheld probe (Eutech Instruments Pty. Ltd., Singapore). Salinity (g L^{-1}, mean ± SD) was monitored using an ISSCO UR-2 hand-held refractometer (model RF20; Extech Instruments Corporation, Nashua, New Hampshire, United States of America). The total ammonia concentration (mg L^{-1}) was determined weekly using a commercial water testing kit (Aquarium Pharmaceuticals, Chalfont, Pennsylvania, United States of America).

3.3.6 Sample collection

Feeding was stopped 24 h before the end of the experiment. Three fish from each tank were euthanized and measured for their body mass (nearest 0.1 g) and fork length (nearest 0.1 mm). The growth performance for this trial was reported by Stone and Booth (2018). All data reported for each treatment for animal performance were based on the mean of the replicate tanks. All calculations using fish weight are based on wet values:

Weight gain = final weight – initial weight

For each fish, the visceral cavity was cut open and the whole removed gut weighed (nearest 0.01 g) to determine the visceral somatic index (VSI; %). The liver was removed and weighed

(nearest 0.01 g) to determine the hepatosomatic index (HSI; %). These indices were calculated as follows:

VSI% = (visceral weight [g] / body mass [g]) × 100

HSI% = (liver weight [g] / body mass [g]) × 100

A 1 cm^3 section of the left lobe of each liver was dissected and immediately fixed with 10% seawater-buffered formalin (pH of 7.2) for histological evaluation. Liver, gallbladder content and foregut were snap frozen in liquid nitrogen and stored at – 80 °C until measurement of total bile acid concentration and taurine concentrations. The liver samples were also used to measure the concentration of HMGCR and CYPA71 enzymes. Blood samples were collected from the caudal vasculature using 21 gauge needles and 5 mL syringes.

3.3.7 Liver histology

The liver samples were fixed for 24 h before being transferred to 70% (v/v) ethanol and stored at room temperature according to Hu et al. (2013). The samples were dehydrated using standard procedures, embedded in paraffin and sectioned at 5 μm on a rotary microtome. A section of liver from each fish was haematoxylin and eosin (H&E) stained and examined at 200-fold magnification using a light microscope (Olympus BF BX43). Three 559,390 μm^2 microphotographs were taken per stained slide using a digital camera (Olympus DP27). Quantitative assessments of the vacuole volume as a proportion of the liver cell volume (VPLC%, Crowe et al. 2021) was determined by applying a H&E stain sensitive colour threshold using Fiji ImageJ processing software (National Institutes of Health, Bethesda, Maryland, United States of America).

3.3.8 Biochemical analyses

Samples from individual fish were partially thawed, weighed, pooled per treatment replicate and homogenised in five volumes of phosphate buffered saline (PBS) with a pH of 7.1. Homogenisation was performed using a Retch MM 400 ball mill at frequency 30.01 s^{-1} for 4 min with Biospec 2 mm zirconia beads (Cat. No. 11079124zx). The suspensions were centrifuged twice at an acceleration of 10,000 *g* for 10 min at 4 °C (Beckman Coulter Microfuge 16 centrifuge) with the supernatants being extracted to new microfuge tubes between cycles. All assay kits included internal standard solutions and manufacturer instructions were followed for assays. Tissue concentrations are reported per tissue weight (µmoles g^{-1}).

3.3.8.1 Total bile acid concentration

The liver, gallbladder content and foregut pooled supernatants were analysed for total bile acid concentrations by use of a commercial fluorometric bile acid assay kit by reading Ex/Em = 560/590 nm after 45 min of dark incubation at room temperature (Catalogue No. MET-5005; Cell Biolabs Inc.®, San Diego, California, United States of America).

3.3.8.2 Taurine concentration

The pooled liver supernatants were analysed for taurine by use of a commercial colorimetric taurine assay kit by reading samples at 405 – 415 nm (Catalogue No. MET-5070; Cell Biolabs Inc.®, San Diego, California, United States of America).

3.3.8.3 HMGCR and CYP7A1 enzyme-linked immunosorbent assays

The pooled liver supernatants were analysed for HMGCR concentration after reading the absorbance of samples at a wavelength of 450 nm (Catalogue No. CSB-E15772m; CusaBio, Texas, United States of America). The pooled supernatants were analysed for CYP7A1

concentration after reading the absorbance of samples at a wavelength of 450 nm (Catalogue No. CSB-EL006395FI; CusaBio, Texas, United States of America).

3.3.9 Blood analyses

Two mL of blood was placed into separate Vacuette® or BD vacutainer® tubes (Z serum clot activator or EDTA tubes) and stored indirectly on ice until same-day analysis of blood parameters by IDEXX Laboratories (Unley, South Australia, Australia). Specifically, the following parameters were observed: total bile acids, cholesterol, triglycerides and total bilirubin.

3.3.10 Statistical analyses

A two-way ANOVA was used to assess the effects of CL level (30%; Diets 1 and 2; and, 20%; Diets 3 and 4) and emulsifier inclusion (Diets 2 and 4). The normality of data was assessed using the Shapiro–Wilk test. Homogeneity of variances among means was assessed using Levene's test for equality of variance errors. Where significant interactions were observed, the data were analysed using Tukey's Honestly Significant Difference (HSD) multiple range test, unless otherwise stated. The significance level was set at $\alpha = 0.05$ for all statistical tests. All data are presented as the mean ± the standard error (SE) of the mean, unless otherwise stated. IBM SPSS Version 22 for Windows (IBM SPSS Inc., Chicago, Illinois, United States of America) software was used for all statistical analyses.

3.4 Results

3.4.1 Water quality

Average values for water temperature, dissolved oxygen, pH and salinity were 15.8 ± 1.4 °C, 100.2 ± 3.9%, 7.8 ± 0.1 and 38 ± 1 g L^{-1}, respectively, across the study period. The total ammonia concentration was found to be below the limits of detection on all occasions.

3.4.2 Growth performance

The growth performance for this trial was reported by Stone and Booth (2018). In short, no interaction was observed between the addition of the emulsifier and the lipid level on final weight. The addition of the emulsifier had no significant influence on the final weight ($P > 0.05$) of the Yellowtail Kingfish. The final weight was significantly greater ($P = 0.031$) in fish fed diets containing 30% CL (Diets 1 and 2) when compared to the final weight of fish fed diets containing 20% CL (Diets 3 and 4).

3.4.3 Somatic indices

No interaction was observed between the addition of the emulsifier and the lipid level on HSI or the VSI ($P > 0.05$; Table 3.2) of the Yellowtail Kingfish. The addition of the emulsifier had no significant effect on the HSI ($P = 0.485$) or the VSI ($P = 0.564$). The HSI was significantly greater ($P = 0.009$) in fish fed diets containing 30% CL (Diets 1 and 2) when compared to the fish fed diets containing 20% CL (Diets 3 and 4; Table 3.2). Likewise, the VSI was significantly greater ($P = 0.006$) in fish fed diets containing 30% CL (Diets 1and 2) when compared to the fish fed diets containing 20% CL (Diets 3 and 4; Table 3.2).

3.4.4 Liver histology

There was no significant interaction between lipid level and the addition of the emulsifier to diets on the VPLC% ($P = 0.476$; Table 3.2). The VPLC% was not significantly affected by either lipid level ($P = 0.321$) or the addition of the emulsifier ($P = 0.529$; Table 3.2). The livers of fish fed all treatments showed extensive large vacuolisation with hepatocytes containing optically empty content identified as intracytoplasmic lipid droplets (Figure 3.1; McFadzen et al., 1997; Hu et al., 2013). All treatments led to extensive large vacuolisation within hepatocytes, although fish fed 20% CL diets presented overall more homogenous hepatocyte distribution and consistently positioned cuboidal cholangiocytes compared with the livers of

fish fed 30% CL diets. Fish fed the 20% CL level diets had consistent homogenous hepatocyte vacuolisation with clear nuclei; whereas fish fed 30% CL level diets had more vacuole fatty deposits with varying hepatocyte size and less distinguishable nuclei. The livers of several fish fed the 20% CL diet with emulsifier contained less hepatocyte fat deposition compared to that of the fish fed the 20% CL diet with no emulsifier. Mild bile duct proliferation was observed in livers of all fish but an increased level of bile duct proliferation was seen in the livers of fish fed diets with a 30% CL level (Figure 3.2). Furthermore, in livers of fish fed the diets with a 30% CL level newly forming bile ducts without lumen were observed.

Table 3.2. Somatic parameters of Yellowtail Kingfish, *Seriola lalandi*, fed different dietary lipid levels and emulsifiers for 84 days.[1]

Diet	1	2	3	4	2 factor ANOVA (*P value*)[2]		
					Lipid level (A)	Emulsifier (B)	A × B
Somatic parameters							
Viscerosomatic index (VSI; %)[3]	7.83±0.30	8.18±0.53	6.73±0.25	6.83±0.41	**0.006**	0.564	0.758
Hepatosomatic index (HSI; %)[4]	1.44±0.05	1.35±0.12	1.15±0.06	1.12±0.11	**0.009**	0.485	0.759
VPLC%[5]	47.22±0.44	50.10±3.24	50.6±0.38	50.98±2.23	0.321	0.529	0.476

[1] Values are mean ± SE; $n = 3$.

[2] A significance level of $\alpha = 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.

[3] Viscerosomatic index (VSI; %) two way-ANOVA results level were significantly affected by lipid level (30% CL > 20% CL).

[4] Hepatosomatic index (HSI; %) two way-ANOVA results level were significantly affected by lipid level (30% CL > 20% CL).

[5] Vacuole volume as a proportion of the liver cell volume (Crowe et al. 2021).

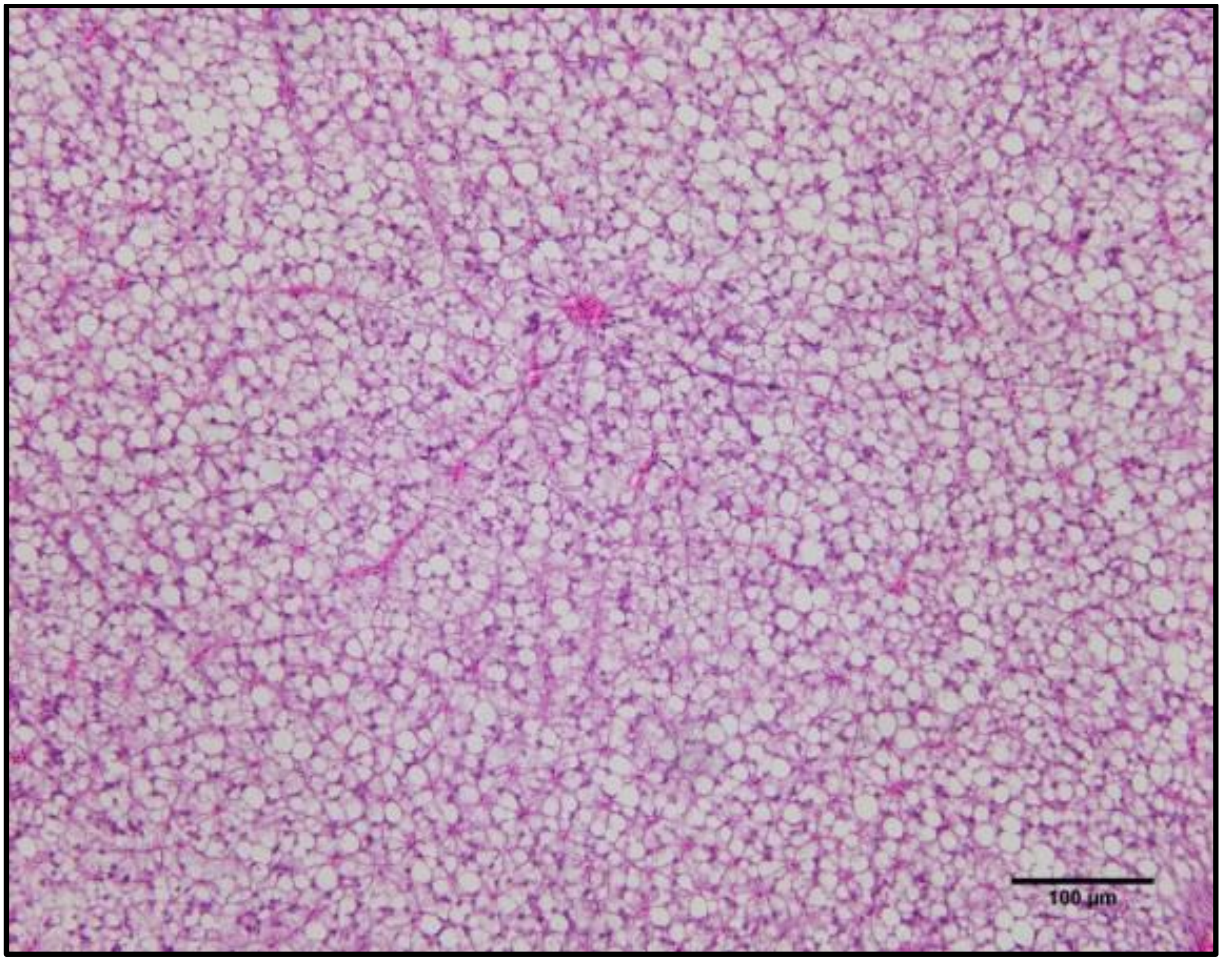

Figure 3.1. Yellowtail Kingfish, *Seriola lalandi*, liver section after an 84-day feeding trial with varied dietary levels of energy and the addition of a commercial emulsifier. Diet 1 contained crude lipid, 29.3 ± 0.7 g $100g^{-1}$ comprising of a fish oil and poultry oil blend. Haematoxylin and eosin stain.

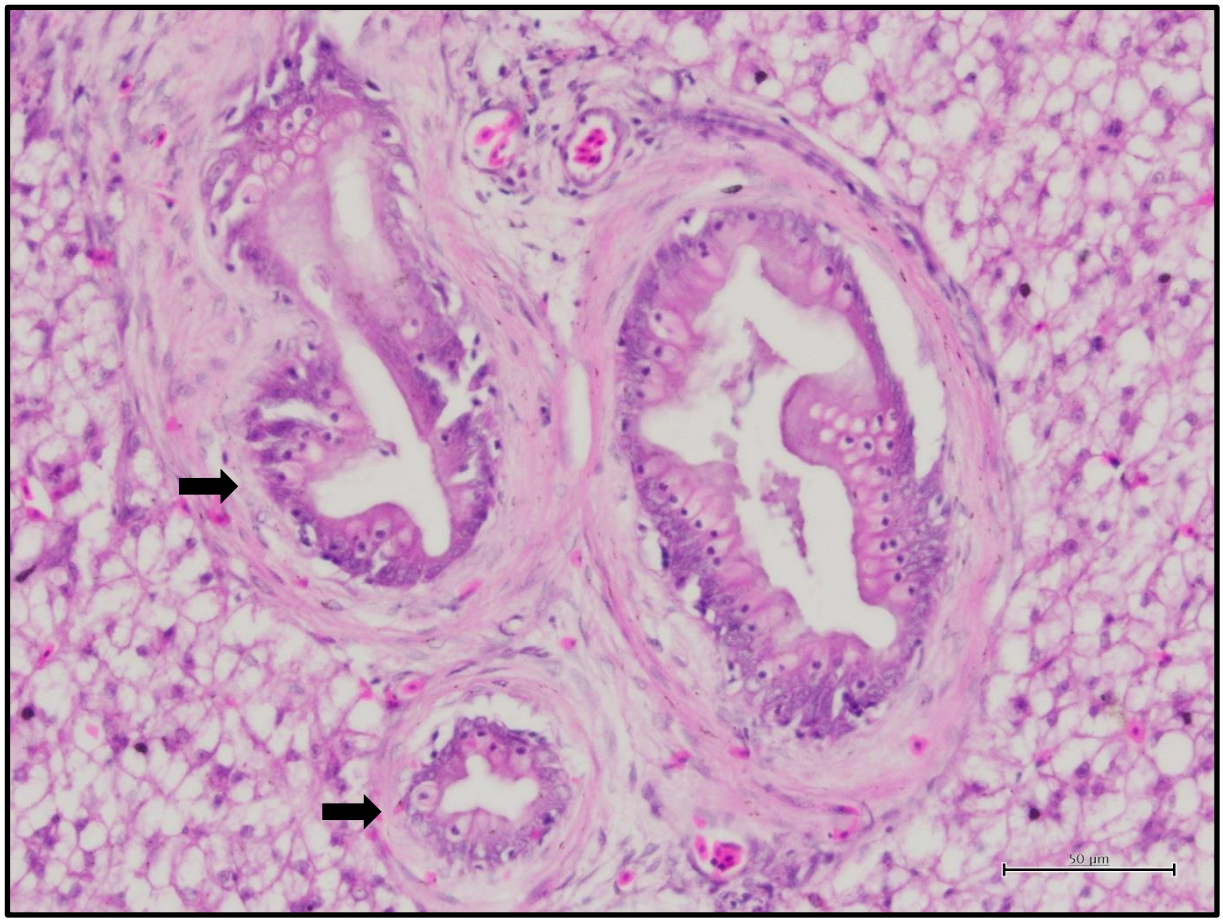

Figure 3.2. Yellowtail Kingfish, *Seriola lalandi*, liver section after an 84-day feeding trial with varied dietary levels of energy and the addition of a commercial emulsifier. Increased bile duct proliferation (*arrows*) was seen in the livers of fish fed diets with a 30% crude lipid level. Haematoxylin and eosin stain.

3.4.5 Biochemical analyses

3.4.5.1 Total bile acid concentration

No significant interaction was observed between CL level and the addition of the emulsifier for liver (P = 0.827), gallbladder content (P = 0.837) or foregut (P = 0.783; Table 3.3) total bile acids. The addition of the emulsifier did not significantly affect total bile acid concentrations of the foregut (P = 0.873), liver (P = 0.647) or gallbladder content (P = 0.888)

in fish fed any of the diets (Table 3.3). Crude lipid level did not significantly affect total bile acid concentrations in the liver (P = 0.395) or gallbladder content (P = 0.615). The total bile acid concentration of the foregut was significantly greater (P = 0.015) in fish fed the 20% CL (Diets 3 and 4) when compared to foregut tissue of fish fed the 30% CL (Diets 1 and 2; Table 3.3).

3.4.5.2 Taurine concentration

There was no significant interaction between CL level and the addition of the emulsifier to diets on the taurine concentration of liver tissue (P = 0.580; Table 3.3). The taurine concentration of liver tissue was not significantly affected by either dietary lipid level (P = 0.579) or the addition of the emulsifier (P = 0.578; Table 3.3).

Table 3.3. Bile acid and taurine concentrations of Yellowtail Kingfish, *Seriola lalandi*, fed different dietary lipid levels and emulsifiers for 84 days.[1]

Diet	1	2	3	4			
Nominal dietary lipid level (%)	30	30	20	20	2 factor ANOVA (*P value*)[2]		
Dietary emulsifier (mg lipid kg^{-1})	0	40	0	40	Lipid level (A)	Emulsifier (B)	A × B
Total bile acid concentration (µmoles g^{-1})							
Liver	22.64±3.86	23.55±4.37	18.51±1.19	21.09±4.27	0.395	0.647	0.827
Gallbladder content	20.66±3.94	19.35±3.82	17.96±2.92	18.20±3.92	0.615	0.888	0.837
Foregut[3]	18.81±0.98	18.38±0.88	21.52±0.27	21.63±1.39	**0.015**	0.873	0.783
Taurine concentration (µmoles g^{-1})							
Liver	0.0032±0.00	0.0027±0.00	0.0032±0.00	0.0032±0.00	0.580	0.579	0.578

[1] Values are mean ± SE; $n = 3$.
[2] A significance level of $\alpha = 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.
[3] Foregut two way-ANOVA results level were significantly affected by lipid level (20% CL > 30% CL).

3.4.5.3 HMGCR concentration

No significant interaction was observed between CL level and the addition of the emulsifier to diets on the concentration of HMGCR in liver tissue (P = 0.379; Table 3.4). The HMGCR concentration of liver tissue was not significantly affected by either dietary lipid level (P = 0.889) or the addition of the emulsifier (P = 0.629; Table 3.4).

3.4.5.4 CYP7A1 concentration

No statistically significant interaction was observed between lipid level and the addition of the emulsifier to diets on the concentration of CYP7A1 in liver tissue (P = 0.638; Table 3.4). Addition of the emulsifier did not significantly affect the CYP7A1 concentration (P = 0.463; Table 3.4). However, these concentrations in fish fed the 20% CL diet with emulsifier (Diet 4) were numerically reduced compared to fish fed the 20% CL without emulsifier (Diet 3). Similarly, the concentrations in fish fed the 30% CL diet with emulsifier (Diet 2) were numerically reduced compared to fish fed the 30% CL diet without emulsifier (Diet 1; Table 3.4). Dietary lipid level did not significantly affect the concentration of CYP7A1 in fish livers (P = 0.095; Table 3.4). The concentration of CYP7A1 in the livers of fish fed diets containing 20% CL were numerically reduced compared to the livers of fish fed diets containing 30% CL (Table 3.4).

Table 3.4. HMGCR and CYP7A1 concentrations in liver tissue of Yellowtail Kingfish, *Seriola lalandi*, fed different dietary lipid levels and emulsifiers for 84 days.[1]

Diet	1	2	3	4			
Nominal dietary lipid level (%)	30	30	20	20	2 factor ANOVA (*P value*)[2]		
Dietary emulsifier (mg lipid kg^{-1})	0	40	0	40	Lipid level (A)	Emulsifier (B)	A × B
HMGCR (µmoles g^{-1})	50.13±9.21	45.29±8.20	37.97±18.64	54.18±3.43	0.889	0.629	0.379
CYP7A1 (µmoles g^{-1})	2594.64±301.54	2182.03±345.72	1813.06±388.83	1720.64±261.22	0.095	0.463	0.638

[1] Values are mean ± SE; $n = 3$.

[2] A significance level of $\alpha = 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.

3.4.6 Blood analyses

None of the blood biochemical parameters was significantly affected by an interaction between the addition of the emulsifier and lipid level ($P > 0.05$; Table 3.5). The addition of the emulsifier had no significant effect on the blood biochemistry parameters measured ($P > 0.05$; Table 3.5). Although not significantly different, the total bile acids level within each CL level reduced with the addition of dietary emulsifier. The triglyceride level was significantly greater ($P = 0.007$) in fish fed diets containing 30% CL (Diets 1 and 2) when compared to the fish fed diets containing 20% CL (Diets 3 and 4; Table 3.5). Likewise, the bile acids level was significantly greater ($P = 0.047$) in fish fed diets containing a 30% CL (Diets 1 and 2) when compared to the fish fed diets containing 20% CL (Diets 3 and 4; Table 3.5). Neither cholesterol nor total bilirubin concentrations were significantly different between any of the treatments.

Table 3.5. Blood biochemistry of Yellowtail Kingfish, *Seriola lalandi*, fed different dietary lipid levels and emulsifiers for 84 days.[1]

Diet	1	2	3	4			
Nominal dietary lipid level (%)	30	30	20	20	2 factor ANOVA (*P value*)[2]		
Dietary emulsifier (mg lipid kg^{-1})	0	40	0	40	Lipid level (A)	Emulsifier (B)	A × B
Biochemistry							
Total Bilirubin (mmol L^{-1})	2±0	5±2	2±0	2±0	0.265	0.176	0.257
Cholesterol (mmol L^{-1})	4.3±0.3	5.0±0.1	4.8±0.3	4.7±0.3	0.712	0.259	0.175
Triglyceride (mmol L^{-1})[3]	5.0±0.5	5.1±0.3	4.1±0.4	3.4±0.1	**0.007**	0.424	0.296
Bile Acids (mmol L^{-1})[4]	29.9±12.8	16.9±5.2	9.7±3.2	5.0±1.4	**0.047**	0.230	0.574

[1] Values are mean ± SE; $n = 3$.
[2] A significance level of $\alpha = 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.
[3] Triglyceride (mmol L^{-1}) two way-ANOVA results level were significantly affected by lipid level (30% CL > 20% CL).
[4] Bile Acids (mmol L^{-1}) two way-ANOVA results level were significantly affected by lipid level (30% CL > 20% CL).

3.5 Discussion

Yellowtail Kingfish liver structure and fat deposition, cholesterol and bile acid metabolism and blood liver function indices in this study were affected by the level of dietary lipid in formulated feeds. The addition of the emulsifier to diets appeared to have no significant effect for animal growth as reported by Stone and Booth (2018) but did affect bile acid metabolism. Here we provide the first report of several enterohepatic-related measures in cultured Yellowtail Kingfish.

Cultured Yellowtail Kingfish have a high metabolic demand which is met by high energy formulated feeds (Hilton et al., 2008; Booth et al., 2010). Fish have a lower feeding activity during sub-optimal water temperatures as they have reduced metabolic energy requirement (Watanabe et al., 1999). At summer water temperature, sub-adult Yellowtail Kingfish require formulated feeds to contain ~18 to 20 MJ kg^{-1} of digestible energy to maintain metabolic functions, with increased dietary levels of energy for continual growth (Booth et al., 2010; Stone et al., 2016). Diets in this study contained well-balanced fatty acid profiles leading to significantly increased final weight in fish fed diets containing 30% CL compared to fish fed diets containing 20% CL (Stone and Booth, 2018). The growth rates in all treatments highlight the commercial relevance of the results. Improved growth and feed utilisation are observed in most fish when fed increased energy diets but an excess of dietary energy may accumulate as fatty deposits in different tissues or be stored as visceral fat (Turchini et al., 2009).

Increased dietary CL level from 20% to 30% significantly increased the HSI and VSI for Yellowtail Kingfish. Greater HSI as a result of increased dietary lipids is a common response in other marine species such as large yellow croaker, *Pseudosciaena crocea* R., Atlantic salmon and European sea bass (Hansen et al., 2008; Hartviksen et al., 2014; Wang et al., 2015; Yan et

al., 2015). This has implications for producers as harvest yield of a whole fish weight is increased but the harvest yield of fillets may not increase accordingly, resulting in expenditure on unnecessarily high lipid feeds. The storage of fats in peripheral organs or visceral tissues can also be influenced by water temperature (Sissener et al., 2017). Understanding the impact dietary FO substitution may have on Yellowtail Kingfish during periods of sub-optimal and optimal water temperature is essential for maintaining year-long production.

Gross and histological examination of fish livers are necessary to assess the nutritional impact that alternative lipid feedstuffs may have on cultured species (Aydin et al., 2015). Dietary changes can distinctly alter the appearance of hepatocytes in cultured marine finfish when fed diets with different energy levels (Crowe et al., 2021). In this study, increasing the dietary lipid level from 20% CL to 30% CL significantly increased the HSI and VSI for Yellowtail Kingfish, indicating increased visceral and specifically liver weight. The energy available in diets appears to have exceeded the dietary requirements resulting in gross changes in the liver. However, the VPLC% and liver histological observations indicated that irrespective of the 20% CL or 30% CL level or inclusion of the emulsifier in diets, livers displayed similar hepatocyte vacuolisation at a considerably greater level than that observed in wild Yellowtail Kingfish (Crowe et al., 2021 and Chapter 2). In mammals, high energy diets can lead to extensive accumulation of intracytoplasmic lipid droplets with varying ranges of hepatocyte size (Scudamore, 2013). Likewise, fish fed high energy diets can get liver steatosis as a result of lipid intake exceeding the dietary requirements (Spisni et al., 1998; Kowalska et al., 2011). This has the potential to impair liver function resulting in limited or reduced animal growth (Twibell et al., 2017). However, increased liver size with no significant difference in the hepatocyte vacuolisation seen in the current study may be an indication of hyperplasia within livers of fish fed the higher lipid level; a common occurrence in mammalian species when fed

a diet with lipid levels above the species' metabolic requirement (Sumiyishi et al., 2010). This is a likely explanation for the increase in HSI and VSI with dietary energy level. A common challenge in the production of formulated feeds is the balance of nutrient composition which is invariably influenced by the type of raw materials and cooking technology. It is known that the digestibility of dietary lipids is affected by the composition of fatty acids (Turchini et al., 2009). As the nutrient compositions of each diet were similarly formulated with a difference in CL level it can be presumed that it is not the digestibility of the dietary ingredients, rather the increase in energy availability that is creating the hyperplastic effect within the livers of fish fed diets with a 30% CL level.

Newly forming bile ducts occur naturally as a process of animal growth. However, the need to increase hepatic processing of higher dietary energy may also be an explanation for the greater bile duct proliferation observed in fish fed the 30% CL diets. Bile duct proliferation can also result from damaged bile ducts from bile stagnation due to ductular blockages by parasitic mucosporozoa, bacterial infections or mycotoxins and could also be a result of the cytotoxic effect of bile acids on the bile ducts (Moschetta et al., 2005; Rohdes et al., 2016; Watanabe et al., 1998). Apart from a few granulomas most likely associated with the age of the fish, no histological observations resulted in finding evidence of parasites, bacteria or inflammation. Diets with increased vegetable oil have the potential to increase some cytotoxic bile acids (such as monohydroxy bile acid lithocholic acid; Flickert et al., 2006). Phosphatidylcholine is the foremost abundant biliary phospholipid of vertebrates and is suggested to protect cholangiocytes in bile duct epithelium from the toxic effect of bile acids (Tocher, 2008; Moschetta et al., 2005). This is thought to be due to the high efficacy of phosphatidylcholine forming mixed micelles with bile acids, reducing activity of bile acid anions, reducing the overall cytotoxicity of bile (Moschetta et al., 2005). Further indication that the liver was

functioning at an adequate level is the total bilirubin concentrations in the blood which did not differ between the treatments. Elevated bilirubin levels are commonly used in liver function test to diagnose biliary tract disease or liver dysfunction (Wang et al., 2012; Higgins, 2013).

Although fish in this study had increased HSI with increased dietary CL, steatosis or necrosis was not evident in sections taken for fish fed either the 20% CL or 30% CL diets regardless of the addition of emulsifier. The lack of steatosis observed and the hyperplastic effect observed in the fish livers indicate that Yellowtail Kingfish have a level of plasticity to dietary changes making them a highly suitable aquaculture species. It is recommended that the long term impact of high energy diets and potential fatty liver or steatosis in the liver of fish fed these diets should be taken into consideration when formulating feed for sub-adult Yellowtail Kingfish fed over longer periods than the present trial.

Cholesterol is essential for bile acid production, disease resistance, gonadal steroid hormone production and cell membrane growth and function (Lecerf and de Logeril, 2011; Liland et al., 2013; Norambuena et al., 2013). Formulated feeds containing FO and FM are high in available cholesterol which is known to inhibit the synthesis of *de novo* cholesterol in fish (Bowyer et al., 2012b; Liland et al., 2013; Norambuena et al., 2013). Dietary cholesterol and *de novo* cholesterol synthesised in the liver, triggered by a decrease in dietary cholesterol, make up plasma cholesterol concentrations (Maita et al., 2006). Removal of cholesterol from the blood can be a result of increased bile acid synthesis or increased removal of cholesterol from the body (Kornter et al., 2013). The cholesterol concentrations in blood were not significantly different between fish fed any of the experimental diets even though the dietary cholesterol levels were approximately 6 – 8% greater in the diets with 30% CL compared to the diets with 20% CL. This suggests that the dietary cholesterol levels were adequate to maintain the

required cholesterol pools within the fish. The blood cholesterol concentrations were between 4.3 ± 0.3 and 5.0 ± 0.1 mmol L^{-1} (mean ± SE; Table 3.5). If mammalian species have plasma cholesterol levels >5.0 mmol L^{-1} (hypercholesterolemia) and plasma triglyceride levels >1.8 mmol L^{-1} (hypertriglyceridemia) they are considered to have hyperlipidaemia (Higgins 2013). There are reports that replacement of FO with alternative oils can lead to hypercholesterolemia in European sea bass, Atlantic salmon and Yellowtail Kingfish (Kaushik et al., 2004; Jordal et al., 2007; Bowyer et al, 2012b). However, naturally feeding wild Yellowtail Kingfish had blood cholesterol concentrations of 9.03 ± 0.59 mmol L^{-1} and triglyceride levels of 1.92 – 5.47 mmol L^{-1} (mean ± SD; Chapter 2). According to mammalian standards, the fish from the present trial are nearly hyperlipidaemic, yet the concentrations are approximately half of that observed in wild caught Yellowtail Kingfish. Care must be taken when interpreting potential hypo- and hyper- cholesterolemic, triglyceridemic or lipidaemic effects of diets for Yellowtail Kingfish until the normal ranges for this species are better understood.

Liver HMGCR enzyme concentration did not differ between fish fed any of the experiment diets. The synthesis of mevalonate is the irreversible committed step of cholesterol synthesis by HMGCR in the liver which is up regulated if *de novo* cholesterol is required (Maita et al., 2006; Kortner et al., 2013). In Atlantic salmon, increased liver HMGCR gene expression is associated with decreased dietary cholesterol (Morais et al., 2011). The stable HMGCR concentration in liver of fish in the present study further suggests that there was an adequate level of dietary cholesterol in each diet to maintain the fish health and growth performance.

The conversion of cholesterol into bile acids involves a series of enzymatic processes. In mammals, this occurs *in vivo* by the introduction of a hydroxyl group at C7a, C27, C24S and C25, with the 7α-hydroxylation of cholesterol being the main pathway for bile acid synthesis

within the liver (Crosignani et al., 2011). Bile acid metabolism in mammals via the neutral or classical pathway, regulated by cholesterol-7α-hydroxylase (CYP7A1), results in the formation of primary bile acids, cholic acid and chenodeoxycholic acid (Kullak-Ublick et al., 2004; de Aguiar Vallim et al., 2013). Blood total bile acid levels were increased in fish fed the 30% CL diet compared with fish fed the 20% CL diets. Under what is considered in literature to be normal enterohepatic circulation, a healthy liver is well confined and will have efficient reabsorption of bile acids from the portal vein to the liver (Morita et al., 1978; Wang et al., 2012). However, when there is disruption to enterohepatic circulation of bile acids via hepatic dysfunction or portosystemic shunting, total bile acids in blood increase (Morita et al., 1978). An increase in liver bile acid concentrations and CYP7A1 enzyme concentration was also observed in fish fed the 30% CL diets compared with fish fed the 20% CL diets. The hydroxylation of cholesterol by CYP7A1 is considered to be of great regulatory importance of the classical pathway of bile acid synthesis (Lefebvre et al., 2009). In Atlantic salmon when plasma cholesterol levels are high, CYP7A1 activity is increased (Kortner et al., 2013). Blood cholesterol levels were constant across treatments, but fish fed increased lipid levels had elevated blood bile acid concentrations, liver bile acid concentration and CYP7A1 enzyme concentration displaying a response whereby: 1) the increased dietary lipid and subsequent dietary cholesterol level led to a stimulation of CYP7A1 resulting in bile acid synthesis; and 2) the subsequent reabsorption of bile acids and circulation around the body.

Bile acid concentrations are highest during the postprandial phase where the gallbladder muscle layer contracts, emptying the content into the intestinal lumen to assist in the digestion and absorption of dietary lipids (Van Waarde, 1988; Yamamoto et al., 2007; Lefebvre et al., 2009; Nguyen et al, 2011). The muscles of the gallbladder relax again to allow for refilling during a period of fasting (Wang et al., 2012). Enterocytes transport bile acids to the basolateral domain

for efflux and they are then transported back to the liver via portal vein circulation (Kullak-Ublick et al., 2004; Murashita et al., 2014). Approximately 95% of bile acids are recycled through the enterohepatic circulating system, with the remaining bile acids removed from the body via faecal excretion (Zhou and Hylemon, 2014). As feed was withheld from experimental animals for 24 h prior to sampling these levels are an indication of a pre-prandial phase whereby the body is just maintaining the animals' bile acid pools (York, 2017). This is an indication that the blood bile acid level, liver bile acid concentration and CYP7A1 enzyme concentration observed in the animals are normal and increases are relative to the amount of lipid in the diet that the animals are metabolising.

It is well known that substituting FO and FM with terrestrial-sourced lipids and proteins in the diets of marine carnivorous fish may only be employed to species-specific thresholds before digestion of nutrients are affected (Gatlin et al., 2007; Hardy, 2010; Bowyer et al., 2012a). This includes the reduced efficiency of enterohepatic circulation of bile acid (Nguyen et al., 2011). For example, soybean meal, commonly used as FM alternative, increases undigested molecular fractions that bind to bile acid which reduces peptide production in the intestine, increasing faecal steroid excretion and inhibiting reabsorption of bile acid, further reducing the ability of nutrient uptake (Sugano et al., 1988; Nguyen et al., 2011). When functioning in normal capacity, bile acids emulsify dietary lipids by breaking large lipid globules into smaller droplets by increasing the surface area and therefore digestive efficiency of fatty acids by pancreatic lipase (Wang et al., 2012). Significantly greater blood triglyceride concentrations in fish fed diets containing 30% CL than fish fed diets containing 20% CL of the present trial indicate that this process was not impeded. A need for addition of a commercial emulsifiers in formulated feeds for the emulsification and digestion of long chain fatty acids, such as triglycerides, may be required if bile acid production is reduced from inclusion of alternative lipid sources beyond

those considered in this study. Increased dietary cholesterol stimulates CYP7A1 and the synthesis of bile acids which is a route for elimination of cholesterol via faeces (Lefebvre et al., 2009). Although, the faeces of the fish were not sampled for bile acid or cholesterol concentrations, the adequate animal growth and health as well as elevated levels of bile acid and stable levels of cholesterol in the blood seen in the current study indicate that reabsorption was not impeded (Stone and Booth, 2018). However, of interest is the reduced blood bile acids levels and CYP7A1 concentration observed in fish fed diets with added emulsifier within each lipid level group.

Emulsifiers aid in the digestion and emulsification of dietary lipids and appear to have decreased metabolic demand for bile acids as evident by lowered plasma bile acid levels and decreased liver bile acid and CYP7A1 concentrations. Membrane-derived signalling molecules such as lysophospholipids are emulsifying and solubilising agents, produced by phospholipases enzymes modifying the structure of phospholipid molecules (Ahmad et al., 2015; Guiotto et al., 2015). Phospholipids are well known to facilitate digestion and absorption of lipids and act as carriers for long-chain polyunsaturated fatty acids (Trushenski et al., 2013). Sunflower, soybean, canola and other oil seed lecithin have been used as dietary emulsifiers in food production, pharmaceuticals and dietetics (Guiotto et al., 2015). In rainbow trout (235 – 375 g), a dietary additive of soy lecithin increased the digestibility of energy (Poston, 1991). Increased specific growth rate was observed in Atlantic salmon fry (1.5 g) fed soy lecithin at a dietary inclusion level of 30 g kg^{-1} compared to fry fed a diet void of the soy lecithin additive (Hung et al., 1997). Furthermore, increased growth was observed in juvenile cobia, *Rachycentron canadum*, (65 ± 1 g) when fed a 12% FM diet with the addition of 1% marine based phospholipid (marine lecithin) when compared with a control diet of 50% FM with no added phospholipid (Trushenski et al., 2013).

3.6 Conclusion

At the water temperatures (15.8 ± 1.4 °C) recorded in this study, the addition of emulsifier at 40 mg lipid kg^{-1} in diets with 30% CL and 20% CL had no significant impact on cholesterol and bile acid metabolism and the liver structure in sub-adult Yellowtail Kingfish. Greater dietary CL% led to increased HSI and VSI as well as increased bile duct proliferation. Likewise, diets with greater CL led to numerically increased liver CYP7A1 concentrations and blood total bile acid and triglyceride levels. The fish have plasticity in this respect, enabling them to grow and maintain enterohepatic function. However, fish fed diets with 30% CL presented livers that did not have as homogenous hepatocyte distribution or as consistent positioned cuboidal cholangiocytes around bile ducts as fish fed 20% CL. The liver is the most important organ for cholesterol metabolism and the synthesis of bile that enables emulsification and uptake of nutrients. Therefore, should an adverse environmental or biological event occur challenging the animals' health further, the impacts this might also have on the liver or whole animal growth and health is unknown. The addition of this commercial emulsifier at 40 mg lipid kg^{-1} in these diets presents no benefit for the production of Yellowtail Kingfish. Emulsifying agents at different concentrations or of different sources may have desired effects beyond those examined in this experiment and their uses should be investigated.

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Chapter 4: Investigating liver structure and function in Yellowtail Kingfish, *Seriola lalandi,* in response to alternative oils

4.1 Abstract

The inclusion of low cholesterol oil alternatives to fish oil (FO) could result in alterations to the biosynthesis and availability of bile acids for the absorption of dietary lipids. In this study the effects that FO substituted with poultry oil (PO) or canola oil (CO) in formulated feeds on Yellowtail Kingfish liver histological structures, hepatic cholesterol and bile acid synthesis, enzyme concentration and the recycling of bile acids were investigated. Dietary FO was replaced with PO at 100%, 75% and 50% inclusion levels (Diets 1, 2 and 3, respectively). The lipid source (PO) was further replaced with CO at 0%, 33.3%, 66.7% and 100% (Diets 2, 4, 5 and 6, respectively). Neither the visceral somatic index (VSI) nor hepatosomatic index (HSI) were statistically different between fish fed diets with graded FO replacement by PO (Diets 1, 2 and 3) nor statistically different between the fish fed diets formulated with FO and a graded PO and CO blend (Diets 2, 4, 5 and 6). The liver vacuole volume as a proportion of the liver cell volume (VPLC%) was significantly increased ($P < 0.05$) in fish fed the diet with 100% PO (Diet 1) compared to that of fish fed diets with greater levels of FO inclusion (Diets 2 and 3). Regression analyses showed a statistically significant ($r^2 = 0.588$; $P < 0.05$) positive relationship between the decrease in FO and an increase in liver VPLC%. Regression analyses showed a significant negative relationship with diets of decreasing PO and increasing CO and the total bile acid concentrations in liver tissue ($r^2 = 0.402$; $P < 0.05$). Regression analyses showed a statistically significant ($r^2 = 0.506$; $P < 0.05$) positive relationship between liver CYP7A1 concentration and increasing PO and decreasing FO. No statistically significant differences were observed between any of the blood biochemistry parameters measured from fish fed diets with decreasing FO and increasing PO nor from fish fed diets formulated with FO and a graded PO and CO blend. Yellowtail Kingfish may be able to be fed a formulated diet containing either: 1) ~25% dietary lipid level (2.12 g $100g^{-1}$ of ΣLC n-3 PUFA) with up to 100% of the FO component replaced by PO; or 2) ~25% dietary lipid level (2.12 g $100g^{-1}$ of

ΣLC n-3 PUFA) with up 100% of the commercially used alternative lipid source (PO) with CO without an apparent negative impact on any of the parameters measured in this study.

4.2 Introduction

In the early 1900s fish oil (FO) content in Atlantic salmon (*Salmo salar*) feed was as high as 25% while more recent FO level of some feeds were as low as 7% (Tacon and Metian, 2008). Reduction in the use of FO is driven by several factors including: 1) increased commodity costs due to demand for the resource in formulated feeds; 2) terrestrial animal feeds and inclusion in human food, with an estimated 460% of the current production levels required by 2030; 3) the aquaculture industry moving towards more sustainable and environmentally responsible practices (Kim et al., 2012; Asche et al., 2013; Rojas and Stein, 2013).

Fish oils have large concentrations of eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA). Eicosapentaenoic acid and DHA are long-chain polyunsaturated fatty acids (LC-PUFA) essential for cultured carnivorous finfish to sustain optimal growth and health (Geay et al., 2011). Furthermore, LC-PUFA are sources of metabolic energy for reproduction, immune defence and precursors for eicosanoids, the hormone-like molecules that assist in lipid and cholesterol metabolism (Tocher, 2003; Tocher et al., 2008; Trushenski and Lochmann, 2009; Martins et al., 2012). In diets with 100% FO substitution, trace amounts of FO may still be present from fish meal used to supply the protein component of the diet. Formulated diets that substitute FO with an alternative oil source reduce the amount of LC-PUFA, particularly EPA and DHA as they are not found in terrestrial plant oils or terrestrial animal fats (Bowyer et al., 2012b).

The inclusion of alternatives to FO, particularly vegetable oils, could result in low cholesterol (Norambuena et al., 2013; Chapter 2). Cholesterol is an essential precursor for bile acids synthesised in liver hepatocytes as well as for disease resistance and is vital to maintain healthy cell membrane growth and function (Mohapatra and Mishra, 2011; Nguyen et al, 2011; Holm

et al., 2013; Liland et al., 2013). Although teleost fish are capable of producing cholesterol *de novo*, the inclusion of low cholesterol alternatives to FO puts demands on *de novo* cholesterol biosynthesis (Norambuena et al., 2013). Cholesterol synthesis and its subsequent conversion into bile acids involve a series of enzymatic processes regulated by 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR). Bile acid synthesis from cholesterol catabolism in mammals is regulated by cholesterol-7α-hydroxylase (CYP7A1) and results in the formation of primary bile acids, cholic acid and chenodeoxycholic acid (Kullak-Ublick et al., 2004; de Aguiar Vallim et al., 2013). After feeding, bile acids are secreted into the small intestine to assist in the micellar solubilisation of dietary lipids, esterification, emulsification and digestion of long chain fatty acids (Van Waarde, 1988; Yamamoto et al., 2007; Nguyen et al, 2011; El-Sayed, 2014; Murashita et al., 2014; Zhou and Hylemon, 2014). Enterocytes transport bile acids to the basolateral domain for efflux and transported back to the liver via the portal vein circulation (Kullak-Ublick et al., 2004; Murashita et al., 2014). Approximately 95% of bile acids are recycled through the enterohepatic circulating system, with the remaining bile acids removed from the body via faecal excretion (Zhou and Hylemon, 2014).

Canola oil (CO), soybean oil, poultry oil (PO) and swine, bovine or ovine fats have all been trialled as FO substitutes in formulated feeds at varied levels of substitution (Drew et al., 2007; Shafaeipour et al., 2008; Stone et al., 2011a and 2011b; Bowyer et al., 2012a and 2012b). The maximum level of FO that can be substituted in formulated feeds is species-dependent. Incorrect inclusion levels can have a negative impact on animal health through anti-nutritional factors including reduced palatability and therefore feed intake resulting in decreased growth performance (Francis et al., 2001; Chen et al., 2011; Lim et al., 2011; Collins et al., 2012). Reduced lipid metabolism and growth have been observed in red seabream, *Pagrus auratus*,

and Yellowtail Kingfish, *Seriola lalandi*, respectively when fed diets with FO substituted by high levels of CO (Glencross et al., 2004; Bowyer et al., 2012b).

In this study the effects that FO substitution in formulated feeds with PO or CO would have on Yellowtail Kingfish liver histological structures, hepatic cholesterol and bile acid synthesis, enzyme concentration and the recycling of bile acids were investigated. This was achieved by investigating the histological changes and nutrient deposition in liver tissue, rate limiting enzymes for hepatic cholesterol synthesis and bile acid synthesis and analyses of blood profiles.

4.3 Methods

4.3.1 Experimental description

In this study, commercial diets with graded FO levels and diets with FO substituted by graded PO and CO blends (Table 4.1A) were used to investigate the effect on liver structure, liver enzyme concentration, blood biochemistry and health of Yellowtail Kingfish. The biochemical composition of the six diets is displayed in Table 4.1B. The experiment was conducted over 84 days at the South Australian Research and Development Institute (SARDI) South Australian Aquatic Sciences Centre (SAASC) at West Beach, Adelaide, Australia. At the commencement of the experiment, individual fish were weighed (kg) and measured for fork length (mm) after the fish had been anaesthetised in seawater using AQUI-S® (AQUI-S® New Zealand Ltd.) at a concentration of 0.02 mL L^{-1}. A total of 342 fish (body mass 1.45 ± 0.12 kg; fork length, 459 ± 14 mm; mean ± SD) were randomly distributed between eighteen 5,000 L fibreglass tanks with 19 fish per tank. Each of the six treatments was randomly assigned to three replicate tanks. Fish were fed to satiation once daily 09:00 h (Stone and Booth, 2018).

4.3.2 Experimental system

The recirculating aquaculture system (RAS) was kept undercover in ambient conditions with supplemental fluorescent lighting provided during the natural light period. Ambient temperature sea water was treated by settlement and sand filtration and circulated through the RAS. The sea water was then returned through filter-screen baffle-boards, pumped through a drum filter with 70 μm^2 filter pores (HDF1603 Hydrotech, Saint-Maurice, France), returned to the biofilter, and finally treated with UV light disinfection (D-32051 Wedeco, Herford, Germany) at a rate of 35,000 L h^{-1} by two electric centrifugal pumps (Grundfos, Regency Park, Australia) before being returned to the tanks. One hundred percent of the water volume of each 5,000 L tank was replaced daily.

4.3.3 Fish source and acclimation

Sub-adult Yellowtail Kingfish were obtained from Clean Seas Pty. Ltd. sea cage facilities at Port Lincoln, Australia. The fish were acclimated for 4 weeks in 5,000 L fibreglass tanks supplied by the RAS at SARDI SAASC and fed a commercial diet (Ridley Corporation Ltd. Pelagica diet; gross energy, 19.3 MJ kg^{-1}, crude lipid [CL], 24%, crude protein [CP], 46%) before the commencement of the experiment.

4.3.4 Experimental diets

In this study, six treatments referred to as Diet 1, Diet 2, Diet 3, Diet 4, Diet 5 and Diet 6 were formulated to contain ~25% dietary lipid level, and contain 2.12 g $100g^{-1}$ of ΣLC n-3 PUFAs (Table 4.1A and 4.1B). Diets 1, 2 and 3 were formulated to replace dietary FO with the commercially used alternative lipid source PO at 100%, 75% and 50% inclusion levels, respectively (Table 4.1A). Diets 2, 4, 5 and 6 were formulated with FO in combination with replacement of the commercially used alternative lipid source (PO) with CO at 0%, 33.3%, 66.7% and 100%, respectively (Table 4.1A). In this study, the pellet kernel (9 mm), fish oil,

poultry oil and canola oil were supplied by Ridley Corporation Ltd. (Narangba, Queensland, Australia). Diets were formulated based on a Yellowtail Kingfish commercial diet (30% fish meal; ~48% crude protein [CP], ~25% crude lipid [CL] and a gross energy [GE] level of ~19.80 MJ kg^{-1}). The pellet kernel utilised in the current study contained ~10% CL, which was top coated at normal atmospheric pressure with an additional 17.3% lipid (fish oil, poultry oil and/or canola oil; total crude lipid level 25%) at Aquafeeds Australia (Mount Barker, South Australia, Australia).

The six experimental diet ingredient formulations were commercially confidential and are not provided; the corresponding proximate compositions of all diets are shown in Table 4.1B. The experimental diets were analysed for biochemical composition according to methods in the British Pharmacopoeia Commission (2004) or German Institute for Standardization (DIN) (2000) by SARDI. A one kg sample of each diet was collected, ground and analysed for proximate composition (moisture, protein, fat, ash, total carbohydrate and energy) and fatty acids profile.

Table 4.1A. Experimental diets with graded fish oil levels (Diets 1, 2 and 3) and diets formulated with fish oil and a graded poultry oil and canola oil blend (Diets 2, 4, 5 and 6) fed to Yellowtail Kingfish, *Seriola lalandi*, for 84 days.

Oil Diet	Fish oil (% added)	Poultry oil (% added)	Canola oil (% added)	Target ΣLC n-3 PUFA (g $100g^{-1}$ diet)	Analysed ΣLC n-3 PUFA (g $100g^{-1}$ diet)
1	0.00	100.00	0.00	1.45	1.42
2	26.71	73.52	0.00	2.12	2.34
3	50.35	50.06	0.00	2.88	3.33
4	27.81	48.26	24.13	2.12	2.39
5	28.68	23.84	47.68	2.12	2.43
6	29.37	0.00	70.86	2.12	2.46

Table 4.1B. The proximate and fatty acid composition of the six experimental diets fed to Yellowtail Kingfish, *Seriola lalandi*.[1]

Item (as fed)	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Proximate composition (g 100 g^{-1})						
Moisture	4.8	4.9	4.8	4.6	4.6	5.0
Crude protein	47.8	47.9	48.4	48.1	48.1	48.1
Crude lipid	24.9	24.6	25.3	24.6	25.1	25.1
Ash	8.2	8.1	8.2	8.1	8.2	8.3
Carbohydrate[2]	14.3	14.5	13.3	14.6	14.0	13.5
Gross energy (MJ kg^{-1})	19.8	19.7	19.9	19.8	19.8	19.8

[1] Diet 1 - 0% fish oil + 100% poultry oil [added oil]; 1.42 g 100 g^{-1} LC n-3 PUFA, Diet 2 - 26.71% fish oil + 73.52% poultry oil [added oil]; 2.34 g 100 g^{-1} LC n-3 PUFA, Diet 3 - 50.35% fish oil + 50.06% poultry oil [added oil]; 3.33 g 100 g^{-1} LC n-3 PUFA, Diet 4 - 27.81% fish oil + 48.26% poultry oil + 24.13% canola oil [added oil], Diet 5 - 28.68% fish oil + 23.84% poultry oil + 47.68% canola oil [added oil], Diet 6 - 29.37% fish oil + 0% poultry oil + 70.86% canola oil [added oil].

[2] Carbohydrate = 100 - (moisture + lipid + protein + ash).

4.3.5 Water quality

Water quality parameters were monitored daily, unless otherwise indicated. Temperature (°C, mean ± SD) and dissolved oxygen (% saturation, mean ± SD) were monitored using an OxyGuard Handy Polaris temperature and dissolved oxygen probe (OxyGuard International A/S, Farum, Denmark). The pH (mean ± SD) was monitored using a Eutech pH Testr 30 multiparameter handheld probe (Eutech Instruments Pty. Ltd., Singapore). Salinity (g L^{-1}, mean ± SD) was monitored using an ISSCO UR-2 hand-held refractometer (model RF20; Extech Instruments Corporation, Nashua, New Hampshire, United States of America). The total ammonia concentration (mg L^{-1}) was determined weekly using a commercial water testing kit (Aquarium Pharmaceuticals, Chalfont, Pennsylvania, United States of America).

4.3.6 Sample collection

Feeding was stopped 24 h before the end of the experiment. Three fish from each tank were euthanized and measured for their body mass (nearest 0.1 g) and fork length (nearest 0.1 mm). The growth performance and feed utilisation for this trial was reported by Stone and Booth (2018). All data reported for each treatment for animal performance were based on the mean of the replicate tanks. All calculations using fish weight and diets are based on wet or as fed values, respectively:

Weight gain = final weight – initial weight

Specific growth rate (SGR; % d^{-1}) = ([ln final weight – ln initial weight] /d) x100

Condition factor (CF) = (fish body mass [g] / fish length $[cm]^3$) × 100

Apparent feed conversion ratio (FCR) = feed consumed / fish weight gain

Blood samples were collected from the caudal vasculature using 21 gauge needles and 5 mL syringes. For each fish, the visceral cavity was cut open and the whole removed gut weighed (nearest 0.01 g) to determine the visceral somatic index (VSI; %). The liver was removed and weighed (nearest 0.01 g) to determine the hepatosomatic index (HSI; %). These indices were calculated as follows:

VSI% = (visceral weight [g] / body mass [g]) × 100

HSI% = (liver weight [g] / body mass [g]) × 100

Samples of the liver, foregut, midgut, hindgut, gallbladder content and faeces were collected, immediately snap frozen in liquid nitrogen and stored at – 80 °C until analysis. Two 1 cm^3 sections of the left lobe of the liver were dissected and immediately placed into a histology cassette and fixed with 10% neutral buffered formalin (pH of 7.2) for 24 h for histological evaluation. One of the liver sections from each fish was used for standard paraffin embedding

and then sectioned by rotary microtome, while the other was preserved in an 18% phosphate buffered saline (PBS) sucrose + 0.01% sodium azide (NaN_3) solution with pH 7.1 for cryostat sectioning. The liver samples were also used to measure the concentration of HMGCR and CYPA71 enzymes.

4.3.7 Liver histology

Once fixed, one liver section from each fish was transferred to 70% (v/v) ethanol and stored at room temperature according to Hu *et al.* (2013). The sample was dehydrated using standard procedures, embedded in paraffin and sectioned at 5 μm on a rotary microtome. This section of liver from each fish underwent several staining techniques: haematoxylin and eosin (H&E) for quantitative assessments of the vacuole volume as a proportion of the liver cell volume (VPLC%) and tissue structure as per Crowe et al. (2021) and periodic acid Schiff (PAS) for glycogen deposition. The second liver section samples were sectioned at 10 μm on a cryostat microtome and mounted on polyethylenimine coated slides. This section of liver from each fish underwent two additional staining techniques: Oil Red O (ORO) for unsaturated neutral lipid deposition; and Sudan Black B (SBB) for total lipid deposition. Stained sections were examined at 400-fold magnification using a light microscope (Olympus BF BX50). Three 559,390 μm^2 microphotographs were taken per stained slide using a digital camera (Olympus DP27). Quantitative assessments of the stained deposition volume as a proportion of the liver cell volume was determined by applying stain sensitive colour thresholds using Fiji ImageJ processing software (National Institutes of Health, Bethesda, Maryland, United States of America).

4.3.8 Biochemical analyses

Samples from individual fish were partially thawed, weighed and homogenised in five volumes of PBS with a pH of 7.1. Homogenisation was performed using a Retch MM 400 ball mill at

frequency 30.01 s^{-1} for 4 min with Biospec 2 mm zirconia beads (Catalogue No.11079124zx). The suspensions were centrifuged twice at an acceleration of 10,000 *g* for 10 min at 4 °C (Beckman Coulter Microfuge 16 centrifuge) with the supernatants being extracted to new microfuge tubes between cycles. All assay kits included internal standard solutions and manufacturer instructions were followed for assays. Tissue concentrations are reported per tissue weight (μmoles g^{-1}).

4.3.8.1 Total bile acid concentration

The liver, gallbladder content, foregut, midgut, hindgut and faeces pooled supernatants were analysed for total bile acid concentrations by use of a commercial fluorometric bile acid assay kit by reading Ex/Em = 560/590 nm after 45 min of dark incubation at room temperature (Catalogue No. MET-5005; Cell Biolabs Inc.®, San Diego, California, United States of America).

4.3.8.2 Taurine concentration

The pooled liver supernatants were analysed for taurine by use of a commercial colorimetric taurine assay kit by reading samples at 405 – 415 nm (Catalogue No. MET-5070; Cell Biolabs Inc.®, San Diego, California, United States of America).

4.3.8.3 HMGCR and CYP7A1 enzyme-linked immunosorbent assays

The pooled liver supernatants were analysed for HMGCR concentration after reading the absorbance of samples at a wavelength of 450 nm (Catalogue No. CSB-E15772m; CusaBio, Texas, United States of America). The pooled supernatants were analysed for CYP7A1 concentration after reading the absorbance of samples at a wavelength of 450 nm (Catalogue No. CSB-EL006395FI; CusaBio, Texas, United States of America).

4.3.9 Blood analyses

Two mL of blood was placed into separate Vacuette® or BD vacutainer® tubes (Z serum clot activator or EDTA tubes) and stored indirectly on ice until same-day analysis of blood parameters by IDEXX Laboratories (Unley, South Australia, Australia). Specifically, the following parameters were observed: total bile acids, bile salts, cholesterol, triglycerides and total bilirubin.

4.3.10 Statistical analyses

A one-way analysis of variance (ANOVA) and regression analysis were used to assess the effects of the different diets. Two planned comparisons of diets formulated with graded FO levels (100%, 75% and 50% inclusion levels) and diets formulated with FO and a graded PO and CO blend (replacement of the commercially used alternative lipid source [PO] with CO at 0%, 33.3%, 66.7% and 100%) were performed. The normality of data was assessed using the Shapiro–Wilk test. Homogeneity of variances among means was assessed using Levene's test for equality of variance errors. The significance level was set at $\alpha = 0.05$ for all statistical tests. All data are presented as the mean ± the standard error (SE) of the mean, unless otherwise stated. IBM SPSS Version 20 for Windows (IBM SPSS Inc., Chicago, Illinois, United States of America) software was used for all statistical analyses.

4.4 Results

4.4.1 Water quality

Average values for water temperature, dissolved oxygen, pH and salinity were 16.2 ± 2.1 ℃, 104.1 ± 4.6%, 7.8 ± 0.2 and 38 ± 0 g L^{-1}, respectively, across the study period. The total ammonia concentration was found to be below the limits of detection on all occasions.

4.4.2 Performance indices

The growth performance and feed utilisation for this trial was reported by Stone and Booth (2018). In short, final weight ($P > 0.05$), SGR ($P > 0.05$), CF ($P > 0.05$), apparent feed intake (% BW d^{-1}; $P > 0.05$) and FCR ($P > 0.05$) of Yellowtail Kingfish were not significantly influenced by diet.

4.4.3 Somatic indices

4.4.3.1 Graded fish oil

Neither the VSI ($P = 0.904$) nor HSI ($P = 0.656$) were statistically different between the fish fed diets formulated to replace FO with PO (Diets 1, 2 and 3; Table 4.2A).

Table 4.2A. Somatic parameters of Yellowtail Kingfish, *Seriola lalandi,* fed experimental diets with graded fish oil levels diets for 84 days.[1]

Diet	1	2	3	ANOVA[2]
Visceral index (VSI; %)	6.8 ± 0.4	6.7 ± 0.3	6.6 ± 0.4	0.904
Hepatosomatic index (HSI; %)	1.1 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	0.656

[1] Values are mean ± SE; $n = 3$

[2] A significance level of $\alpha = 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.

4.4.3.2 Graded poultry and canola oil blends

Neither the VSI ($P = 0.938$) nor HSI ($P = 0.954$) were statistically different between the fish fed diets formulated with FO and a graded PO and CO blend (Diets 2, 4, 5 and 6; Table 4.2B).

Table 4.2B. Somatic parameters of Yellowtail Kingfish, *Seriola lalandi*, fed experimental diets formulated with fish oil and a graded poultry oil and canola oil blend for 84 days.[1]

Diet	2	4	5	6	ANOVA[2]
Visceral index (VSI; %)	6.7 ± 0.3	6.7 ± 0.3	6.7 ± 0.3	6.5 ± 0.3	0.938
Hepatosomatic index (HSI; %)	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.0	0.954

[1] Values are mean ± SE; $n = 3$

[2] A significance level of $\alpha = 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.

4.4.4 Liver histology

4.4.4.1 Graded fish oil

The liver VPLC% was significantly increased ($P = 0.008$) in fish fed the diet with 100% PO (Diet 1) compared to that of fish fed diets with reduced PO and increased levels of FO (Diets 2 and 3). Regression analyses showed a statistically significant positive relationship between the decrease in FO and an increase in liver VPLC% ($r^2 = 0.588$; $P = 0.016$; Table 4.3A). The increasing dietary FO had no significant effect on the proportion of stain in livers for neutral lipids ($P = 0.306$), total lipids ($P = 0.553$) or glycogen ($P = 0.253$; Table 4.3A). Similarly, regression analyses revealed no significant relationship between graded FO and proportion of stain in livers for neutral lipids ($r^2 = 0.223$; $P = 0.199$), total lipids ($r^2 = 0.081$; $P = 0.458$) or glycogen ($r^2 < 0.001$; $P = 0.988$; Table 4.3A).

Table 4.3A. Vacuole area and proportion of histology stain on liver sections of Yellowtail Kingfish, *Seriola lalandi*, fed experimental diets with graded fish oil levels diets for 84 days.[1]

					Regression[#]	
Diet	1	2	3	ANOVA (*P value*)[*]	r^2	*P* value[2]
VPLC%[†]	41.8±1.2	29.7±0.8	29.9±3.1	**0.008**	0.588	**0.016**
Neutral lipids	4.6±0.4	4.3±1.2	7.6±2.3	0.306	0.223	0.199
Total lipids	90.1±0.1	91.1±1.1	87.9±3.3	0.553	0.081	0.458
Glycogen	0.4±0.2	7.1±6.6	0.5±0.4	0.253	>0.000	0.988

[1] Values are mean ± SE; *n* = 3.

[*] A significance level of α = 0.05 was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.

[†] Vacuole volume as a proportion of the liver cell volume (Crowe et al. 2021). Regression results were significantly affected by crude protein inclusion (Diet 1^b, Diet 2^a, Diet 3^a).

[#] Regression values generated from treatment groups; *n* = 9.

4.4.4.2 Graded poultry and canola oil blends

The proportion of stain observed in livers of fish fed diets formulated with FO and a graded PO and CO blend were not significantly different between treatments for VPLC% (*P* =0.713), neutral lipids (*P* = 0.272), total lipids (*P* = 0.524) or glycogen (*P* = 0.290; Table 4.3B). Similarly, regression analyses did not reveal any significant relationship between treatments formulated with FO and a graded PO and CO blend and the proportion of stain in livers for VPLC% (r^2 = 0.001; *P* = 0.933), neutral lipids (r^2 = 0.048; *P* = 0.495), total lipids (r^2 = 0.120; *P* = 0.269) or glycogen (r^2 = 0.201; *P* = 0.144; Table 4.3B).

Table 4.3B. Vacuole area and proportion of histology stain on liver sections of Yellowtail Kingfish, *Seriola lalandi*, fed experimental diets formulated with fish oil and a graded poultry oil and canola oil blend for 84 days.[1]

Diet	2	4	5	6	ANOVA (*P value*)[*]	Regression[#]	
						r^2	*P* value[2]
VPLC%[†]	29.7±0.8	34.8±4.9	34.8±1.9	30.2±6.2	0.713	0.001	0.933
Neutral lipids	4.3±1.2	6.8±1.6	3.3±0.7	4.1±1.1	0.272	0.048	0.495
Total lipids	91.1±1.1	92.0±1.2	86.9±3.5	88.4±3.5	0.524	0.120	0.269
Glycogen	7.1±2.1	11.3±3.4	0.1±0.2	0.1±0.4	0.290	0.201	0.144

[1] Values are mean ± SE; $n = 3$.

[*] A significance level of $\alpha = 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.

[†] Vacuole volume as a proportion of the liver cell volume (Crowe et al. 2021).

[#] Regression values generated from treatment groups; $n = 9$.

4.4.5 Biochemical analyses

4.4.5.1 Total bile acid concentration

4.4.5.1.1 Graded fish oil

Decreasing dietary FO had no significant effect on the total bile acid concentration in tissue samples from the liver ($P = 0.301$), gallbladder content ($P = 0.592$), foregut ($P = 0.543$), midgut ($P = 0.552$), hindgut ($P = 0.807$) or faeces ($P = 0.586$; Table 4.4A). Similarly, regression analyses showed no significant relationship between decreasing dietary FO and total bile acid concentration in tissue samples from the liver ($r^2 = 0.121$; $P = 0.360$), gallbladder content ($r^2 = 0.089$; $P = 0.437$), foregut ($r^2 = 0.151$; $P = 0.301$), midgut ($r^2 = 0.075$; $P = 0.475$), hindgut ($r^2 = 0.036$; $P = 0.627$) or faeces ($r^2 = 0.006$; $P = 0.838$; Table 4.4A).

Table 4.4A. Total bile acid and taurine concentrations from sampling points within the enterohepatic bile acid cycling and excretory system of Yellowtail Kingfish, *Seriola lalandi*, fed experimental diets with graded fish oil levels diets for 84 days.[1]

					Regression[†]	
Diet	1	2	3	ANOVA (*P value*)[*]	r^2	*P* value[2]
Total bile acid concentration (µmoles g^{-1})						
Liver	12.1±0.3	12.4±0.1	11.5±0.5	0.301	0.121	0.360
Gallbladder content	12.0±0.9	12.2±0.9	11.0±0.8	0.592	0.089	0.437
Foregut	9.8±1.0	10.1±1.8	12.7±2.7	0.543	0.151	0.301
Midgut	11.1±0.4	10.7±0.3	11.8±1.1	0.552	0.075	0.475
Hindgut	10.8±0.6	10.5±0.6	11.6±2.0	0.807	0.036	0.627
Faeces	28.4±0.4	20.8±7.0	26.8±5.7	0.586	0.006	0.838
Taurine concentration (µmoles g^{-1})						
Liver	0.08 ± 0.01	0.08 ± 0.00	0.08 ± 0.01	0.757	n.d.	n.d.

[1] Values are mean ± SE; $n = 3$.

[*] A significance level of $\alpha = 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.

[†] Regression values generated from treatment groups; $n = 9$.

4.4.5.1.2 Graded poultry and canola oil blends

Total bile acid concentrations were not significantly affected by diets formulated with FO and a graded PO and CO blend in liver (P = 0.137), gallbladder content (P = 0.914), foregut (P = 0.753), midgut (P = 0.472), hindgut (P = 0.166) or faeces (P = 0.657; Table 4.4B). Regression analyses showed a significant negative relationship with diets of decreasing PO and increasing CO and the total bile acid concentrations in liver tissue (r^2 = 0.402; P = 0.027; Table 4.4B). Regression analyses showed no significant relationship between total bile acid concentrations and treatments formulated with FO and a graded PO and CO blend in tissue samples of gallbladder content (r^2 = 0.002; P = 0.885), foregut (r^2 = 0.058; P = 0.452), midgut (r^2 < 0.001; P = 0.992), hindgut (r^2 = 0.050; P = 0.832) or faeces (r^2 = 0.027; P = 0.608; Table 4.4B).

Table 4.4B. Total bile acid and taurine concentrations from sampling points within the enterohepatic bile acid cycling and excretory system of Yellowtail Kingfish, *Seriola lalandi*, fed experimental diets formulated with fish oil and a graded poultry oil and canola oil blend for 84 days.[1]

						Regression†	
Diet	2	4	5	6	ANOVA (*P value*)[2]	r^2	*P* value[2]
Total bile acid concentration (µmoles g^{-1})							
Liver	12.4±0.1	11.7±0.4	11.8±0.4	10.7±0.7	0.137	0.402	**0.027**
Gallbladder content	12.24±0.9	12.66±0.6	12.18±0.1	12.2±0.4	0.914	0.002	0.885
Foregut	10.1±1.8	12.8±2.3	13.2±2.5	11.8±2.1	0.753	0.058	0.452
Midgut	10.7±0.3	10.1±0.3	10.2±0.4	10.8±0.4	0.472	0.000	0.992
Hindgut	10.5±0.6	9.3±0.7	9.3±0.6	11.1±0.5	0.166	0.050	0.832
Faeces	20.8±7.0	23.0±2.4	18.2±3.0	26.2±4.3	0.657	0.027	0.608
Taurine (µmoles g^{-1})							
Liver	0.08 ± 0.00	0.07 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.324	n.d.	n.d.

[1] Values are mean ± SE; $n = 3$.

[2] A significance level of $\alpha = 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.

† Regression values generated from treatment groups; $n = 9$.

4.4.5.2 Taurine concentration

4.4.5.2.1 Graded fish oil

No statistically significant differences were observed between the liver taurine concentration of fish fed diets with graded FO (P = 0.757; Table 4.4A).

4.4.5.2.2 Graded poultry and canola oil blends

No statistically significant difference was observed between the liver taurine concentration of fish fed diets formulated with FO and a graded PO and CO blend (P = 0.324; Table 4.4B).

4.4.5.3 HMGCR and CYP7A1 enzymes

4.4.5.3.1 Graded fish oil

No statistically significant differences were observed for either HMGCR concentration (P = 0.882) or CYP7A1 concentration (P = 0.102) in liver tissue of fish fed diets with graded FO (Table 4.5A). Regression analyses showed no significant relationship between graded FO and HMGCR concentration in liver tissue (r^2 =0.041; P = 0.604; Table 4.5A). Regression analyses showed a statistically significant relationship between diets with graded PO and CYP7A1 concentration in liver tissue whereby CYP7A1 concentration increased with increasing PO (r^2 =0.506; P = 0.032; Table 4.5A). Although not significantly different, a numerical decrease in HMGCR concentration was observed with increasing PO.

Table 4.5A. HMGCR and CYP7A1 concentrations of liver tissue from Yellowtail Kingfish, *Seriola lalandi*, fed experimental diets with graded fish oil levels diets for 84 days.[1]

					Regression†	
Diet	1	2	3	ANOVA (*P value*)[2]	r^2	*P* value[2]
HMGCR (µmoles g^{-1})	11.50±3.15	14.21±3.47	16.41±10.92	0.882	0.041	0.601
CYP7A1 (µmoles g^{-1})	3221.09±370.59	2816.32±348.19	1864.51±407.00	0.102	0.506	**0.032**

[1] Values are mean ± SE; $n = 3$.

[2] A significance level of $\alpha = 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.

† Regression values generated from treatment groups; $n = 9$.

4.4.5.3.2 Graded poultry and canola oil blends

No statistically significant difference was observed for either HMGCR concentration ($P = 0.849$) or CYP7A1 concentration ($P = 0.433$) on liver tissue of fish fed diets formulated with FO and a graded PO and CO blend (Table 4.5B). Regression analyses showed no significant relationship between diets formulated with FO and a graded PO and CO blend and HMGCR concentration ($r^2 = 0.013$; $P = 0.727$) or CYP7A1 concentration ($r^2 = 0.433$; $P = 0.205$) in liver tissue (Table 4.5B). A numerical reduction in CYP7A1 concentration was observed with increasing CO (Table 4.5B).

Table 4.5B. HMGCR and CYP7A1 concentrations of liver tissue from Yellowtail Kingfish, *Seriola lalandi*, fed experimental diets formulated with fish oil and a graded poultry oil and canola oil blend for 84 days.[1]

						Regression†	
Diet	2	4	5	6	ANOVA (*P value*)[2]	r^2	*P* value[2]
HMGCR (µmoles g^{-1})	14.21±3.47	20.25±11.80	10.93±3.20	22.26±16.00	0.849	0.013	0.727
CYP7A1 (µmoles g^{-1})	2816.32±284.30	2840.06±201.94	2414.99±62.24	2266.99±301.71	0.433	0.205	0.140

[1] Values are mean ± SE; $n = 3$.

[2] A significance level of $\alpha = 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.

† Regression values generated from treatment groups; $n = 9$.

4.4.6 Blood analyses

4.4.6.1 Graded fish oil

No statistically significant differences ($P > 0.05$) were observed between any of the blood biochemistry parameters measured from fish fed diets with graded FO (Diets 1, 2 and 3; Table 4.6A).

Table 4.6A. Blood biochemistry from Yellowtail Kingfish, *Seriola lalandi*, fed experimental diets with graded fish oil levels diets for 84 days.[1]

Diet	1	2	3	ANOVA (*P value*)[2]
Biochemistry				
Protein (g L^{-1})	38.1 ± 1.0	37.2 ± 1.2	36.8 ± 0.7	0.634
Albumin (g L^{-1})	11.3 ± 0.4	10.7 ± 0.2	10.7 ± 0.4	0.332
Globulin (g L^{-1})	26.8 ± 0.6	26.4 ± 1.0	26.1 ± 0.4	0.805
Total Bilirubin (mmol L^{-1})	2.8 ± 1.4	1.7 ± 0.5	1.4 ± 0.3	0.565
ALT (IU L^{-1}) [3]	14.8 ± 2.7	10.9 ± 1.0	9.9 ± 1.0	0.198
ALP (IU L^{-1}) [3]	22.4 ± 2.5	27.0 ± 2.3	31.0 ± 3.4	0.176
Cholesterol (mmol L^{-1})	3.9 ± 0.2	4.0 ± 0.3	4.5 ± 0.2	0.243
Triglyceride (mmol L^{-1})	2.0 ± 0.5	2.3 ± 0.3	1.7 ± 0.0	0.449
Bile Acids (mmol L^{-1})	2.9 ± 0.1	5.4 ± 1.9	5.2 ± 1.1	0.358

[1] Values are mean ± SE; $n = 3$.

[2] A significance level of $\alpha = 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.

[3] ALT, alanine aminotransferase; ALP, alkaline phosphatase.

4.4.6.2 Graded poultry and canola oil blends

No statistically significant differences ($P > 0.05$) were observed between any of the blood biochemistry parameters measured from fish fed diets formulated with FO and a graded PO and CO blend (Diets 2, 4, 5 and 6; Table 4.6B).

Table 4.6B. Blood biochemistry from Yellowtail Kingfish, *Seriola lalandi*, fed experimental diets formulated with fish oil and a graded poultry oil and canola oil blend for 84 days.[1]

Diet	2	4	5	6	ANOVA (*P value*)[2]
Biochemistry					
Protein (g L^{-1})	37.2 ± 1.2	35.9 ± 0.1	37.2 ± 0.1	36.4 ± 1.6	0.727
Albumin (g L^{-1})	10.7 ± 0.2	10.7 ± 0.4	10.9 ± 0.2	10.2 ± 0.2	0.403
Globulin (g L^{-1})	26.4 ± 1.0	25.2 ± 0.3	26.2 ± 0.1	26.2 ± 1.4	0.748
Total Bilirubin (mmol L^{-1})	1.7 ± 0.5	1.1 ± 0.1	1.2 ± 0.1	1.0 ± 0.0	0.369
ALT (IU L^{-1}) [3]	10.9 ± 1.0	9.4 ± 1.3	9.1 ± 1.5	9.8 ± 3.4	0.931
ALP (IU L^{-1}) [3]	27.0 ± 2.3	24.2 ± 3.8	29.8 ± 3.4	30.7 ± 2.7	0.490
Magnesium (mmol L^{-1})	1.2 ± 0.0	1.3 ± 0.0	1.3 ± 0.1	1.3 ± 0.1	0.941
Cholesterol (mmol L^{-1})	4.0 ± 0.3	4.3 ± 0.2	4.2 ± 0.4	4.1 ± 0.1	0.908
Triglyceride (mmol L^{-1})	2.3 ± 0.3	1.8 ± 0.5	1.9 ± 0.1	2.3 ± 0.1	0.560
Bile Acids (mmol L^{-1})	5.4 ± 1.9	11.1 ± 8.4	6.2 ± 3.3	5.5 ± 0.6	0.790

[1] Values are mean ± SE; $n = 3$.

[2] A significance level of $\alpha = 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.

[3] ALT, alanine aminotransferase; ALP, alkaline phosphatase.

4.5 Discussion

The growth performance for this trial is reported by Stone and Booth (2018). In short, fish fed diets with CO had reduced performance and feed utilisation. Conversely, in terms of growth performance indices, PO was a suitable lipid replacement for FO up to 73.5% of total added lipid.

The VSI and HSI of all fish from the present trial were consistent with the mean VSI and HSI for wild Yellowtail Kingfish (Chapter 2). Both VSI and HSI were less than cultured Yellowtail Kingfish fed experimental diets with 30% crude lipid levels but consistent with those fed 20% crude lipid levels in the same experiment (Chapter 3). This indicates that the ΣLC n-3 PUFA

(g $100g^{-1}$ diet) levels achieved for the oil type of each experiment diet was at least equivalent to this intake in wild diets.

4.5.1 Liver histology

Diets formulated with FO and a graded PO and CO blend (Diet 4, 5 and 6) did not alter the VPLC% of fish fed. However, the VPLC% of fish fed a diet with 100% PO (Diet 1) was significantly increased compared to that of fish fed decreased PO and increased FO diets (Diets 2 and 3). This did not have an effect on the proportion of neutral lipids, total lipids or glycogen within hepatocytes. Considerable literature has been presented on how FO contains high levels of essential LC-PUFA, particularly eicosapentaenoic acid (20:5n-3, EPA), docosahexaenoic acid (22:6n-3, DHA) and arachidonic acid (20:4n6, AA) (Turchini et al., 2003; Turchini et al., 2009). Likewise, it is well known that these essential LC-PUFA are required for maintaining optimum growth and health for cultured marine finfish (Bowyer et al., 2012a; Bowyer et al., 2012b; Castro et al., 2015). Substituting FO with CO or PO will alter the dietary and fatty acid composition and influence β-oxidation. The liver is an important organ for β-oxidation with different species' ability to catabolise fatty acids being dependent on environmental factors, size and life stage (Turchini et al., 2009; Bowyer et al., 2012a). Fatty acid digestion is greatest in order from LC-PUFA (C_{20} and C_{22}), C_{18} PUFA, monounsaturated fatty acids (MUFA) to saturated fatty acids (SFA) being the least digestible. Long-chain polyunsaturated fatty acids (e.g. EPA and DHA) are most easily digested, absorbed and preferentially selected as energy substrates compared with LA and ALA (Turchini et al., 2009). Linoleic acid and ALA are some of the least efficiently absorbed fatty acids, although are readily oxidised in Atlantic salmon when present in high concentrations (Turchini et al., 2009).

Yellowtail Kingfish are marine carnivorous species well adapted to feeding on LC-PUFA and PUFA diets in the wild and when cultured, require these in formulated feeds (Chapter 2). As

such Yellowtail Kingfish may be limited in their ability to convert linoleic acid (18:2 n-6) and α-linolenic acid (18:3 n-3) to corresponding C_{20} and C_{22} n-6 and n-3 LC-PUFA (Turchini et al., 2009). Replacing FO with vegetable oil decreases LC-PUFA content and increases dietary LA and ALA which is reflected in the whole fish and organ composition (Turchini et al., 2009). Depending on source, PO generally contains high levels of SFA and up to ~20% PUFA, primarily linoleic acid (18:2 n-6). Linoleic acid is difficult to oxidise and considered the most detrimental fatty acid to fillet flesh quality (Turchini et al., 2009). Regardless of the level of FO substitution trace amounts of FO may still be present from a fish meal component of the diet unless fish meal is also fully substituted. In diets with 100% FO substitution a deficiency in essential fatty acids can occur if the fish meal component of the diet is too low to meet the species' minimum requirement. In the present study we propose that increased VPLC% observed in fish fed 100% PO (Diet 1) is due to the increased SFA levels and reduced n-3 PUFA compared to the other diets. Although digested, the lipid content was not as readily utilised and therefore excess is stored. Substituting FO and fish meal with terrestrial sourced lipids may only be replaced to certain levels before digestive processes are affected (Bowyer et al., 2012a).

4.5.2 Total bile acid concentration

Fish fed diets containing up to 100% PO as a replacement for FO had no significant difference in total bile acid concentrations in the different tissues. However, total bile acid concentrations decreased in the liver of fish fed diets with increased CO. As ~30% of the lipid in each diet was FO (Diets 2, 4, 5 & 6) the bile acid concentration observed in the liver is most likely due to either: 1) PO allowing for increasing or maintaining bile acid concentration; or 2) CO reducing the bile acid concentration. These are among the first recorded liver bile acid concentrations for Yellowtail Kingfish with limited studies for comparison, particularly for

comparison with dietary CO inclusion. However, the reductions observed are consistent with reduced liver bile acids in Atlantic salmon when fed diets with terrestrial plant ingredients substituting fish-based ingredients (Kortner et al., 2013). Furthermore, it has been previously noted that substitution of marine ingredients with terrestrial plant ingredients reduces the enterohepatic bile acid levels in Japanese yellowtail, *Seriola quinqueradiata* (Khaoian et al., 2014), rainbow trout *Oncorhynchus mykiss* (Iwashita et al., 2009), and red seabream, *Pagrus major* (Goto et al., 2001).

Bile acid production and cholesterol are intrinsically linked. Dietary cholesterol is obtained from animal cells with plant cells being void of cholesterol and rich in other phytosterols; stiosterol, campesterol and stigmasterol (Lecerf and de Logeril, 2011). Furthermore, phytosterols within plant ingredients directly compete with cholesterol at the mixed micelle level, decreasing intestinal cholesterols absorption (Lecerf and de Logeril, 2011). Bile acid production is dependent on the total amount of absorbed cholesterol from the intestine; the net of intestinal absorption efficiency of intestinal cholesterol and the amount of dietary cholesterol (Wang et al., 2012). In this study, dietary CO reduced liver bile acid concentrations through compounded effects of limiting dietary cholesterol available for bile acid synthesis and the introduction of phytosterols competing for micellular position reducing intestinal cholesterol absorption. This compounded effect resulted in a greater net reduction of cholesterol compared with the other diets and was not compensated through *de novo* synthesis.

4.5.3 Taurine concentration

Liver taurine concentrations of fish fed the graded FO diets and fish fed diets with FO and a graded PO and CO blend had no significant differences observed. Liver taurine concentrations in this study were greater than that seen in Japanese yellowtail when fed with either fish-based diets or taurine supplemented plant-based diets (Khaoian et al., 2014). Fish-based ingredients

are reported to contain high levels of taurine, a β-sulphonic amino acid, however, terrestrial plants contain minimal amounts (<1% of the taurine level observed in animal ingredients (Nguyen et al., 2011; Salze and Davis, 2015). Taurine is essential for the conjugation with cholesterol to from bile acids in hepatocytes (Nguyen et al., 2011).

Taurine supplementation at varying inclusion levels is reported to improve the growth performance of cobia, *Rachycentron canadum*, red seabream, and several *Seriola* spp. when fed diets with terrestrially sourced ingredients as the main lipid and protein sources (Nguyen et al., 2011; El-Sayed, 2014). Likewise, juvenile Yellowtail Kingfish have a compromised ability to process bile from the liver to intestine when 100% of dietary FO is replaced with CO resulting in green liver syndrome (Bowyer et al., 2012b). Green liver is the accumulations of bile pigments in the liver caused by a dietary taurine deficiency, often when fish-based ingredients are replaced with terrestrial plant ingredients (Goto, et al., 2001). However, the absence of green liver and the high level of liver taurine concentration confirm that all diets in the present study were formulated to contain suitable taurine levels for growth and animal health (Stone and Booth, 2018).

4.5.4 HMGCR and CYP7A1

Within hepatocytes, mevalonate synthesis is the irreversible step of cholesterol biosynthesis and is controlled via feedback mechanisms (Maita et al., 2006; Deng et al., 2014; Chapter 3). Up-regulation of HMGCR genes for cholesterol synthesis occurs when *de novo* cholesterol is required and is down-regulated when dietary cholesterol is available in excess (Kortner et al., 2013). Plant-based proteins and oils, generally void of cholesterol and high in phytosterols, decrease dietary cholesterol and up-regulate HMGCR gene expression in Atlantic salmon and European sea bass, *Dicentrarchus labrax* (Morais et al., 2011; Torrecillas et al., 2018). The opposite down-regulation of HMGCR gene expression is also observed when cholesterol

supplementation is provided in formulated feeds for rainbow trout (Deng et al., 2014). Cholesterol synthesis and liver HMGCR increases in fish when fed diets where FM replacement by plant or animal proteins and oils reduce the overall cholesterol content of formulated feed. The FM component of diets in the present study did not differ. Consistent with other species, liver HMGCR concentration decreased within the livers of Yellowtail Kingfish fed diets with greater PO inclusion indicating that a sufficient amount of cholesterol was available within the diet, potentially provided by the increased level of the PO. Conversely, HMGCR concentration, although variable, increased numerically when CO was the predominant oil source. Thus, the CO added to these diets did not provide sufficient dietary cholesterol when substituting FO and PO in Yellowtail Kingfish diets and HMGCR activation was required for cholesterol synthesis. Dietary cholesterol content should be carefully considered when CO is used in Yellowtail Kingfish formulated feeds.

Cholesterol-7α-hydroxylase is considered the main rate-limiting enzyme contributing to bile acid synthesis by regulating the conversion of cholesterol to 7α-hydroxycholesterol (Crosignani et al., 2011; Guerra-Olvera and Viana, 2015). Dietary cholesterol above the body's metabolic requirements stimulates CYP7A1 and therefore bile acid synthesis which is a primary route for elimination of cholesterol via faeces (Lefebvre et al., 2009). Cholesterol supplementation in diets with terrestrial plant-based ingredients added to Atlantic salmon feed increased CYP7A1 activity (Kortner et al., 2013). Deng et al. (2014) reported total bile acid levels increasing in intestinal content of rainbow trout fed plant-based diets with cholesterol supplementation at 9 g kg^{-1} but did not affect the hepatic CYP7A1 activity. This suggested the increased levels of cholesterol were responsible for stimulation of the cholesterol catabolic pathway. The current study is in line with previous research whereby replacement of FO with an alternative terrestrial oil source (PO) increased liver CYP7A1 concentration. This is a result

of a greater need for bile acid synthesis, possibly for the removal of cholesterol that exceeded the dietary requirement within the fish fed diets with increasing PO inclusion. This may be due to remnant FO being present and provided to the animals in the fish meal component of the diet. The present study supports previous research by observing reduced CYP7A1 concentration in fish fed diets of increased CO, a plant-based diet without cholesterol supplementation. Reduced lipid metabolism and growth have been observed in red seabream (*P. auratus*) and Yellowtail Kingfish respectively when fed diets with FO substituted by high levels of CO (Glencross et al., 2004; Bowyer et al., 2012b). Overall, as dietary PO decreased and CO increased a numerical reduction in CYP7A1 concentration highlights that: 1) PO may have had increased levels of cholesterol available for bile acid synthesis; or 2) CO reduced the availability of cholesterol for bile acid synthesis.

4.5.5 Blood analyses

Cholesterol concentrations and FO inclusion level were greatest in Diet 3 and treatments were within the cholesterol concentration range expected for cultured Yellowtail Kingfish (Crowe et al., 2021). These levels were half that observed in wild caught Yellowtail Kingfish (Chapter 2). Plasma cholesterol concentrations are a combination of exogenous and endogenous cholesterol synthesised in the liver (Maita et al., 2006; Bowyer et al., 2012b). The consumption of FO, containing high levels of EPA and DHA, generally reduces the level of available cholesterol through stimulating fatty acid oxidation (Hosomi et al., 2011). Plasma cholesterol concentrations are reduced when cultured fish are fed formulated feeds containing FO high in available cholesterol (Bowyer et al., 2012b; Liland et al., 2013). Interestingly, the plasma cholesterol numerically decreased with increased PO. This reduction may have been due to cholesterol being utilised for synthesis of bile acids and elimination from the body as indicated by the significant increase in CYP7A1 concentration. This could provide explanation as to

cholesterol being supressed and increased bile acids synthesis for excretion from the body. Furthermore, plasma bile acid concentrations also numerically decreased with increasing PO and may be an indication of an overall reduced body pool of bile acids. This is possibly due to reduced recycling of bile acids and increased excretion of the bile acids due to increased synthesis for cholesterol elimination. Increased bile acid in the faeces was observed in fish fed Diet 1 (100% PO) but was not significantly different from that of Diet 2 (27% FO and 73% PO) or Diet 3 (50% FO / 50% PO).

Previous research into alternative feed ingredients for Yellowtail Kingfish has shown that complete substitution for FO by CO in diets produces a hypercholesterolemic effect on plasma cholesterol levels (Bowyer et al., 2012b). In the present study reduced plasma cholesterol concentrations were observed when CO inclusion increased but did not significantly differ in fish fed diets with up to 71% CO and 29% FO. Additionally, these levels were within the cholesterol concentration range for cultured Yellowtail Kingfish and approximately half that seen in wild caught Yellowtail Kingfish (Crowe et al., 2021; Chapter 3). Plasma bile acid concentrations also numerically decreased with increasing CO. Increased HMGCR concentration, decreasing plasma cholesterol and bile acid concentrations and increased bile acid in faeces of fish fed diets with increasing CO indicates that the addition of CO reduces the overall cholesterol pool within the fish and facilitated the removal of cholesterol via the enterohepatic system.

4.6 Conclusion

At the water temperatures (16.2 ± 2.1 °C) recorded in this study, Yellowtail Kingfish may be able to be fed a formulated diet containing ~25% dietary lipid level (2.12 g $100g^{-1}$ of ΣLC n-3 PUFA) with up to 100% of the FO component replaced by PO without apparent effects on: VSI, HSI, liver lipid and glycogen storage, total bile acid concentration of the liver, gallbladder

content, foregut, midgut, hindgut or faeces, nor liver taurine concentrations, liver HMGCR concentrations or any of the blood parameters measured in this study. However, in this short term study, hepatocyte vacuolisation increased with increasing levels of PO. Similarly, CYP7A1 concentration numerically increased with increasing PO indicating a compensatory synthesis of bile acids for micellular solubilisation of dietary lipids or for increased cholesterol excretion. The long-term feeding of diets containing high inclusion levels of PO to Yellowtail Kingfish and the effect this has on their liver function and structure should be closely monitored. Similarly, Yellowtail Kingfish may be able to be fed a formulated diet containing ~25% dietary lipid level (2.12 g $100g^{-1}$ of ΣLC n-3 PUFA) with up 100% of the commercially used alternative lipid source (PO) with CO without an apparent negative impact on: VSI, HSI, liver lipid and glycogen storage, hepatocyte vacuolisation, total bile acid concentration of the gallbladder content, foregut, midgut, hindgut or faeces, nor liver taurine concentrations, liver HMGCR or CYP7A1 concentrations or any of the blood parameters measured in this study. However, fish fed diets with decreasing PO and increasing CO had decreasing total bile acid concentrations in liver tissue which did not appear to be metabolically compensated for. Reduction of dietary FO and replacement with either PO, CO or a PO-CO blend at high inclusion levels in formulated feeds may have desired effects beyond those examined in this experiment and their use should be investigated.

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Chapter 5: Investigating liver structure and function in Yellowtail Kingfish, *Seriola lalandi,* in response to reduced fish meal diets

5.1 Abstract

Alterations to the enterohepatic system, the storage of lipids and the consequent effects on liver structure need to be considered when replacing wild derived fish meal (wd-FM) with alternative ingredients in formulated diets for commercial Yellowtail Kingfish, *Seriola lalandi*. Wild derived-FM is known to contain high levels of cholesterol and taurine relative to many other protein sources. Cholesterol and taurine are important precursors in bile acids synthesis. The replacement of wd-FM with other alternative ingredients is likely to alter the available level of cholesterol and taurine for bile acid production, its availability for absorption of dietary lipids and fat-soluble vitamins, and excretion. In this 252-day study, Yellowtail Kingfish (2.52 ± 0.25 kg; 546 ± 20 mm [fork length; mean ± standard deviation]) were fed five different diets formulated on a digestible nutrient content basis that contained highly palatable and digestible ingredients at realistic commercial inclusion levels. A 30% wd-FM diet served as a control. Dietary wd-FM inclusions were reduced to 20% and 10%, replaced with FM by-product meal. In addition, dietary wd-FM levels were reduced to 20% and replaced with poultry meal or soy protein concentrate in two other separate diets. Fat deposition in the liver was similar between all treatment groups but severe bile duct proliferation and periductular fibrosis was observed in the livers of fish fed diets with wd-FM replacement with 10% poultry meal or 10% soy protein concentrate. Yellowtail Kingfish fed a 30% wd-FM diet may have the FM replaced by up to 66.67% fish meal by-product, 33.3% poultry meal or 33.3% soy protein concentrate without any significant impact on total bile acid synthesis, storage and excretion and liver lipid storage.

5.2 Introduction

Total fish meal (FM) production for global aquaculture feed peaked in 1994 at 30.1 million tonnes with an oscillating but overall trend of decline since (FAO, 2020b), fuelling an increase in the cost of FM (FAO, 2020b). This downward production trend is driving research to find alternative feed ingredients, thereby reducing global fisheries catch, which contributes to over 70% of FM production (FAO, 2020a). Inclusions of FM in aquaculture feeds were as high as 50% in the early 1990s, whereas in comparison current feeds contain minimal amounts, with some feeds reduced to 7.5% FM inclusion (Kousoulaki et al., 2012; Asche et al., 2013). Fish meal reduction in formulated feeds is also largely due to increased cost as a result of competition for its use. Poultry and pig industries were led to reduced dietary FM inclusion levels and finding alternative feed protein sources as a result of competition and rising costs from use in formulated feeds for aquaculture, terrestrial animal feeds and human food (Kim et al., 2012; Asche et al., 2013; Rojas and Stein, 2013). Further reduction for FM inclusion is inevitable in the face of this demand and as the aquaculture industry moves towards more sustainable and responsible environmental practices.

Thorough understanding of the nutritional requirement of a cultured species is critical to the success of an aquaculture operation (Stone and Bellgrove, 2013). Growth, development and health of cultured marine carnivorous fish are dependent on the correct dietary levels of protein to energy ratio, amino acid and fatty acid profiles, as well as other essential dietary nutrients such as taurine, cholesterol and other minerals and vitamins (Stone and Bellgrove, 2013). The substitution of aquatic based ingredients with terrestrial ingredients has been a highly successful approach used to minimise the use of FM in formulated feeds for Yellowtail Kingfish (Stone and Bowyer, 2013; Stone et al., 2016; Stone and Booth, 2018). However, substitution of aquatic based ingredients with terrestrial based ingredients in formulated feeds may result in a reduction or absence of essential amino acids and long-chain polyunsaturated

fatty acids (LC-PUFA) increasing adverse health and growth impacts (Francis et al., 2001; Chen et al., 2011; Lim et al., 2011; Collins et al., 2012). Terrestrial ingredients can negatively impact the absorption of essential nutrients by altering digestive enzymatic processes and the morphometry of the digestive tract and liver (Chapter 3; Chapter 4; Bowyer et al., 2012a; Bowyer et al., 2012b; Stone and Bellgrove, 2013; Bansemer et al., 2015). Substitution level of alternative feed ingredients to FM and the resulting growth performance indices are species-specific (Trushenski and Lochmann, 2009; Wang et al., 2008; Lim et al., 2011; Santigosa et al., 2011).

Considerable literature has been published on how fish oil and FM contain high levels of essential amino acids and LC-PUFA that are required for maintaining optimum growth and health for cultured marine finfish (Turchini et al., 2003; Turchini et al., 2009; Bowyer et al., 2012a; Bowyer et al., 2012b; Castro et al., 2015). A known effect of substituting FM with terrestrial sourced proteins is the reduced efficiency of enterohepatic circulation of bile acids (Nguyen et al., 2011). For example, soybean meal, commonly used as a FM alternative, increases undigested molecular fractions that bind to bile acids, reducing the production of peptides in the intestine and increasing faecal steroid excretion (Sugano et al., 1988; Nguyen et al., 2011). The increased faecal excretion has the potential to reduce absorption of bile acids which limits the absorption of essential dietary lipids and vitamins (Nguyen et al., 2011).

Bile acids are essential for facilitating the absorption of dietary lipids and fat-soluble vitamins (Lefebvre et al., 2009; Bowyer et al., 2012b). Primary bile acids are steroids synthesised from cholesterol in liver hepatocytes (Mohapatra and Mishra, 2011; Nguyen et al, 2011; Holm et al., 2013). Once synthesised, they are secreted into the canaliculi, drained into bile ducts and finally after being conjugated with taurine or glycine are stored in the gallbladder as bile salts (Mohapatra and Mishra, 2011; Nguyen et al, 2011; Holm et al., 2013; El-Sayed, 2014; Guerra-

Olvera and Viana, 2015). The gallbladder empties its content into the intestinal lumen to assist in the digestion and absorption of dietary lipids (Van Waarde, 1988; Yamamoto et al., 2007; Lefebvre et al., 2009; Nguyen et al, 2011; York, 2017). Enterocytes transport bile acids to the basolateral domain for efflux and they are then transported back to the liver via the portal vein circulation (Kullak-Ublick et al., 2004; Murashita et al., 2014). Fish meal is reported to contain high levels of taurine, a *β*-sulphonic amino acid required for this conjugation. Teleost fish either acquired cholesterol from the diet or can synthesise it *de novo* in the liver, although in a lesser capacity than mammals (Kortner et al., 2013). For cultured fish, dietary cholesterol can be obtained from terrestrial animal meals as plant meals are devoid of cholesterol and rich in other phytosterols such as sitosterol and stigmasterol (Lecerf and de Logeril, 2011).

In Atlantic salmon, *Salmo salar*, bile acid metabolism is controlled by several nuclear receptors and mRNA species in the liver and intestine, including: liver X receptor (LXR), farnesoid X receptor (FXR), cytochrome P4507A1 (CYP7A1), bile acid transporters (ABCB11) and apical Na-dependent bile acid transporter (ASBT, gene symbol SLC10A2) (Kortner et al., 2013). These genes are key regulators in the enterohepatic circulating system responsible for recycling of bile acids through intestinal reabsorption. Approximately 95% of bile acids are recycled through this system (Zhou and Hylemon, 2014). The remaining bile acids and unrequired or excess cholesterol are removed from the body via faecal excretion (Lefebvre et al., 2009).

The Yellowtail Kingfish, *Seriola lalandi*, is a carnivorous marine species cultured in Australia for its fast growth and premium flesh quality. In Australia, wild Yellowtail Kingfish predominantly feed on smaller fish species which contain high levels of essential amino acids and LC-PUFA, particularly eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3; Chapter 2; Fowler et al., 2003; Turchini et al., 2003; Turchini et al., 2009). The aim of this study was to understand the effect that partial substitution of FM in formulated feeds would

have on Yellowtail Kingfish liver structure, hepatic cholesterol and bile acid synthesis, enzyme concentration and the recycling of bile acids. This was achieved by investigating the histological changes and nutrient deposition in liver tissue, rate limiting enzymes for hepatic cholesterol synthesis and bile acid synthesis and analyses of blood profiles.

5.3 Methods

5.3.1 Experimental description

In this study, commercial diets with differing dietary protein sources provided by the substitution of FM with three other protein sources were used (Table 5.1). The effect on liver structure, liver cholesterol and bile acid metabolism, blood biochemistry, growth and health of Yellowtail Kingfish were investigated. The biochemical composition of the five diets is displayed in Table 5.1. The experiment was conducted over 252 days at the South Australian Research and Development Institute (SARDI) South Australian Aquatic Sciences Centre (SAASC) at West Beach, Adelaide, Australia. At the commencement of the experiment, individual fish were weighed (kg) and measured (fork length; mm) after the fish had been anaesthetised in seawater using AQUI-S® (AQUI-S® New Zealand Ltd.) at a concentration of 0.02 mL L^{-1}. A total of 306 fish (body mass 2.52 ± 0.25 kg; fork length, 546 ± 20 mm; mean ± SD) were randomly distributed between fifteen 5,000 L fibreglass tanks with 17 per tank. Each of the 5 treatments were randomly assigned to three replicate tanks. Fish were fed to satiation once daily at 09:00 h (Stone and Booth, 2018).

Table 5.1. The proximate composition and alternative protein inclusion (%) of experimental diets fed to Yellowtail Kingfish, *Seriola lalandi*, for 252 days.

Diet	Diet 1 Control 30% FM	Diet 2	Diet 3	Diet 4	Diet 5
Fish meal replacement (%)	0	33.33	66.67	33.3	33.33
Fish by-product meal (%)	0	10.7	21.4	0	0
Poultry meal (%)	0	0	0	11.32	0
Soy protein concentrate (%)	0	0	0	0	10.88
Analysed proximate composition (g 100 g^{-1})					
Moisture	8.7	7.5	7.4	7.7	7.8
Crude protein	45.4	45.7	46.0	44.9	46.1
Crude lipid	24.1	24.8	23.9	24.7	24.3
Ash	8.9	9.0	9.8	8.4	7.8
Carbohydrate[1]	12.9	13.0	12.9	14.3	14.0
Gross energy (MJ kg^{-1})	18.8	19.1	18.9	19.1	19.2

[1] Carbohydrate = 100 - (moisture + lipid + protein + ash).

5.3.2 Experimental system

The recirculating aquaculture system (RAS) was kept undercover in ambient conditions with supplemental fluorescent lighting provided during the natural light period. Ambient temperature sea water was treated by settlement and sand filtration and circulated through the RAS. The sea water was then returned through filter-screen baffle-boards, pumped through a drum filter with 70 μm^2 filter pores (HDF1603 Hydrotech, Saint-Maurice, France), returned to the biofilter, and finally treated with UV light disinfection (D-32051 Wedeco, Herford, Germany) at a rate of 35,000 L h^{-1} by two electric centrifugal pumps (Grundfos, Regency Park, Australia) before being returned to the tanks. One hundred percent of the water volume of each 5,000 L tank was replaced daily.

5.3.3 Fish source and acclimation

Sub-adult Yellowtail Kingfish were obtained from Clean Seas Pty. Ltd. sea cage facilities at Port Lincoln, Australia. The fish were acclimated for 4 weeks in 5,000 L fibreglass tanks supplied by the RAS at SARDI SAASC and fed a commercial diet (Ridley Corporation Ltd. Pelagica diet; gross energy, 19.3 MJ kg^{-1}, crude lipid [CL], 24%, crude protein [CP], 46%) before the commencement of the experiment.

5.3.4 Experimental diets

In this study, five diets were formulated on a digestible basis to contain a mix of protein sources to replace FM with ~45 – 47% CP (39% digestible protein) and ~25% CL (24% digestible lipid; Table 5.1). Wild derived FM (wd-FM), fish by-product meal (FBM), poultry meal (PM) and soy protein concentrate (SPC) were investigated in this study. The FBM, PM, and SPC ingredients were substituted into the base formulation by reducing the wd-FM levels to 20% and 10%. This resulted in five separate diets: control Diet 1 (30% wd-FM), Diet 2 (20% wd-FM + 10.7% FBM), Diet 3 (10% wd-FM + 21.4% FBM), Diet 4 (20% wd-FM + 11.32% PM) and Diet 5 (20% wd-FM + 10.88% SPC; Table 5.1). All diets were formulated by SARDI, Clean Seas Pty. Ltd. and Skretting Australia Pty Ltd. staff and manufactured using cooking extrusion technology (9 mm pellets) by Skretting Australia Pty Ltd (Cambridge, TAS, Australia). The experimental diets were analysed for biochemical composition according to methods in the British Pharmacopoeia Commission (2004) or German Institute for Standardization (DIN) (2000) by SARDI. A one kg sample of each diet was collected, ground and analysed for proximate composition (moisture, protein, fat, ash, total carbohydrate and energy) and fatty acids profile. The five experimental diet ingredient formulations were commercially confidential; however, the corresponding proximate diet composition, fatty acid profiles and proximate amino acid profiles are shown in Table 5.1.

5.3.5 Water quality

Water quality parameters were monitored daily, unless otherwise indicated. Temperature (°C, mean ± SD) and dissolved oxygen (% saturation, mean ± SD) were monitored using an OxyGuard Handy Polaris temperature and dissolved oxygen probe (OxyGuard International A/S, Farum, Denmark). The pH (mean ± SD) was monitored using a Eutech pH Testr 30 multiparameter handheld probe (Eutech Instruments Pty. Ltd., Singapore). Salinity (g L^{-1}, mean ± SD) was monitored using an ISSCO UR-2 hand-held refractometer (model RF20; Extech Instruments Corporation, Nashua, New Hampshire, United States of America). The total ammonia concentration (mg L^{-1}) was determined weekly using a commercial water testing kit (Aquarium Pharmaceuticals, Chalfont, Pennsylvania, United States of America).

5.3.6 Sample collection

Feeding was stopped 24 h before the end of the experiment. Three fish from each tank were euthanized and measured for their body mass (nearest 0.1 g) and fork length (nearest 0.1 mm). The growth performance and feed utilisation for this trial was reported by Stone and Booth (2018). All data reported for each treatment for animal performance were based on the mean of the replicate tanks. All calculations using fish weight and diets are based on wet or as fed values, respectively:

Weight gain = final weight – initial weight

Specific growth rate (SGR; % d^{-1}) = ([ln final weight – ln initial weight] /d) x100

Condition factor (CF) = (fish body mass [g] / fish length $[cm]^3$) × 100

Apparent feed conversion ratio (FCR) = feed consumed / fish weight gain

Blood samples were collected from the caudal vasculature using 21 gauge needles and 5 mL syringes. For each fish, the visceral cavity was cut open and the whole removed gut weighed (nearest 0.01 g) to determine the visceral somatic index (VSI; %). The liver was removed and

weighed (nearest 0.01 g) to determine the hepatosomatic index (HSI; %). These indices were calculated as follows:

VSI% = (visceral weight [g] / body mass [g]) × 100

HSI% = (liver weight [g] / body mass [g]) × 100

Liver, gallbladder content, foregut, midgut, hindgut and faeces were collected, immediately snap frozen in liquid nitrogen and stored at – 80 ℃ until analysis. The liver samples were used to measure the concentration of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) and CYPA71 enzymes. Two 1 cm^3 sections of the left lobe of the liver were also dissected and immediately placed into a histology cassette and fixed with 10% neutral buffered formalin (pH of 7.2) for 24 h for histological evaluation. One of the liver sections from each fish was used for standard paraffin embedding and then sectioned by rotary microtome, while the other was preserved in an 18% phosphate buffered saline (PBS) sucrose + 0.01% sodium azide (NaN_3) solution with pH 7.1 for cryostat sectioning.

5.3.7 Liver histology

Once fixed, one liver section from each fish was transferred to 70% (v/v) ethanol and stored at room temperature according to Hu et al. (2013). The sample was dehydrated using standard procedures, embedded in paraffin and sectioned at 5 μm on a rotary microtome. This section of liver from each fish underwent several staining techniques: haematoxylin and eosin (H&E) for quantitative assessments of the vacuole volume as a proportion of the liver cell volume (VPLC%) and tissue structure as per Crowe et al. (2021) and periodic acid Schiff (PAS) for glycogen deposition. The second liver section samples were sectioned at 10 μm on a cryostat microtome and mounted on polyethylenimine coated slides. This section of liver from each fish underwent two staining techniques: Oil Red O (ORO) for unsaturated neutral lipid deposition and Sudan Black B (SBB) for total lipid deposition. Stained sections were examined at 400-

fold magnification using a light microscope (Olympus BF BX50). Three 559,390 μm^2 microphotographs were taken per stained slide using a digital camera (Olympus DP27). Quantitative assessments of the stained deposition volume as a proportion of the liver cell volume were determined by applying stain sensitive colour thresholds using Fiji ImageJ processing software (National Institutes of Health, Bethesda, Maryland, United States of America).

5.3.8 Biochemical analyses

Samples from individual fish were partially thawed, weighed and homogenised in five volumes of PBS with a pH of 7.1. Homogenisation was performed using a Retch MM 400 ball mill at frequency 30.01 s^{-1} for 4 min with Biospec 2 mm zirconia beads (Catalogue No.11079124zx). The suspensions were centrifuged twice at an acceleration of 10,000 *g* for 10 min at 4 °C (Beckman Coulter Microfuge 16 centrifuge) with the supernatants being extracted to new microfuge tubes between cycles. All assay kits included internal standard solutions and manufacturer instructions were followed for assays. Tissue concentrations are reported per tissue weight (μmoles g^{-1}).

5.3.8.1 Total bile acid concentration

The liver, gallbladder content, foregut, midgut, hindgut and faeces pooled supernatants were analysed for total bile acid concentrations by use of a commercial fluorometric bile acid assay kit by reading Ex/Em = 560/590 nm after 45 min of dark incubation at room temperature (Catalogue No. MET-5005; Cell Biolabs Inc.®, San Diego, California, United States of America).

5.3.8.2 Taurine concentration

The pooled liver supernatants were analysed for taurine by use of a commercial colorimetric taurine assay kit by reading samples at 405 – 415 nm (Catalogue No. MET-5070; Cell Biolabs Inc.®, San Diego, California, United States of America).

5.3.8.3 HMGCR and CYP7A1 enzyme-linked immunosorbent assays

The pooled liver supernatants were analysed for HMGCR concentration after reading the absorbance of samples at a wavelength of 450 nm (Catalogue No. CSB-E15772m; CusaBio, Texas, United States of America). The pooled supernatants were analysed for CYP7A1 concentration after reading the absorbance of samples at a wavelength of 450 nm (Catalogue No. CSB-EL006395FI; CusaBio, Texas, United States of America).

5.3.9 Blood analyses

Two mL of blood was placed into separate Vacuette® or BD vacutainer® tubes (Z serum clot activator or EDTA tubes) and stored indirectly on ice until same-day analysis of blood parameters by IDEXX Laboratories (Unley, South Australia, Australia). Biochemistry parameters were measured but of specific interest were the following parameters: total bile acids, bile salts, cholesterol, triglycerides and total bilirubin.

5.3.10 Statistical analyses

A one-way analysis of variance (ANOVA) and regression analysis were used to assess the effects of the different diets. Two planned comparisons of diets formulated with: 1) graded wd-FM (30% control, 20% and 10% inclusion levels) replaced with FBM; and 2) 30% wd-FM control diet reduced to 20% with the inclusion of 10% FBM, 10% PM or 10% SPC in 3 separate diets, were conducted. The normality of data was assessed using the Shapiro–Wilk test. Homogeneity of variances among means was assessed using Levene's test for equality of variance errors. The significance level was set at $\alpha = 0.05$ for all statistical tests. Where

significant interactions were observed, the data were analysed using Tukey's Honestly Significant Difference (HSD) multiple range test, unless otherwise stated. All data are presented as the mean ± the standard error (SE) of the mean, unless otherwise stated. IBM SPSS Version 20 for Windows (IBM SPSS Inc., Chicago, Illinois, United States of America) software was used for all statistical analyses.

5.4 Results

5.4.1 Water quality

Average values for temperature, dissolved oxygen, pH and salinity were 16.7 ± 2.8 °C, 102.9 ± 5.3%, 7.8 ± 0.2 and 38 ± 0 g L^{-1}, respectively, across the study period. The ammonia concentration was found to be below the limits of detection on all occasions.

5.4.2 Performance indices

The growth performance and feed utilisation for this trial was reported by Stone and Booth (2018). In short, final weight ($P > 0.05$), SGR ($P > 0.05$), CF ($P > 0.05$), apparent feed intake (% BW d^{-1}; $P > 0.05$) and FCR ($P > 0.05$) of Yellowtail Kingfish were not significantly influenced by diet.

5.4.3 Somatic indices

5.4.3.1 Increasing wd-FM replacement with fish by-product meal

Increasing wd-FM replacement with FBM had no statistically significant effect on the VSI ($P = 0.107$) or HSI ($P = 0.684$; Table 5.2A).

Table 5.2A. Somatic parameters of Yellowtail Kingfish, *Seriola lalandi*, fed increasing wild derived fish meal replacement with fish by-product meal for 252 days.[1]

Diet	1	2	3	ANOVA (*P value*)[2]
Visceral index (VSI; %)	5.9 ± 0.2	5.9 ± 0.3	6.7 ± 0.2	0.107
Hepatosomatic index (HSI; %)	0.8 ± 0.1	0.8 ± 0.0	0.9 ± 0.1	0.684

[1] Values are mean ± SE; $n = 3$.
[2] A significance level of $\alpha = 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.

5.4.3.2 Replacement of wd-FM with either fish by-product meal, poultry meal or soy protein concentrate

Replacement of 33.3% wd-FM with either FBM, PM or SPC had no statistically significant effect on the VSI ($P = 0.684$) or HSI ($P = 0.900$; Table 5.2B).

Table 5.2B. Somatic parameters of Yellowtail Kingfish, *Seriola lalandi*, fed a wild derived fish meal diet partially substituted with fish by-product meal, poultry meal or soy protein concentrate on a digestible protein basis for 252 days.[1]

Diet	1	2	4	5	ANOVA (*P value*)[2]
Visceral index (VSI; %)	5.9 ± 0.2	5.9 ± 0.3	6.2 ± 0.2	6.2 ± 0.4	0.684
Hepatosomatic index (HSI; %)	0.8 ± 0.1	0.8 ± 0.0	0.9 ± 0.1	0.9 ± 0.1	0.900

[1] Values are mean ± SE; $n = 3$.
[2] A significance level of $\alpha = 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.

5.4.4 Liver histology

The livers of fish fed diets with wd-FM displayed typical hepatocyte structure. Bile ducts had consistent cuboidal cholangiocyte positioning surrounded by hepatocytes filled with vacuolar fat. The livers of fish fed diets containing ~10% FBM replacement (Diet 2) and ~20% FBM replacement (Diet 3) displayed similar hepatocyte structure to that of those fish fed the control diet (Diet 1; 30% wd-FM) but overall less vacuolar fat and slightly congested sinusoids (Figures 5.1 and 5.4). Fat deposition in the livers of fish fed 10% PM replacement (Diet 4) and 10% SPC replacement (Diet 5) were similar to that of the control diet (Diet 1; 30% wd-FM). Severe bile duct proliferation and periductular fibrosis was observed in the livers of fish fed 10% PM replacement (Diet 4; Figures 5.5) and the 10% SPC replacement (Diet 5; Figure 5.7, 5.8 and 5.9). The bile ducts had significant periductular fibrosis characterised by sclerosing fibrous layering around the bile duct giving an "onion-skin" appearance. A fibrous sheath and the fibrous bridging connected multiple bile ducts. Newly forming bile ducts without lumen were also present (Figure 5.6).

5.4.4.1 Increasing wd-FM replacement with fish by-product meal

Increasing wd-FM replacement with FBM had no significant effect on liver VPLC% (P = 0.643) and regression analysis showed no significant relationship between increasing wd-FM replacement with FBM and VPLC% (r^2 = 0.088; P = 0.439; Table 5.3A). Almost negligible quantities of glycogen within hepatocytes were detected. Increasing wd-FM replacement with FBM had no significant effect on the proportion of liver glycogen between treatments (P = 0.892; Table 5.3A). Similarly, regression analysis showed no significant relationship between increasing wd-FM replacement with FBM and glycogen concentration in the liver (r^2 = 0.018; P = 0.734; Table 5.3A). Neutral lipids were not significantly different between treatments with increasing wd-FM replacement with FBM (P = 0.528) and regression analysis showed no

relationship between increasing wd-FM replacement with FBM and neutral lipid content in the liver (r^2 = 0.001; *P* = 0.941; Table 5.3A). Increasing wd-FM replacement with FBM had no significant effect on the proportion of liver total lipid between treatment (*P* = 0.126). However, regression analyses revealed a significant decrease in total lipid deposition with increasing wd-FM replacement by FBM (r^2 = 0.471; *P* = 0.041; Table 5.3A).

Table 5.3A. Vacuole area and proportion of histology stain on liver sections of Yellowtail Kingfish, *Seriola lalandi*, fed increasing wild derived fish meal replacement with fish by-product meal for 252 days.[1]

				ANOVA	Regression[#]	
Diet	1	2	3	*P value*[*]	r^2	*P* value
VPLC%[†]	38.8±4.7	39.5±1.1	33.8±6.1	0.643	0.088	0.439
Neutral lipids	5.9±1.1	4.6±0.7	5.8±0.7	0.528	0.001	0.941
Total lipids	7.9±0.9	7.0±0.9	4.6±1.1	0.126	0.471	**0.041**
Glycogen	0.1±0.1	0.1±0.0	0.1±0.1	0.892	0.018	0.734

[1] Values are mean ± SE; *n* = 3.
[*] A significance level of α = 0.05 was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.
[†] Vacuole volume as a proportion of the liver cell volume (Crowe et al. 2021).
[#] Regression values generated from treatment groups; *n* = 9.

5.4.4.2 Replacement of wd-FM with either fish by-product meal, poultry meal or soy protein concentrate

Replacement of wd-FM (Diet 1) with either ~10% FBM (Diet 2), PM (Diet 4) or SPC (Diet 5) had no significant effect on the liver VPLC% (*P* = 0.539; Table 5.3B; Figure 5.1). The proportion of liver glycogen was not significantly different between fish fed diets with wd-FM (Diet 1) with either ~10% FBM (Diet 2), PM (Diet 4) or SPC (Diet 5) (*P* = 0.340; Table 5.3B). Liver neutral lipids were not significantly different between treatments of fish fed diets with wd-FM (Diet 1) with either ~10% FBM (Diet 2), PM (Diet 4) or SPC (Diet 5; *P* = 0.292; Table 5.3B; Figure 5.2). Stained total lipids of liver tissue were not significantly different between

treatments of fish fed diets with wd-FM (Diet 1) with either ~10% FBM (Diet 2), PM (Diet 4) or SPC (Diet 5; $P = 0.495$; Table 5.3B; Figure 5.3).

Table 5.3B. Vacuole area and proportion of histology stain on liver sections of Yellowtail Kingfish, *Seriola lalandi*, fed a wild derived fish meal diet partially substituted with fish by-product meal, poultry meal or soy protein concentrate for 252 days on a digestible protein basis.[1]

					ANOVA
Diet	1	2	4	5	*P value*[*]
VPLC%[†]	38.8±4.7	39.5±1.1	42.3±3.8	45.3±2.9	0.539
Neutral lipids	5.9±1.1	4.6±0.7	5.8±0.3	4.1±0.6	0.292
Total lipids	7.9±0.9	7.0±0.9	9.2±1.2	7.8±0.9	0.495
Glycogen	0.1±0.1	0.1±0.0	0.1±0.0	0.0±0.0	0.340

[1] Values are mean ± SE; $n = 3$.
[*] A significance level of $\alpha = 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.
[†] Vacuole volume as a proportion of the liver cell volume (Crowe et al. 2021).

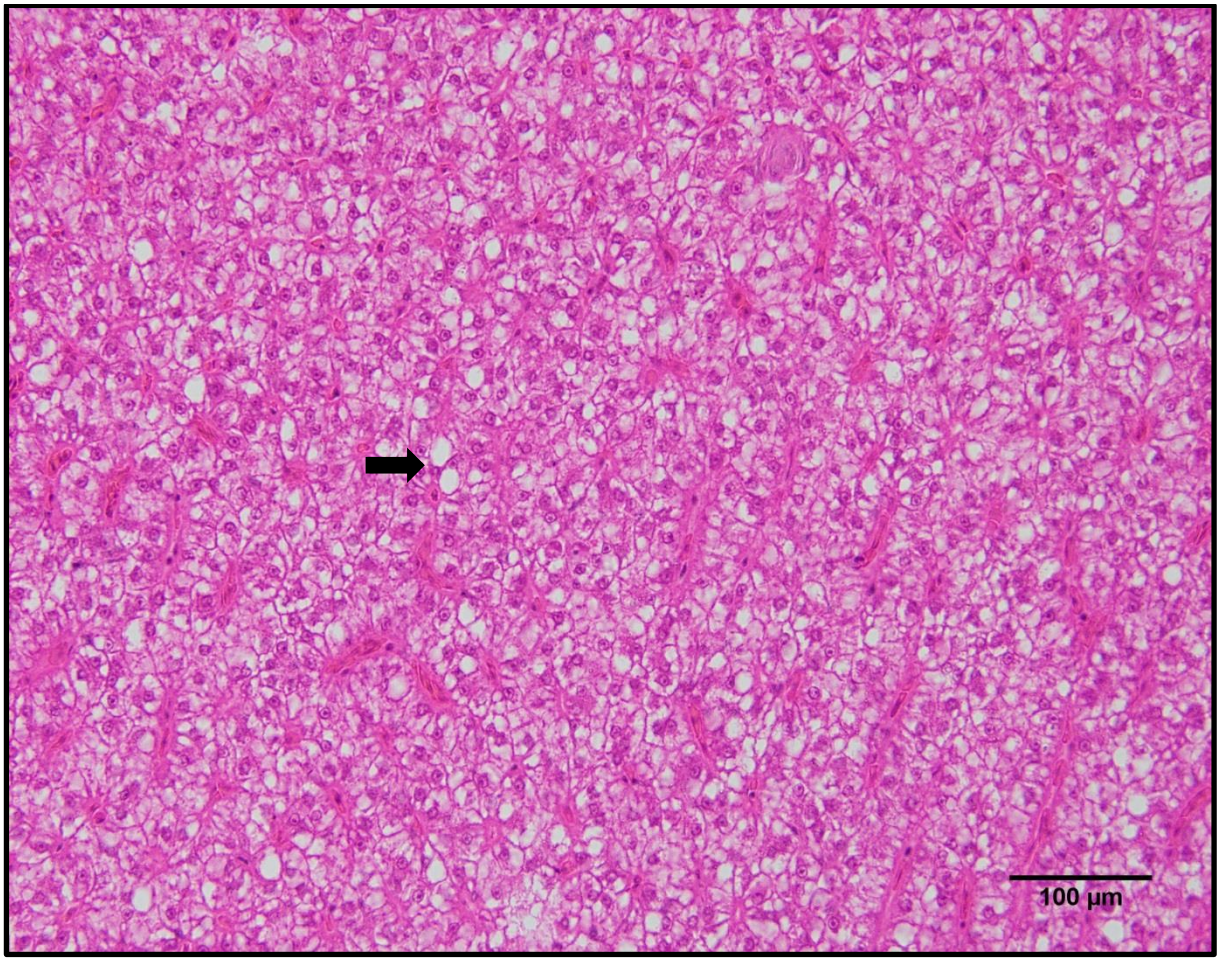

Figure 5.1. Yellowtail Kingfish, *Seriola lalandi*, liver section after a 252-day fish meal replacement trial. Liver hepatocytes displaying typical structure and vacuolar fat (*arrow*). Diet 1 contained 30% wild derived fish meal. 400x magnification. Haematoxylin and eosin stain.

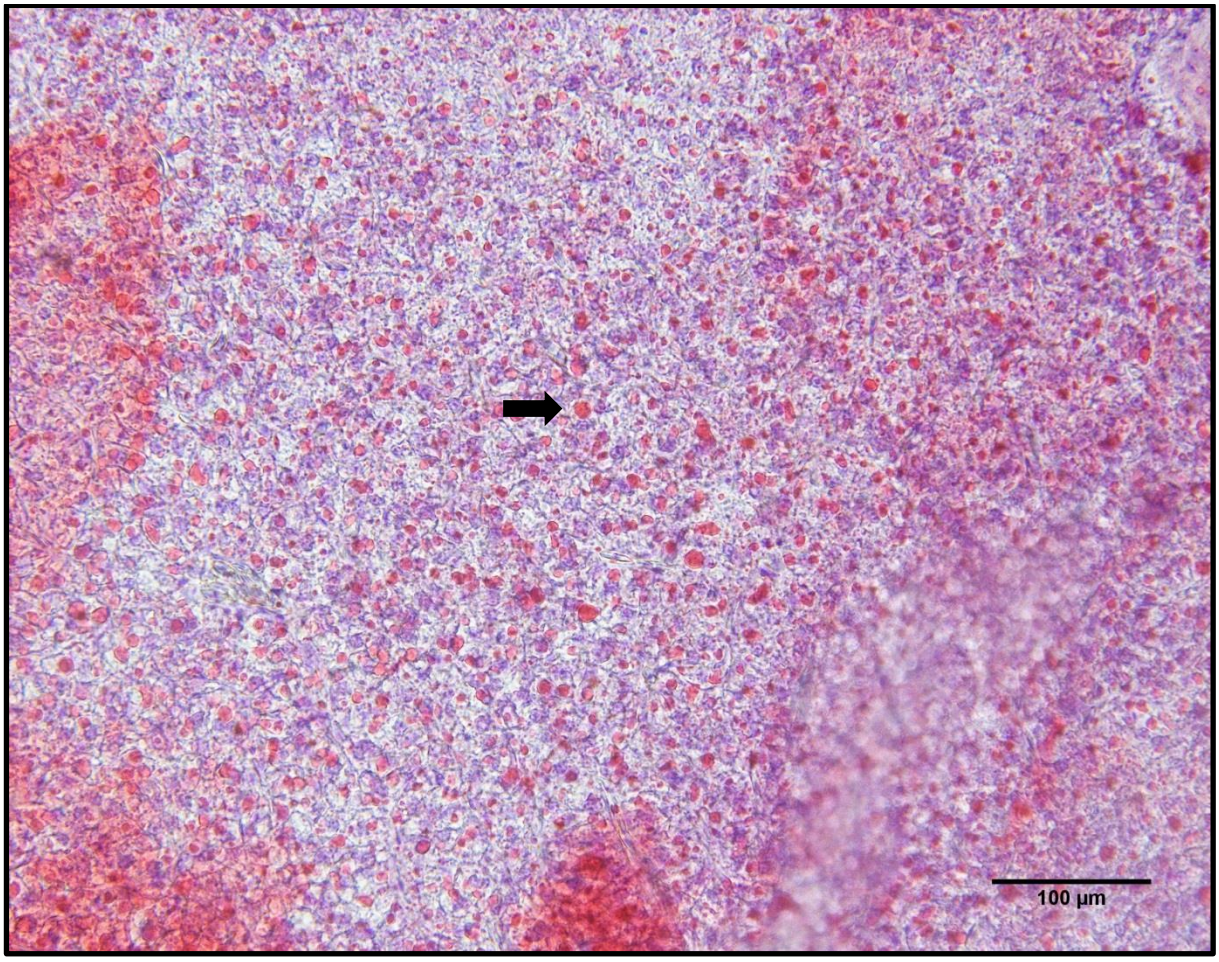

Figure 5.2. Yellowtail Kingfish, *Seriola lalandi*, liver section after a 252-day fish meal replacement trial. Liver hepatocytes containing unsaturated neutral lipid deposition (*arrow*). Diet 1 contained 30% wild derived fish meal. 400x magnification. Oil Red O stain.

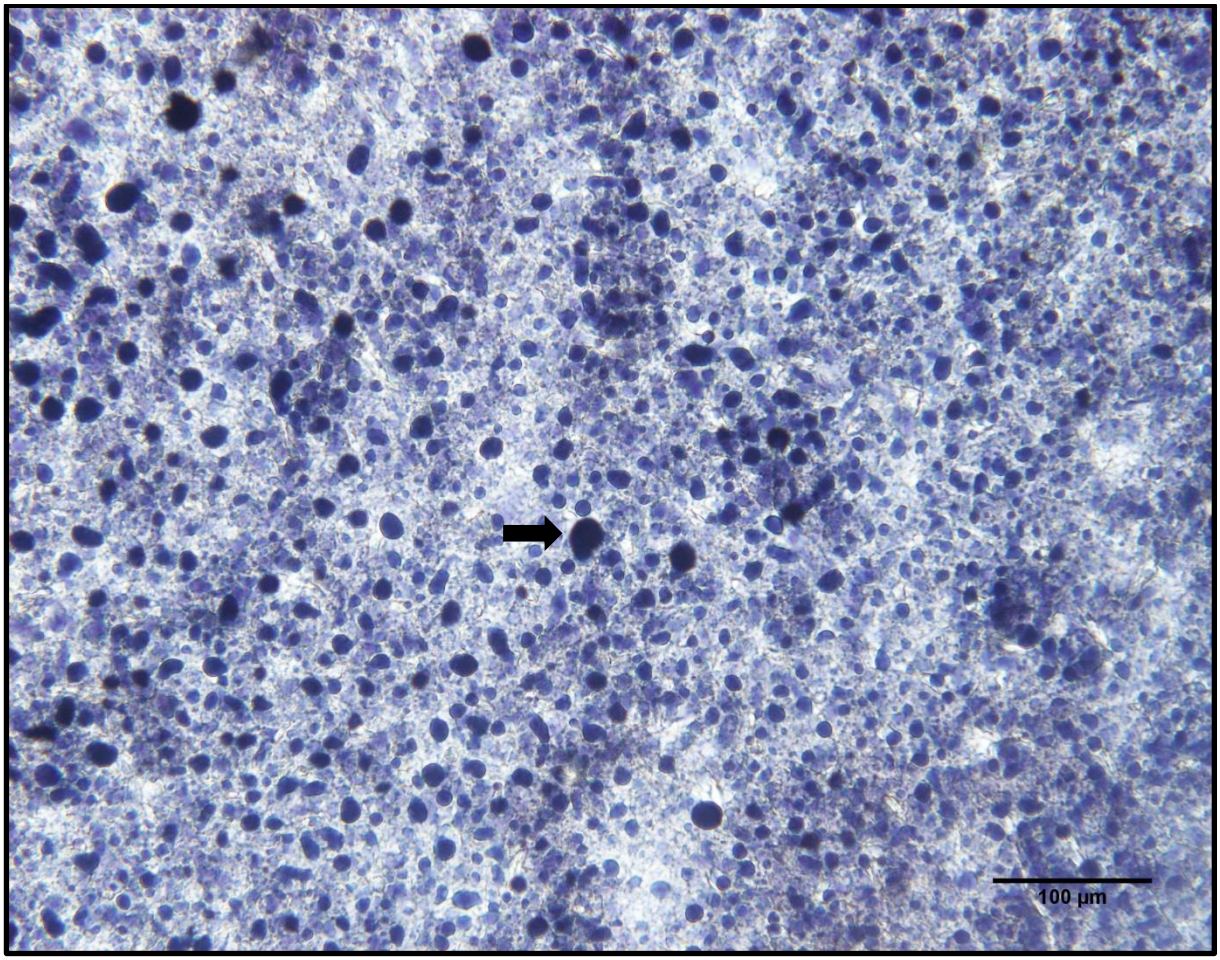

Figure 5.3. Yellowtail Kingfish, *Seriola lalandi*, liver section after a 252-day fish meal replacement trial. Liver hepatocytes containing total lipid deposition (*arrow*). Diet 1 contained 30% wild derived fish meal. 400x magnification. Sudan Black B stain.

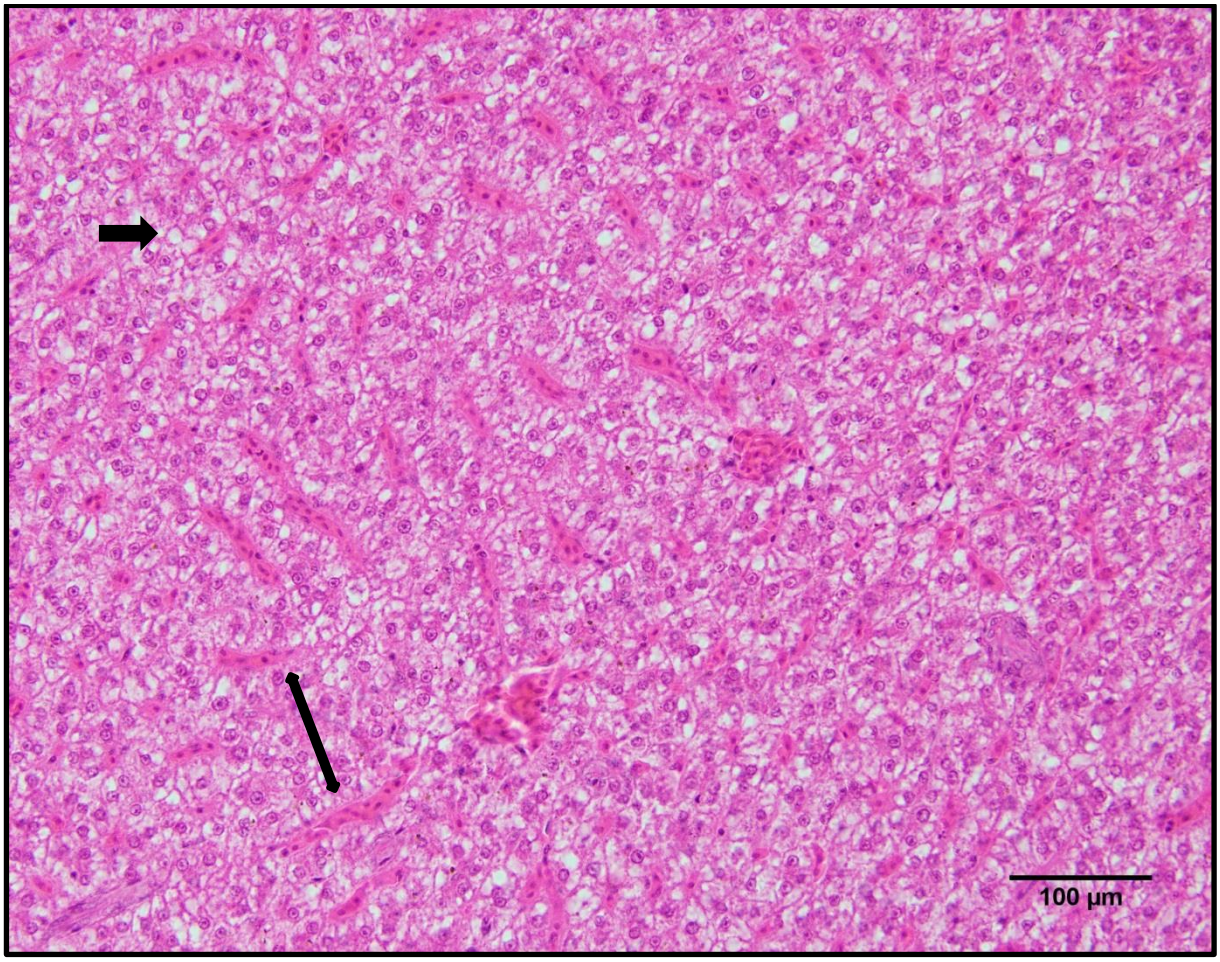

Figure 5.4. Yellowtail Kingfish, *Seriola lalandi*, liver section after a 252-day fish meal replacement trial. Liver hepatocytes displaying typical structure and vacuolar fat (compared to Diet 1 containing 30% wild derived fish meal) but overall less vacuolar fat (*arrow*) and slightly congested sinusoids (*double-headed arrow*). Diet 3 contained ~20% fish by-product meal. 400x magnification. Haematoxylin and eosin stain.

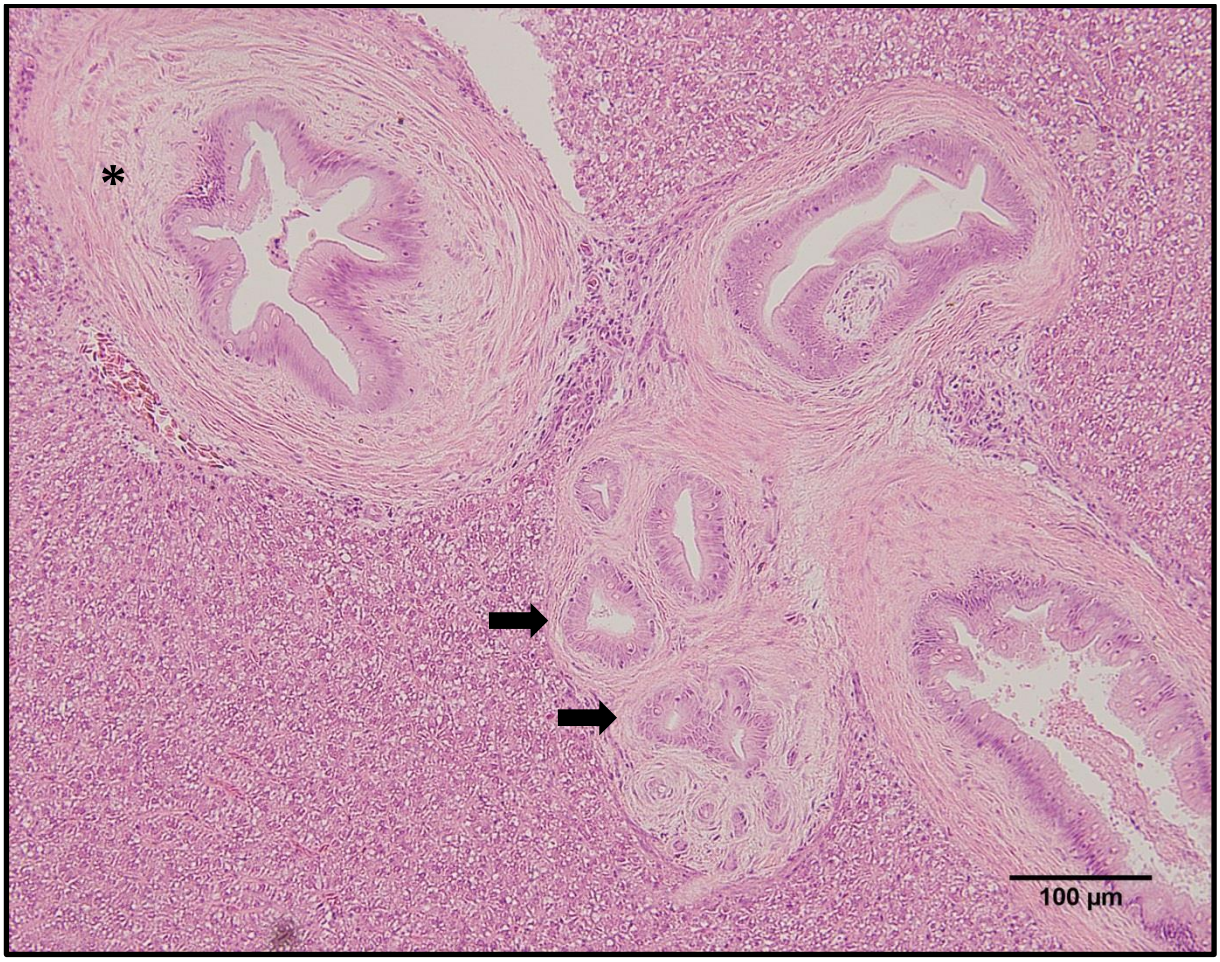

Figure 5.5. Yellowtail Kingfish, *Seriola lalandi*, liver section after a 252-day fish meal replacement trial showing severe bile duct proliferation (*arrows*) and periductular fibrosis (*asterisk*). Diet 4 contained ~10% poultry meal. 200x magnification. Haematoxylin and eosin stain.

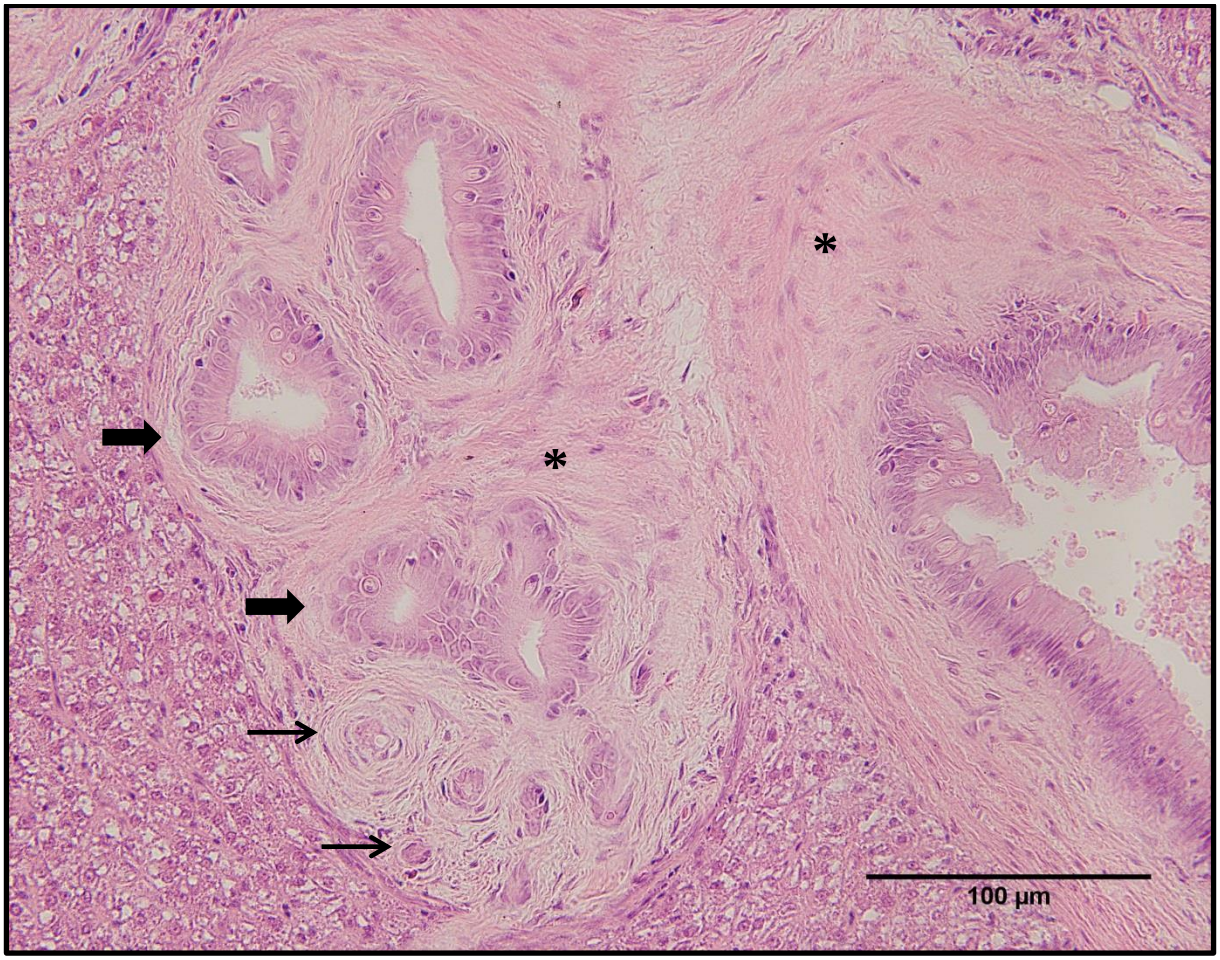

Figure 5.6. Yellowtail Kingfish, *Seriola lalandi*, liver section after a 252-day fish meal replacement trial showing severe bile duct proliferation (*arrows*) and periductular fibrosis (asterisk). Note newly forming bile ducts without lumen (*thin arrow*). Diet 5 contained ~10% soy protein concentrate. 400x magnification. Haematoxylin and eosin stain.

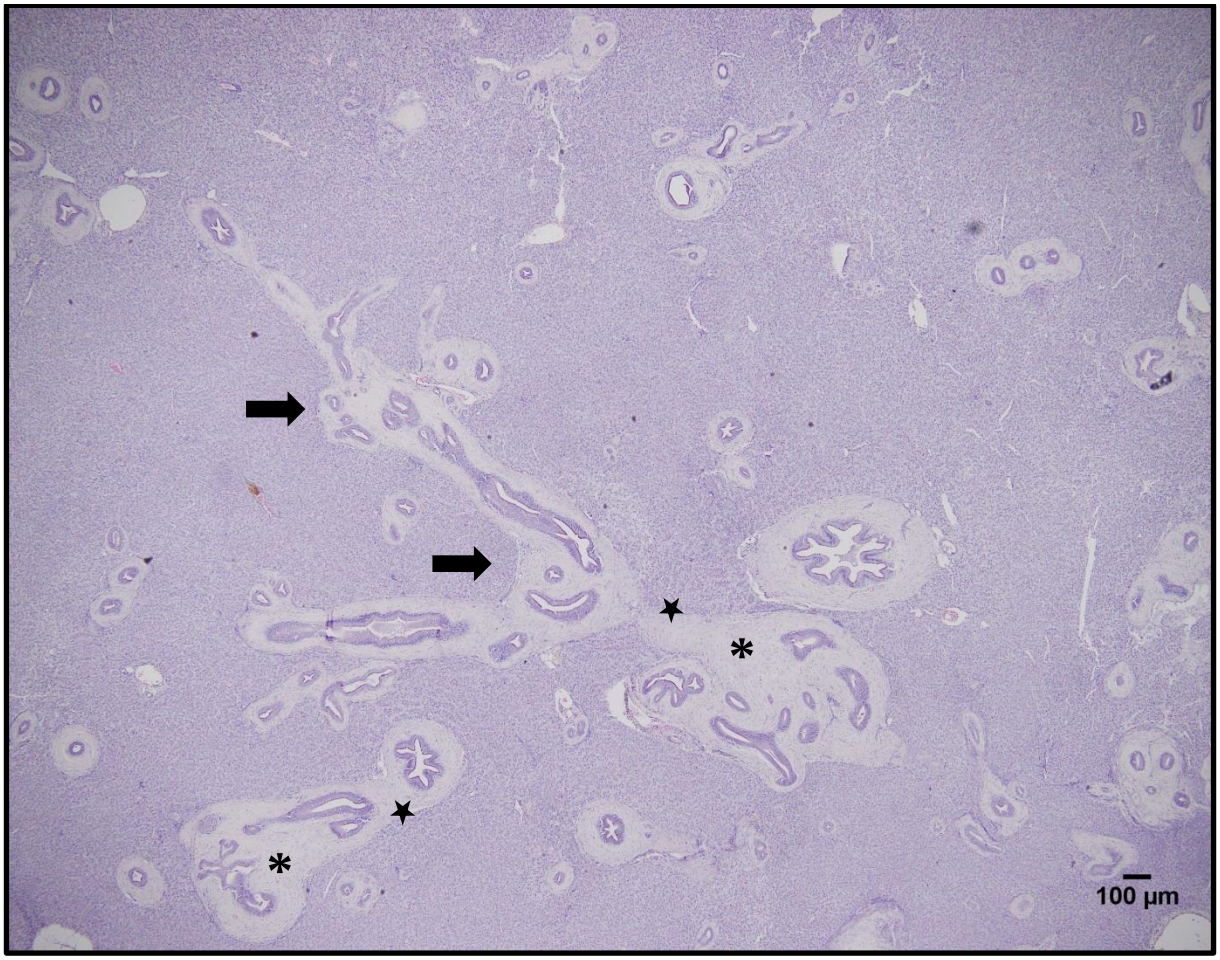

Figure 5.7. Yellowtail Kingfish, *Seriola lalandi*, liver section after a 252-day fish meal replacement trial showing severe bile duct proliferation (*arrows*) and periductular fibrosis (*asterisk*) characterised by sclerosing fibrous layering around the bile duct giving an "onion-skin" appearance. A fibrous sheath and the fibrous bridging (star) connected multiple bile ducts. Diet 5 contained ~10% soy protein concentrate. 40x magnification. Haematoxylin and eosin stain.

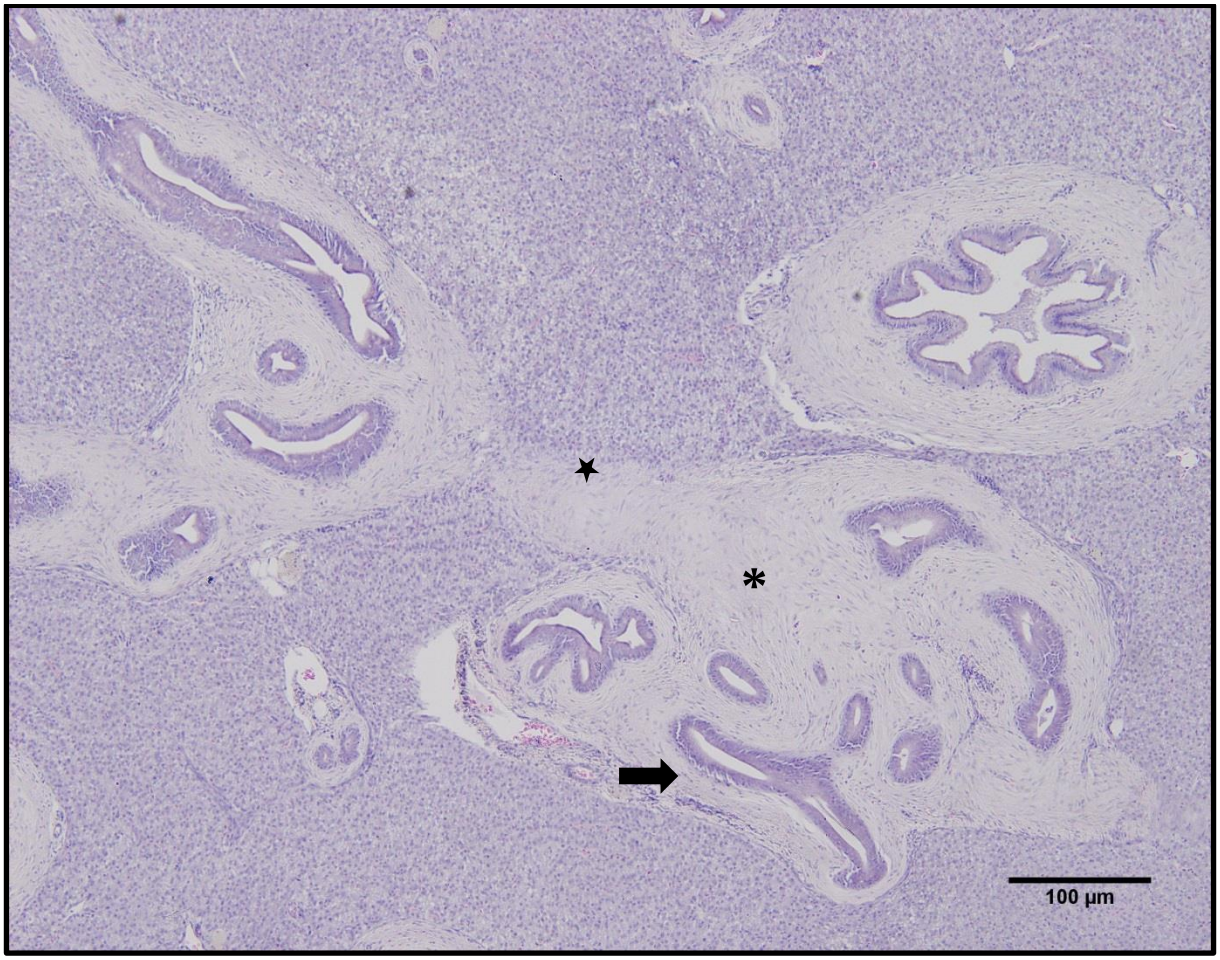

Figure 5.8. Yellowtail Kingfish, *Seriola lalandi*, liver section after a 252-day fish meal replacement trial showing severe bile duct proliferation (*arrow*) and periductular fibrosis (*asterisk*) characterised by an "onion-skin" type fibrous layer around the bile duct. Fibrous sheath and the fibrous bridging connected (*star*) multiple bile ducts. Diet 5 contained ~10% soy protein concentrate. 200x magnification. Haematoxylin and eosin stain.

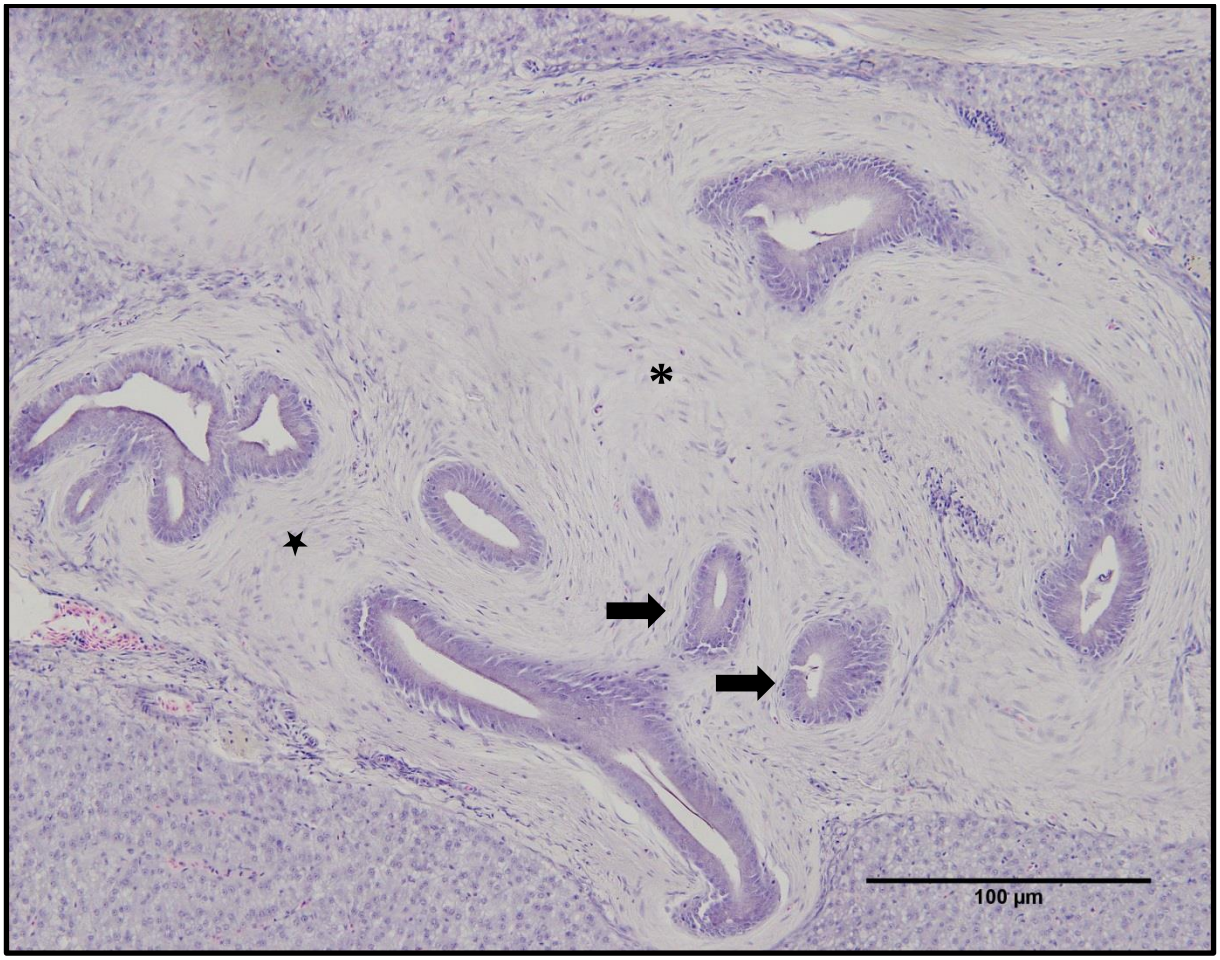

Figure 5.9. Yellowtail Kingfish, *Seriola lalandi*, liver section after a 252-day fish meal replacement trial showing severe bile duct proliferation (*arrow*) and periductular fibrosis (*asterisk*) characterised by an "onion-skin" type fibrous layer around the bile duct. Fibrous sheath and the fibrous bridging (*star*) connected multiple bile ducts. Diet 5 contained ~10% soy protein concentrate. 400x magnification. Haematoxylin and eosin stain.

5.4.5 Biochemical analyses

5.4.5.1 Total bile acid concentration

5.4.5.1.1 Increasing wd-FM replacement with fish by-product meal

Increasing wd-FM replacement with FBM had no significant effect on the total bile acid concentration in tissue samples from the liver (P = 0.249), gallbladder content (P = 0.402), foregut (P = 0.914), midgut (P = 0.178), hindgut (P = 0.353) or faeces (P = 0.298; Table 5.4A). Similarly, regression analyses showed no significant relationship between increasing wd-FM replacement with FBM and total bile acid concentration in tissue samples from the liver (r^2 = 0.350; P = 0.093), gallbladder content (r^2 = 0.019; P = 0.722), foregut (r^2 = 0.027; P = 0.913), midgut (r^2 = 0.017; P = 0.735), hindgut (r^2 = 0.001; P = 0.933) or faeces (r^2 = 0.293; P = 0.733; Table 5.4A).

Table 5.4A. Total bile acid and taurine concentrations from sampling points within the enterohepatic system of Yellowtail Kingfish, *Seriola lalandi*, fed increasing wild derived fish meal replacement with fish by-product meal for 252 days.[1]

				ANOVA	Regression[†]	
Diet	1	2	3	*P value*[*]	r^2	*P* value
Total bile acid concentration (*μmoles* g^{-1})						
Liver	13.30±0.5	12.33±0.8	11.94±0.2	0.249	0.350	0.093
Gallbladder content	12.35±0.4	12.69±0.9	12.02±0.6	0.402	0.019	0.722
Foregut	10.68±1.2	10.57±0.6	10.17±0.8	0.914	0.027	0.913
Midgut	10.63±0.8	12.96±1.2	10.02±1.0	0.178	0.017	0.735
Hindgut	10.08±0.5	12.29±2.0	9.92±0.4	0.353	0.001	0.933
Faeces	37.17±1.0	27.86±2.8	25.80±8.0	0.298	0.293	0.733
Taurine concentration (*μmoles* g^{-1})						
Liver	0.06±0.03	0.06±0.03	0.10±0.01	0.450	n.d.	n.d.

[1] Values are mean ± SE; n = 3.

[*] A significance level of α = 0.05 was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.

[†] Regression values generated from treatment groups; n = 9.

5.4.5.1.2 Replacement of wd-FM with either fish by-product meal, poultry meal or soy protein concentrate

Replacement of wd-FM (Diet 1) with either ~10% FBM (Diet 2), PM (Diet 4) or SPC (Diet 5) had no significant effect on the total bile acid concentration in tissue samples from the liver (P = 0.434), gallbladder content (P = 0.402), foregut (P = 0.984), midgut (P = 0.100) or hindgut (P = 0.431; Table 5.4B). The post-hoc tests revealed no significant difference in the faecal total bile acids concentration between diets; although it appears that fish fed ~10% FBM (Diet 2) and PM (Diet 4) showed lower concentrations than fish fed wd-FM (Diet 1; P = 0.041; Table 5.4B).

Table 5.4B. Total bile acid and taurine concentrations from sampling points within the enterohepatic bile acid cycling and excretory system of Yellowtail Kingfish, *Seriola lalandi*, fed a wild derived fish meal diet partially substituted with fish by-product meal, poultry meal or soy protein concentrate on a digestible protein basis for 252 days.[1]

					ANOVA
Diet	1	2	4	5	*P value*[*]
Total bile acid concentration (*μmoles* g^{-1})					
Liver	13.30±0.5	12.33±0.8	11.82±0.6	12.93±0.7	0.434
Gallbladder content	12.35±0.4	12.69±0.9	12.27±1.0	14.27±9.5	0.402
Foregut	10.68±1.2	10.57±0.6	10.76±0.4	10.38±0.5	0.984
Midgut	10.63±0.8	12.96±1.2	10.29±0.3	10.09±0.5	0.100
Hindgut	10.08±0.5	12.29±2.0	10.30±0.4	10.19±0.3	0.431
Faeces	37.17±1.0	27.86±2.8	32.73±1.0	36.73±2.6	**0.041**
Taurine concentration (*μmoles* g^{-1})					
Liver	0.06±0.03	0.06±0.03	0.05±0.02	0.05±0.02	0.989

[1] Values are mean ± SE; n = 3.

[*] A significance level of α = 0.05 was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.

5.4.5.2 Taurine concentration

5.4.5.2.1 Increasing wd-FM replacement with fish by-product meal

Increasing wd-FM replacement with FBM had no statistically significant effect on the taurine concentration of liver tissue (P = 0.450; Table 5.4A). A numerical increase in taurine concentration in liver tissue of fish fed Diet 3 (10% wd-FM + 21.4% FBM) was observed compared to that of fish fed Diet 1 (30% wd-FM; Table 5.4A).

5.4.5.2.2 Replacement of wd-FM with either fish by-product meal, poultry meal or soy protein concentrate

No statistically significant differences were observed in the liver taurine concentrations between any of the fish fed diets with replacement of wd-FM (Diet 1) with either ~10% FBM (Diet 2), PM (Diet 4) or SPC (Diet 5; P = 0.989; Table 5.4B).

5.4.5.3 HMGCR and digestive processes enzymes

5.4.5.3.1 Increasing wd-FM replacement with fish by-product meal

Increasing wd-FM replacement with FBM had no statistically significant effect on either HMGCR concentration (P = 0.723) or CYP7A1 concentration (P = 0.177) of liver tissue (Table 5.5A). A numerical increase in CYP7A1 concentrations in liver tissue of fish fed Diet 1 (30% wd-FM) and Diet 3 (10% wd-FM + 21.4% FBM) was observed (Table 5.5A). Similarly, regression analyses showed no significant relationship between increasing wd-FM replacement with FBM and HMGCR concentration (r^2 = 0.084; P = 0.448) and CYP7A1 concentrations (r^2 = 0.101; P = 0.405) in liver tissue (Table 5.5A).

Table 5.5A. HMGCR and CYP7A1 concentrations of liver tissue from Yellowtail Kingfish, *Seriola lalandi*, fed increasing wild derived fish meal replacement with fish by-product meal for 252 days.[1]

				ANOVA	Regression[†]	
Diet	1	2	3	*P value*[*]	r^2	*P* value
HMGCR (µmoles g^{-1})	37.72±11.33	30.43±5.93	29.62±3.35	0.723	0.084	0.448
CYP7A1 (µmoles g^{-1})	1.23±0.11	0.98±0.04	1.11±0.08	0.177	0.101	0.405

[1] Values are mean ± SE; $n = 3$.

[*] A significance level of $P < 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.

[†] Regression values generated from treatment groups; $n = 9$.

5.4.5.3.2 Replacement of wd-FM with either fish by-product meal, poultry meal or soy protein concentrate

No statistically significant differences were observed in either HMGCR concentration (P = 0.720) or CYP7A1 (P = 0.059) concentration of liver tissue between fish fed diets with replacement of wd-FM (Diet 1) with either ~10% FBM (Diet 2), PM (Diet 4) or SPC (Diet 5; Table 5.5B). Although, an overall numerical decrease in CYP7A1 concentration was observed with the inclusion of a replacement of wd-FM (Diet 1) with either ~10% FBM (Diet 2), PM (Diet 4) or SPC (Diet 5; Table 5.5B).

Table 5.5B. HMGCR and CYP7A1 concentrations of liver tissue from Yellowtail Kingfish, *Seriola lalandi*, fed a wild derived fish meal diet partially substituted with fish by-product meal, poultry meal or soy protein concentrate for 252 days on a digestible protein basis.[1]

					ANOVA
Diet	1	2	4	5	*P value*[*]
HMGCR (µmoles g^{-1})	37.72±11.33	30.43±5.93	39.63±4.28	27.33±10.86	0.720
CYP7A1 (µmoles g^{-1})	1.23±0.11	0.98±0.04	1.06±0.08	1.06±0.04	0.439

[1] Values are mean ± SE; $n = 3$.

[*] A significance level of $\alpha = 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.

5.4.6 Blood analyses

5.4.6.1 Increasing wd-FM replacement with fish by-product meal

No statistically significant differences ($P > 0.05$) were observed between any of the blood biochemistry parameters measured from fish fed diets of increasing wd-FM replacement with FBM (Table 5.6A).

Table 5.6A. Blood biochemistry from Yellowtail Kingfish, *Seriola lalandi*, fed increasing wild derived fish meal replacement with fish by-product meal for 252 days.[1]

Diet	1	2	3	ANOVA (*P value*)[2]
Biochemistry				
Protein (g L^{-1})	37.7 ± 0.3	39.4 ± 2.3	39.3 ±1.2	0.662
Albumin (g L^{-1})	11.1 ± 0.3	11.6 ± 0.6	11.7 ± 1.2	0.623
Globulin (g L^{-1})	26.6 ± 0.1	27.9 ± 1.7	27.7 ± 0.9	0.688
Total Bilirubin (mmol L^{-1})	1.3 ± 0.2	1.7 ± 0.2	1.9 ± 0.1	0.145
ALT (IU L^{-1}) [3]	7.8 ± 0.9	7.9 ± 0.8	9.0 ± 1.2	0.639
ALP (IU L^{-1}) [3]	27.6 ± 2.4	29.4 ± 4.2	30.9 ± 0.4	0.713
Cholesterol (mmol L^{-1})	5.4 ± 0.2	5.8 ± 0.2	5.2 ± 0.3	0.270
Triglyceride (mmol L^{-1})	2.3 ± 0.3	2.5 ± 0.2	2.5 ± 0.1	0.788
Bile Acids (mmol L^{-1})	21.7 ± 15.6	12.1 ± 5.4	17.6 ± 12.0	0.851

[1] Values are mean ± SE; $n = 3$.
[2] A significance level of $\alpha = 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.
[3] ALT, alanine aminotransferase; ALP, alkaline phosphatase.

5.4.6.2 Replacement of wd-FM with either fish by-product meal, poultry meal or soy protein concentrate

No statistically significant differences ($P > 0.05$) were observed between any of the blood biochemistry parameters measured from fish fed diets with replacement of wd-FM (Diet 1) with either ~10% FBM (Diet 2), PM (Diet 4) or SPC (Diet 5; Table 5.6B).

Table 5.6B. Blood biochemistry from Yellowtail Kingfish, *Seriola lalandi*, fed a wild derived fish meal diet partially substituted with fish by-product meal, poultry meal or soy protein concentrate for 252 days on a digestible protein basis.[1]

Diet	1	2	4	5	ANOVA (*P value*)[2]
Biochemistry[3]					
Protein (g L^{-1})	37.7 ± 0.3	39.4 ± 2.3	40.0 ± 1.1	38.3 ± 0.8	0.618
Albumin (g L^{-1})	11.1 ± 0.3	11.6 ± 0.6	11.8 ± 0.2	11.3 ± 0.3	0.637
Globulin (g L^{-1})	26.6 ± 0.1	27.9 ± 1.7	28.2 ± 0.9	27.0 ± 0.5	0.642
Total Bilirubin (mmol L^{-1})	1.3 ± 0.2	1.7 ± 0.2	1.3 ± 0.2	1.2 ± 0.1	0.370
ALT (IU L^{-1})	7.8 ± 0.9	7.9 ± 0.8	9.1 ± 0.7	8.8 ± 0.2	0.493
ALP (IU L^{-1})	27.6 ± 2.4	29.4 ± 4.2	32.3 ± 2.5	26.0 ± 2.8	0.532
Magnesium (mmol L^{-1})	1.6 ± 0.1	1.6 ± 0.1	1.5 ± 0.0	1.6 ± 0.1	0.837
Cholesterol (mmol L^{-1})	5.4 ± 0.2	5.8 ± 0.2	5.3 ± 0.2	5.4 ± 0.1	0.300
Triglyceride (mmol L^{-1})	2.3 ± 0.3	2.5 ± 0.2	2.3 ± 0.3	1.8 ± 0.3	0.388
Bile Acids (mmol L^{-1})	21.7 ± 15.6	12.1 ± 5.4	14.4 ± 10.6	18.5 ± 23.2	0.910

[1] Values are mean ± SE; $n = 3$.
[2] A significance level of $\alpha = 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.
[3] ALT, alanine aminotransferase; ALP, alkaline phosphatase.

5.5 Discussion

The growth performance for this trial is reported by Stone and Booth (2018). In short, there were no significant differences in growth or feed utilisation between fish fed the different diets.

5.5.1 Liver histology and indices

5.5.1.1 Increasing wd-FM replacement with fish by-product meal

Historically by-products from aquatic species have been discarded as low value commodities. However, due to the increases in FM commodity prices, its replacement or partial substitution in formulated feeds with FBM is emerging as an economic and environmentally conscious practice (Turchini et al, 2009). Fish by-product meals are known to contain mineral, fatty acid and amino acid levels generally in the range of fish meals (Plante et al., 2017). In the present study, the replacement of up to 66.67% of dietary FM with FBM had no significant impact on

the fish liver health. Similarly, previous studies on Korean rockfish, *Sebastes schlegeli*, showed up to 75% of dietary FM can be replaced with a tuna FBM without impacting overall growth, indicating proper functionality of the digestive and excretory systems (Kim et al., 2018). The essential and non-essential amino acids profiles of different sources of FBM will result in varying profiles in the final feed delivered on farm (Plante et al., 2017). The geographical location, species source (cultured or wild) and quality of FBM (head, whole carcase, gonads or visceral content) must be a consideration when formulating diets.

In the present study, fish fed increasing wd-FM replacement by FBM had decreasing total lipid deposition in the liver. Histological observations revealed less vacuolar fat in the hepatocytes of fish fed diets containing ~10% FBM replacement (Diet 2) and ~20% FBM replacement (Diet 3) but similar structures to that of the control diet (Diet 1; 30% wd-FM). Along with no significant difference in HSI or VSI, this indicates that fish fed diets with increasing levels of FM replacement by FBM may have reduced ability to maintain energy storage levels within hepatocytes.

5.5.1.2 Replacement of wd-FM with either fish by-product meal, poultry meal or soy protein concentrate

Severe bile duct proliferation and periductular fibrosis was observed in the livers of fish fed the ~10% PM replacement (Diet 4) and ~10% SPC replacement (Diet 5) diets. Varying degrees of bile duct proliferation and periductular fibrosis are observed in wild Yellowtail Kingfish but what is considered to be normal fibrous layering for *Seriola* species is still being established (Chapter 2). Despite this, the connective tissue sheaths that surrounded the bile ducts and veins or arterioles of fish in the present trial were increased compared to Diet 1 (30% wd-FM). Likewise, these connective tissue sheaths were greater in comparison to known examples of wild and cultured *Seriola* species and wild yellowtail flounder, *Laimanda ferruginea* and

rainbow trout, *Salmo gairdneri* Richardson (Chapter 2; Hampton et al., 1989; Fahraes-Van and Spurell, 2003; Crowe et al., 2021). Furthermore, a greater number of newly forming bile ducts without lumen were present in fish fed ~10% PM replacement (Diet 4) and ~10% SPC replacement (Diet 5) diets in regions already characterised by duct proliferation and periductular fibrosis. Newly forming bile ducts are a part of normal liver growth but the liver's requirement for more bile ducts in these regions may indicate that the established ducts are not functioning as required.

Liver taurine concentrations in fish fed diets with marine ingredients were consistent with those observed in wild Yellowtail Kingfish, whilst fish fed diets containing terrestrial proteins were marginally less (Chapter 2). Studies on several species have indicated that bile acids may only conjugate with taurine in teleosts (Vessey et al., 1990; Kim et al., 2008). Taurine supplementation at varying inclusion levels, is reported to improve the growth performance of Cobia, *Rachycentron canadum*, Red sea bream, *Pagrus major*, and several *Seriola* spp. when fed diets with terrestrially sourced ingredients as the main lipid and protein sources (Nguyen et al., 2011; El-Sayed, 2014). Taurine supplementation was equally added to each diet; however, terrestrial ingredients replacing the marine component in the diets could have reduced the overall dietary taurine. Reduced taurine can lead to an increase in unconjugated bile acids being more hydrophobic in nature increasing their ability to enter cells and cause apoptosis (Rhodes et al., 2016). Furthermore, reduced availability of dietary taurine for bile acid conjugation can results in poor lipid emulsification, absorption and metabolism of dietary lipids (Bowyer et al., 2012b).

Deficient absorption of dietary long chain essential fatty acids may have promoted periductular fibrosis and bile duct proliferation in the Yellowtail Kingfish of the present trial. Similar bile duct proliferation and fibrosis has been observed in sablefish, *Anoplopoma fimbria*, fed diets

containing primarily terrestrial plant proteins and corn oil and flaxseed oil replacing the fish oil component of the diet (Rhodes et al., 2016). This may be species-specific as Atlantic salmon fed PM as a partial FM replacement at an inclusion up to 200 g kg^{-1} did not show differences in their liver histology (Hartviksen et al., 2014).

5.5.2 Total bile acid concentration

Assessment of enterohepatic tissues in fish fed each of the diets revealed consistent total bile acid concentrations within range or marginally less than those observed in wild Yellowtail Kingfish (Chapter 2). The bile acid concentrations in faeces of wild Yellowtail Kingfish are currently unknown. Although not statistically significant, the total bile acid concentration within the faeces did differ between several diets. When compared to Diet 1 (30% FM), Diet 2 (~10% FBM) and Diet 3 (~20% FBM) may have had less dietary cholesterol available for bile acid synthesis leading to reduced bile acid levels in the faeces.

Similarly, Diet 2 (~10% FBM) and Diet 4 (~10% PBM) liver total bile acid concentrations were reduced compared to that of Diet 1 (30% FM) and Diet 5 (10% SPC). Similar to Diet 2 (~10% FBM), Diet 4 (~10% PBM) faecal total bile acids concentrations are most likely less due to having reduced dietary cholesterol available for bile acid synthesis creating more need to retain them. In fish fed Diet 1 (30% FM), a greater production of bile acids may have been the result of increased dietary cholesterol and therefore, excess above metabolic requirements were removed via faeces. However, unlike Diet 1 (30% FM), the increase in faecal bile acid concentrations of Diet 5 (10% SPC) are likely due to increased dietary phytosterols competing for micellular position leading to increased faecal excretion (Chapter 3). When fed soybean protein-based diets reduced liver total bile acid concentrations have been reported for rainbow trout, *Oncorhynchus mykiss,* and yellowtail, *Seriola quinqueradiata* (Yamamoto et al., 2007; Nguyen et al., 2011). Atlantic salmon fed diets with soybean protein inclusion have inhibited

biosynthesis of bile in- and secretion from- the liver and impairment of circulation of bile via the enterohepatic system (Yamamoto et al., 2007).

In this study, fish were sampled 24 h after feeding, therefore, these observations may be of a resting pool of bile acids within the blood and tissues of different organs of the enterohepatic system. As the resting pool of bile acids was constant in other enterohepatic tissues, an adequate level of dietary cholesterol or *de novo* synthesis may have been supplied for general metabolic function. This is despite the measured excretion of bile acids in the faeces, whether from metabolic excess or micellular competition.

Animal cells are rich in cholesterol but plant cells are void of cholesterol and rich in other phytosterols (Lecerf and de Logeril, 2011). Fish acquire cholesterol from their diet or can synthesise it *de novo* in the liver and synthesis is up-regulated when dietary intake does not meet metabolic demand (Lecerf and de Logeril, 2011; Kortner et al., 2013). If *de novo* cholesterol is required HMGCR genes are up-regulated and control the committed step of mevalonate synthesis the precursor in cholesterol synthesis (Maita et al., 2006; Kortner, et al., 2013). In wild Yellowtail Kingfish HMGCR enzyme concentration can range between 2.40 – 9.91 µmoles g^{-1} (Chapter 2). For all treatment groups in the present study the HMGCR concentration was much greater than that seen in wild counterparts. Therefore, when compared to the wild counterparts, this indicates that all fish in the present trial required *de novo* cholesterol synthesis regardless of diet. However, this also indicated that it is highly likely that the combination of *de novo* cholesterol synthesis and the dietary cholesterol supplied in the diets were at an adequate level for the animals to maintain a suitable level for metabolic processes.

5.5.3 HMGCR and CYP7A1 enzymes

Aquaculture is foremost about producing a healthy, fast growing animal. If the level of dietary cholesterol supplied by the FM component of each diet was suitable for consistent growth and partial substitution of FM did not influence the HMGCR concentration, then the reduced HMGCR is of no consequence from a production perspective. Conversely, if the growth rate of the animal is suppressed compared to the control diet (Diet 1; 30% wd-FM) then reduced HMGCR concentration is indicative that precursors for cholesterol synthesis were not present or HMGCR concentration was suppressed by other mechanisms. Reduced HMGCR concentrations in livers of fish fed diets with partial substitution by SPC may be due to anti-nutritional factors including reduced palatability and increases undigested molecular fractions binding to bile acids which increases faecal steroid excretion (Sugano et al., 1988; Francis et al., 2001; Chen et al., 2011; Lim et al., 2011; Nguyen et al., 2011; Collins et al., 2012). This is further explanation for increased bile acid concentration observed in faeces of fish fed diets partially substituted with SPC (Diet 5; ~10% SPC).

Excess cholesterol stimulates CYP7A1 enzyme activity in the liver which controls cholesterol catabolism and its conversation for bile acids synthesis (Lefebvre et al., 2009; Crosignani et al., 2011). Liver CYP7A1 enzyme concentrations fish fed all diets were lower than that of the control diet (Diet 1; 30% wd-FM). Faecal total bile acids concentrations in fish fed the SPC diet (Diet 5; ~10% SPC) were similar to those of the control diet (Diet 1; 30% wd-FM). However, the concentration of CYP7A1 in fish fed the SPC diet (Diet 5; ~10% SPC) was similar to all other treatment groups. This suggests that fish fed diets with SPC inclusion were receiving enough cholesterol for metabolic processes from the FM component but any cholesterol that could have been utilised for growth was being excreted from the body (Sugano et al., 1988; Nguyen et al., 2011). Yamamoto et al. (2007) suggested soybean meal and alcohol extracts of soybean meal led to reduce fat digestibility by impairment of lipid metabolism from

inhibition of bile salts which activate the lipase-colipase system. This ultimately led to inferior feed utilisation and growth. Further investigation into the effect of SPC inclusion on the enterohepatic system is necessary if being utilised in formulated feeds for Yellowtail Kingfish.

5.5.4 Blood analyses

All blood parameters were similar between fish fed the different diets. Combined, interpretation of bilirubin and albumin concentrations is a relative indicator of liver synthetic function and overall nutritional status with increased levels indicating liver dysfunction (Hoboken, 2008; Lee et al., 2009). By comparison, albumin concentrations were lower and bilirubin concentrations were greater in fish fed each of the experiment diets compared to levels observed in wild Yellowtail Kingfish. Wild Yellowtail Kingfish have albumin concentration of 15.00 ± 1.15 g L^{-1} (mean ± SD) and bilirubin levels under the range of the laboratory test's limit of detection (Chapter 2). Satiation trials on cultured Japanese yellowtail, *S. quinqueradiata*, show albumin concentrations up to 20.00 ± 2.00 g L^{-1} (mean ± SD) and bilirubin concentration of 4.00 ± 1.00 g L^{-1} (mean ± SD; Kawanago et al., 2014). Total bilirubin levels in the present trial were above that of wild counterparts but below that observed in this cultured, closely related Japanese yellowtail. Overall, the liver synthetic function of fish in the present trial was reduced compared to wild Yellowtail Kingfish (Chapter 2). However, for the period of this study the fish maintained adequate growth and health status indicating an equally adequate liver synthetic function (Stone and Booth, 2018).

Liver and biliary tract cells require alkaline phosphatase (ALP) for normal function (Kawanago et al., 2014). Elevated ALP is an indicator of cell injury and cholangitis (Higgins, 2013; Nakanuma et al., 2017). The ALP blood concentrations of fish from all treatment groups were consistent with that observed in wild Yellowtail Kingfish at 31.15 ± 10.53 IU L^{-1} (mean ± SD) and cultured Japanese yellowtail at 28.2 ± 4.3 – 41.6 ± 6.3 IU L^{-1} (mean ± SD; Chapter 2;

Kawanago et al., 2014). Nutritional status can also be determined by hepatic aminotransferase (ALT) with increase often observed in carnivorous fish fed high protein-low carbohydrate diets (Metón et al., 2015). The ALT blood concentration of fish from all treatment groups of the present trial were considerably less compared to wild Yellowtail Kingfish at 35.57 ± 16.56 IU L^{-1} (mean ± SD) and similar to that of cultured Japanese yellowtail which had ALT concentrations of 6.7±1.5 – 9.0±2.8 IU L^{-1} (mean ± SD; Chapter 2; Kawanago et al., 2014). Reduced ALT activity is observed within cultured carnivorous fish fed diets containing increased levels of carbohydrates (Metón et al., 2015). Increased carbohydrates are inevitably found in formulated feeds compared to natural diets of Yellowtail Kingfish due to their contribution to pellet binding in the manufacturing process (Booth et al., 2013).

Plasma cholesterol and triglyceride concentration are a combination of dietary cholesterol and *de novo* cholesterol synthesised in the liver (Maita et al., 2006). Substitution of FM with terrestrial proteins has been reported to reduce plasma cholesterol and triglyceride concentrations (Yamamoto et al., 2007; Morais et al., 2011). Irrespective of the diet, fish plasma cholesterol and triglyceride concentration were similar to the control diet (Diet 1; 30% FM). Wild Yellowtail Kingfish have mean cholesterol concentrations of 9.03 ± 0.59 mmol L^{-1} (mean ± SD), approximately twice that observed for fish within this study and mean triglyceride concentrations of 3.37 ± 1.18 mmol L^{-1} (mean ± SD; Chapter 2). Interestingly, SPC substitution at a level of 33.33% (Diet 5; ~10% SPC) did not reduce plasma cholesterol or triglyceride levels compared to the control. However, feed was withheld from experimental animals for 24 h prior to sampling thus fish may have entered into the pre-prandial phase whereby the body is maintaining the plasma cholesterol or triglyceride levels.

Blood bile acid concentrations from fish fed each of the diets were not significantly different from one another and were all above wild Yellowtail Kingfish mean reported at 8.01 ± 7.84

mmol L^{-1} (mean ± SD; Chapter 2). Previously we showed that increased dietary lipid level increases blood bile acid concentration in Yellowtail Kingfish (Chapters 2, 3 and 4). Regular feeding of experiment fish may explain elevated bile acid concentrations as adequate quantities of essential amino acids and LC-PUFA were available despite the reduction in FM with terrestrial ingredients.

5.6 Conclusion

At the water temperatures (16.7 ± 2.8 °C) recorded in this study, Yellowtail Kingfish, fed a 30% wd-FM diet may have the FM replaced by up to 66.67% FBM, 33.3% PM or 33.3% SPC without any significant impact on total bile acid synthesis, storage or excretion. Likewise, hepatocyte vacuolisation, unsaturated neutral lipid storage and total lipid storage within hepatocytes are unaffected by dietary FM replaced with up to 66.67% FBM, 33.3% PM or 33.3% SPC. Yellowtail Kingfish producers may be able to reduce the level of wd-FM in formulated diets by substitution with FBM, PM and SPC without impacting factors measured in the current study, however, the long-term feeding of diets containing PM and SPC to Yellowtail Kingfish and the effect this has on their liver function and structure should be closely monitored. These fish have nutritional plasticity enabling them to grow and maintain enterohepatic function on a variety of diet formulations. However, fish fed PM and SPC have livers that presented overall less homogenous hepatocyte distribution and less consistently positioned cuboidal cholangiocytes, severe bile duct proliferation and periductular fibrosis compared around bile ducts compared to fish fed wd-FM and FBM. The liver is the most important organ for cholesterol regulation and synthesis of bile that enables emulsification and uptake of nutrients. Therefore, should an adverse environmental or biological event occur challenging the animals' health further, the impacts this might also have on the liver or whole animal growth and health are unknown. Reduction of dietary wd-FM levels in commercial

production diets with FBM, PM or SPC may have desired effects beyond those examined in this experiment and should also be investigated.

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Chapter 6: Investigating liver structure and function in Yellowtail Kingfish, *Seriola lalandi*, in response to diet protein and lipid level manipulation

6.1 Abstract

Protein sparing has become an established practice in feed manufacturing where relatively inexpensive ingredients, such as lipids, are incorporated to reduce or 'spare' expensive protein ingredients. Incorrect lipid source, fatty acid profile or dietary inclusion level may produce inadequate energy levels required for maintaining animal health and growth. Changes in either protein or lipid level or both have the potential to reduce essential fatty acids, amino acids, and other nutrients such as the cholesterol required for adequate cell function and growth. Bile acid synthesised from cholesterol assists in the facilitation of the micellar solubilisation, esterification, emulsification and digestion of long chain fatty acids and may be reduced by altering lipid and protein levels. In this 84-day study, Yellowtail Kingfish, *Seriola lalandi* (2.13 ± 0.23 kg, mean body mass ± SD; 504 ± 19 mm, fork length ± SD) were fed six different diets formulated on a digestible basis that contained highly palatable and digestible ingredients at realistic commercial inclusion levels. Diets were formulated to graded crude protein levels (40, 44 and 48%) and either 25% or 30% crude lipid. Fat deposition in the liver and viscera was similar between all treatment groups. Lipid and glycogen storage were not significantly affected by crude protein or crude lipid level. However, a negative relationship between increased crude protein and hepatocyte vacuolisation was observed. Crude protein did not influence the bile acid concentrations in digestive tissues, whereas diets with a 30% crude lipid level significantly decreased ($P < 0.05$) total bile acid concentration of gallbladder content compared to that of fish fed diets with 25% crude lipid level, but no other digestive parameters were affected. Fish fed a dietary crude lipid level of 25% had significantly elevated ($P < 0.05$) taurine concentrations in the liver compared with fish fed diets containing 30% crude lipid level. Liver 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) concentration of fish fed diets containing 40% crude protein level was significantly reduced ($P < 0.05$) compared to fish fed diets containing 48% crude protein levels. Total bilirubin of fish fed 48% CP was

significantly less ($P < 0.05$) than that of fish fed 44% CP but not fish fed 40% CP, which were significantly similar to that of fish fed both 44% CP and 48% CP. At the water temperatures recorded in this study, Yellowtail Kingfish may be able to be fed diets with up to 30% CL and 48% CP without affecting total bile acid synthesis, storage and liver lipid storage.

6.2 Introduction

Optimal levels of dietary protein and lipid are critical for maintaining growth and health of a cultured marine species (Tocher, 2003). Over several decades, 'protein sparing' has become an established practice in feed manufacturing, whereby relatively inexpensive ingredients are incorporated to reduce or 'spare' more expensive protein ingredients (Francis and Turchini, 2017). Fish oil is in continuous demand with the increasing commodity price reflecting its limited supply (Francis and Turchini, 2017; FAO, 2020a). This has led to fish oil substitution and reduction being high priority areas for feed development within the aquaculture industry (Gatlin et al., 2007; Stone and Bowyer, 2013; Stone et al., 2016). Incorrect lipid source, fatty acid profile or insufficient dietary inclusion level may produce inadequate energy levels required for maintaining animal health and growth, resulting in expensive protein being utilised for energy (Stone and Bowyer, 2013). Conversely, high lipid diets can lead to extensive accumulation of intracytoplasmic lipid droplets with varying ranges of hepatocyte size in both mammal and teleost species, known as steatosis (Scudemore, 2013; Crowe et al., 2021). Liver steatosis has been associated with the use of lipids exceeding the dietary requirements and being stored in the liver resulting in a limited or reduced growth of cultured species (Spisni et al., 1998; Kowalska et al., 2011; Twibell et al., 2017).

The nutritional value of a diet is only as good as the quality and source of ingredients it is made from and is only suitable if those ingredients meet the nutritional value for optimal growth of the target species. Changes in either protein or lipid level or both have the potential to reduce essential, amino acids, fatty acids and other nutrients such as cholesterol which is required for adequate cell function and growth. Cholesterol is essential for disease resistance, gonadal steroid hormones, cell membrane growth and function and necessary for bile acid production (Lecerf and de Logeril, 2011; Liland et al., 2013). Teleost fish can either acquire cholesterol

from the diet or can up-regulate *de novo* cholesterol synthesis in the liver when dietary intake does not meet metabolic demand, although at a reduced capacity compared to mammals (Lecerf and de Logeril, 2011; Kortner et al., 2013).

Bile acid synthesised from cholesterol assists in the facilitation of the micellar solubilisation, esterification, emulsification and digestion of long chain fatty acids (Van Waarde, 1988; Yamamoto et al., 2007; Nguyen et al, 2011; El-Sayed, 2014; Murashita et al., 2014; Zhou and Hylemon, 2014). The conversion of cholesterol into bile acids involves a series of enzymatic processes that occur in liver hepatocytes (Mohapatra and Mishra, 2011; Nguyen et al, 2011; Holm et al., 2013). Taurine is required for conjugation with bile before the resultant bile acid is stored in the gallbladder (Takagi et al. 2005; Nguyen et al., 2011). In both mammals and fish, cholesterol-7α-hydroxylase (CYP7A1) is considered the regulatory rate-limiting enzyme contributing to bile acid synthesis, by regulating the conversion of cholesterol to 7α-hydroxycholesterol (Kullak-Ublick et al., 2004; de Aguiar Vallim et al., 2013; Crosignani et al., 2011; Deng et al., 2014; Guerra-Olvera and Viana, 2015).

The Yellowtail Kingfish, *Seriola lalandi*, is a carnivorous marine species with a high metabolic demand (Bowyer et al., 2012). The Yellowtail Kingfish is cultured in Australia for its excellent meat quality and fast growth, reaching a harvest size of 3.0 – 3.5 kg in 18 – 24 months (Miegel et al., 2010; Bowyer et al., 2012). Feed development is a high priority for the for Australian Yellowtail Kingfish aquaculture industry. The aim of this study was to understand the effect that graded protein levels and graded lipid levels have on liver structure and function through cholesterol and bile acid metabolism in sub-adult Yellowtail Kingfish at warm water temperatures.

6.3 Methods

6.3.1 Experimental description

In this study, commercial diets with increasing dietary protein with either a high or low lipid level (Table 6.1) were used to understand the effect on liver structure, liver enzyme concentration, blood biochemistry, growth and health of Yellowtail Kingfish. This was achieved by measuring the concentration of rate limiting enzymes for cholesterol and bile acid synthesis in the liver. Total bile acid concentrations of liver, gallbladder content, foregut, midgut and hindgut were determined using a fluorometric assay. Histological samples of the liver were observed for potential structural changes and differentially stained for indication of neutral and total lipid as well as glycogen deposition. Blood samples were also taken to observe biochemical changes that may have been induced by the different treatments. The experiment was conducted over 84 days at the South Australian Research and Development Institute (SARDI) South Australian Aquatic Sciences Centre (SAASC) at West Beach, Adelaide, Australia. At the commencement of the experiment, individual fish were weighed (kg) and measured for fork length (mm) after the fish had been anaesthetised in seawater using AQUI-S® (AQUI-S® New Zealand Ltd.) at a concentration of 0.02 mL L^{-1}. A total of 360 fish (body mass 2.13 ± 0.23 kg; fork length 504 ± 19 mm; mean ± SD) were randomly distributed between eighteen 5,000 L fibreglass tanks with 20 fish per tank. Each of the six treatments was randomly assigned to three replicate tanks. Fish were fed to satiation once daily at 09:00 h (Stone and Booth, 2018).

Table 6.1. The experimental diets of graded protein levels with low crude lipid level (Diet 1, 2 and 3) and high lipid level (Diet 4, 5 and 6) fed to Yellowtail Kingfish, *Seriola lalandi*, for 84 days.

Diet	1	2	3	4	5	6
Nominal crude lipid level (%)	25	25	25	30	30	30
Nominal crude protein level (%)	40	44	48	40	44	48
Analysed proximate composition (g 100 g^{-1})						
Moisture	6.8	5.7	6.0	5.6	4.6	4.5
Crude protein	38.5	42.9	46.4	40.5	44.2	46.9
Calculated digestible protein	34.2	36.9	40.0	33.8	37.0	40.1
Crude lipid	25.4	25.3	25.7	29.3	32.0	29.0
Calculated digestible lipid	24.9	24.4	24.3	29.8	29.8	29.8
Ash	7.6	7.3	6.9	6.9	6.8	6.6
Carbohydrate[1]	21.7	18.8	15.0	17.7	12.4	13.0
Gross energy (MJ kg^{-1})	19.7	19.9	19.9	20.8	21.4	20.9
Calculated digestible energy (MJ kg^{-1})	16.89	16.94	17.21	18.6	18.79	19.0
Protein:energy ratio (g CP MJ^{-1} GE)	19.5	21.6	23.3	19.5	20.7	22.4
Calculated digestible Protein:digestible energy ratio (g DP MJ^{-1} DE)	20.3	21.8	23.2	18.2	19.7	21.1
Analysed amino acids						
Taurine (g 100 g^{-1})	1.2	1.3	1.4	1.2	1.4	1.3
Cholesterol (mg 100 g^{-1})	290	250	280	270	280	330

[1] Carbohydrate = 100 - (moisture + lipid + protein + ash).

6.3.2 Experimental system

The recirculating aquaculture system (RAS) was kept undercover in ambient conditions with supplemental fluorescent lighting provided during the natural light period. Ambient temperature sea water was treated by settlement and sand filtration and circulated through the RAS. The sea water was then returned through filter-screen baffle-boards, pumped through a drum filter with 70 μm^2 filter pores (HDF1603 Hydrotech, Saint-Maurice, France), returned to the biofilter, and finally treated with UV light disinfection (D-32051 Wedeco, Herford, Germany) at a rate of 35,000 L h^{-1} by two electric centrifugal pumps (Grundfos, Regency Park,

Australia) before being returned to the tanks. One hundred percent of the water volume of each 5,000 L tank was replaced daily.

6.3.3 Fish source and acclimation

Sub-adult Yellowtail Kingfish, *Seriola lalandi*, were obtained from Clean Seas Pty. Ltd. sea cage facilities at Port Lincoln, Australia. The fish were acclimated for 4 weeks in 5,000 L fibreglass tanks supplied by the RAS at SARDI SAASC and fed a commercial diet (Ridley Corporation Ltd. Pelagica diet; gross energy, 19.3 MJ kg^{-1}, crude lipid [CL], 24%, crude protein [CP], 46%) before the commencement of the experiment.

6.3.4 Experimental diets

In this study, six treatments, referred to as Diet 1, Diet 2, Diet 3, Diet 4, Diet 5 and Diet 6, were formulated to three graded CP levels (40, 44 or 48%) and two CL levels (25% or 30%; Table 6.1). Diets were formulated with input from all project participants, and then manufactured by Ridley Corporation Ltd. (Narangba, Queensland, Australia). The proximate composition analyses of diets and whole body tissue were conducted according to methods in the British Pharmacopoeia Commission (2004) or German Institute for Standardization (DIN) (2000). A one kg sample of each diet was collected, ground and analysed for proximate composition (moisture, protein, fat, ash, carbohydrate and energy), taurine and cholesterol (Table 6.1). The six experimental diet ingredient formulations were commercially confidential however; the corresponding proximate diet composition, fatty acid profiles and proximate amino acid profiles are shown in Table 6.1.

6.3.5 Water quality

Water quality parameters were monitored daily, unless otherwise indicated. Temperature (°C, mean ± SD) and dissolved oxygen (% saturation, mean ± SD) were monitored using an OxyGuard Handy Polaris temperature and dissolved oxygen probe (OxyGuard International

A/S, Farum, Denmark). The pH (mean ± SD) was monitored using a Eutech pH Testr 30 multiparameter handheld probe (Eutech Instruments Pty. Ltd., Singapore). Salinity (g L^{-1}, mean ± SD) was monitored using an ISSCO UR-2 hand-held refractometer (model RF20; Extech Instruments Corporation, Nashua, New Hampshire, United States of America). The total ammonia concentration (mg L^{-1}) was determined weekly using a commercial water testing kit (Aquarium Pharmaceuticals, Chalfont, Pennsylvania, United States of America).

6.3.6 Sample collection

Feeding was stopped 24 h before the end of the experiment. Three fish from each tank were euthanized and measured for their body mass (nearest 0.1 g) and fork length (nearest 0.1 mm). The growth performance and feed utilisation for this trial was reported by Stone and Booth (2018). All data reported for each treatment for animal performance were based on the mean of the replicate tanks. All calculations using fish weight and diets are based on wet or as fed values, respectively:

Weight gain = final weight – initial weight

Specific growth rate (SGR; % d^{-1}) = ([ln final weight – ln initial weight] /d) x100

Condition factor (CF) = (fish body mass [g] / fish length $[cm]^3$) × 100

Apparent feed conversion ratio (FCR) = feed consumed / fish weight gain

Blood samples were collected from the caudal vasculature using 21 gauge needles and 5 mL syringes. For each fish, the visceral cavity was cut open and the whole removed gut weighed (nearest 0.01 g) to determine the visceral somatic index (VSI; %). The liver was removed and weighed (nearest 0.01 g) to determine the hepatosomatic index (HSI; %). These indices were calculated as follows:

VSI% = (visceral weight [g] / body mass [g]) × 100

HSI% = (liver weight [g] / body mass [g]) × 100

Liver, gallbladder content, foregut, midgut and hindgut were collected, immediately snap frozen in liquid nitrogen and stored at – 80 °C until measurement of total bile acid concentration and taurine concentrations. The liver samples were also used to measure the concentration of HMGCR and CYPA71 enzymes. Two 1 cm^3 sections of the left lobe of the liver were dissected and immediately placed into a histology cassette and fixed with 10% neutral buffered formalin (pH of 7.2) for 24 h for histological evaluation. One of the liver sections from each fish was used for standard paraffin embedding and then sectioned by rotary microtome, while the other was preserved in an 18% phosphate buffered saline (PBS) sucrose + 0.01% sodium azide (NaN_3) solution with pH 7.1 for cryostat sectioning.

6.3.7 Liver histology

Once fixed, one liver section from each fish was transferred to 70% (v/v) ethanol and stored at room temperature according to Hu et al. (2013). The sample was dehydrated using standard procedures, embedded in paraffin and sectioned at 5 μm on a rotary microtome. This section of liver from each fish underwent several staining techniques: haematoxylin and eosin (H&E) for quantitative assessments of the vacuole volume as a proportion of the liver cell volume (VPLC%) and tissue structure as per Crowe et al. (2021) and periodic acid Schiff (PAS) for glycogen deposition. The second liver section samples were sectioned at 10 μm on a cryostat microtome and mounted on polyethylenimine coated slides. This section of liver from each fish underwent several staining techniques: Oil Red O (ORO) for unsaturated neutral lipid deposition and Sudan Black B (SBB) for total lipid deposition. Stained sections were examined at 400-fold magnification using a light microscope (Olympus BF BX50). Three 559,390 μm^2 microphotographs were taken per stained slide using a digital camera (Olympus DP27). Quantitative assessments of the stained deposition volume as a proportion of the liver cell

volume was determined by applying stain sensitive colour thresholds using Fiji ImageJ processing software (National Institutes of Health, Bethesda, Maryland, United States of America).

6.3.8 Biochemical analyses

Samples from individual fish were partially thawed, weighed and homogenised in five volumes of PBS with a pH of 7.1. Homogenisation was performed using a Retch MM 400 ball mill at frequency 30.01 s^{-1} for 4 min with Biospec 2 mm zirconia beads (Catalogue No.11079124zx). The suspensions were centrifuged twice at an acceleration of 10,000 *g* for 10 min at 4 °C (Beckman Coulter Microfuge 16 centrifuge) with the supernatants being extracted to new microfuge tubes between cycles. All assay kits included internal standard solutions and manufacturer instructions were followed for assays. Tissue concentrations are reported per tissue weight (μmoles g^{-1}).

6.3.8.1 Total bile acid concentration

The liver, gallbladder content and foregut pooled supernatants were analysed for total bile acid concentrations by use of a commercial fluorometric bile acid assay kit by reading Ex/Em = 560/590 nm after 45 min of dark incubation at room temperature (Catalogue No. MET-5005; Cell Biolabs Inc.®, San Diego, California, United States of America).

6.3.8.2 Taurine concentration

The pooled liver supernatants were analysed for taurine by use of a commercial colorimetric taurine assay kit by reading samples at 405 – 415 nm (Catalogue No. MET-5070; Cell Biolabs Inc.®, San Diego, California, United States of America).

6.3.8.3 HMGCR and CYP7A1 enzyme-linked immunosorbent assays

The pooled liver supernatants were analysed for HMGCR concentration after reading the absorbance of samples at a wavelength of 450 nm (Catalogue No. CSB-E15772m; CusaBio, Texas, United States of America). The pooled supernatants were analysed for CYP7A1 concentration after reading the absorbance of samples at a wavelength of 450 nm (Catalogue No. CSB-EL006395FI; CusaBio, Texas, United States of America).

6.3.9 Blood analyses

Two mL of blood was placed into separate Vacuette® or BD vacutainer® tubes (Z serum clot activator or EDTA tubes) and stored indirectly on ice until same-day analysis of blood parameters by IDEXX Laboratories (Unley, South Australia, Australia). Specifically, the following parameters were observed: total bile acids, cholesterol, triglycerides and total bilirubin. Haematocrit values were obtained by centrifuging (Clements Orbital 160, Lidcombe, New South Wales, Australia) blood in pre-heparinised capillary tubes at 1,100 *g* for 1 min.

6.3.10 Statistical analyses

A two-way ANOVA analysis were used to assess the effects of the different diets. The normality of data was assessed using the Shapiro–Wilk test. Homogeneity of variances among means was assessed using Levene's test for equality of variance errors. The significance level was set at $\alpha = 0.05$ for all statistical tests. Where significant interactions were observed, the data were analysed using Tukey's Honestly Significant Difference (HSD) multiple range test. Where significant differences were observed for blood parameters, the data were analysed using Student Newman-Keul's post-hoc tests to detect differences between treatments. All data are presented as the mean ± the standard error (SE) of the mean, unless otherwise stated. IBM SPSS Version 20 for Windows (IBM SPSS Inc., Chicago, Illinois, United States of America) software was used for all statistical analyses.

6.4 Results

6.4.1 Water quality

Average values for water temperature, dissolved oxygen, pH and salinity were 21.7 ± 1.2 °C, 97.3 ± 6.9%, 7.94 ± 0.12 and 38 ± 0 g L^{-1}, respectively, across the study period. The ammonia concentration was found to be below the limits of detection on all occasions.

6.4.2 Performance indices

The growth performance and feed utilisation for this trial was reported by Stone and Booth (2018). In short, CP level did not significantly affect final weight ($P > 0.05$), SGR ($P > 0.05$), CF ($P > 0.05$) and FCR ($P > 0.05$) of Yellowtail Kingfish. These growth parameters were significantly influenced by CL level and the interaction between CP level and CL level ($P < 0.05$). Apparent feed intake (% BW d^{-1}) was significantly influenced by CP level ($P < 0.05$), CL level ($P < 0.05$) and their interaction ($P < 0.05$).

6.4.3 Somatic indices

Neither the CP level ($P = 0.443$) nor the CL level ($P = 0.105$) had a statistically significant effect on the HSI and no significant interaction was observed ($P = 0.912$; Table 6.2). Likewise, CP level ($P = 0.663$) and CL ($P = 0.392$) had no statistically significant effect on VSI and no significant interaction was observed ($P = 0.669$; Table 6.2).

Table 6.2. Somatic parameters of Yellowtail Kingfish, *Seriola lalandi*, experimental diets with graded protein and lipid levels for 84 days.[1]

Diet	1	2	3	4	5	6			
Nominal crude protein level (%)	40	44	48	40	44	48	2 factor ANOVA (*P value*)[2]		
Nominal crude lipid level (%)	25	25	25	30	30	30	Protein level (A)	Lipid level (B)	A × B
Visceral somatic index (VSI; %)	7.40±0.38	7.80±0.55	7.43±0.19	6.96±0.22	7.58±0.33	7.70±0.57	0.683	0.392	0.669
Hepatosomatic index (HSI; %)	1.42±0.12	1.34±0.05	1.22±0.04	1.44±0.03	1.43±0.11	1.27±0.13	0.443	0.105	0.912

[1] Values are mean ± SE; $n = 3$.
[2] A significance level of $\alpha = 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.

6.4.4 Liver histology

No interaction was observed between CP level and CL level in liver VPLC% (P = 0.464), neutral lipids (P = 0.361), total lipids (P = 0.963) or glycogen (P = 0.557; Table 6.3). Crude protein level did not significantly affect VPLC% (P = 0.056), neutral lipids (P = 0.470), total lipids (P = 0.330) or glycogen (P = 0.582; Table 6.3). Similarly, CL level did not significantly affect VPLC% (P = 0.681), neutral lipids (P = 0.666), total lipids (P = 0.919) or glycogen (P = 0.640; Table 6.3). The livers of fish fed all diets displayed typical hepatocyte structure. Bile ducts had consistent cuboidal cholangiocyte positioning surrounded by hepatocytes filled with vacuolar fat. The livers showed no visible signs of fatty liver disease or steatosis.

Table 6.3. Vacuole area and proportion of histology stain on liver sections of Yellowtail Kingfish, *Seriola lalandi*, fed experimental diets with graded protein levels containing two lipid levels for 84 days.[1]

Diet	1	2	3	4	5	6			
Nominal crude protein level (%)	40	44	48	40	44	48	2 factor ANOVA (P value)*		
Nominal crude lipid level (%)	25	25	25	30	30	30	Protein level (A)	Lipid level (B)	A × B
VPLC%†	43.3±3.6	39.7±0.8	35.8±4.6	44.3±1.5	46.4±5.3	32.2±5.5	0.056	0.681	0.464
Neutral lipids	14.2±4.9	10.7±3.7	10.9±1.1	10.4±1.2	14.7±2.6	7.6±2.2	0.470	0.666	0.361
Total lipids	93.5±1.9	96.4±0.5	93.9±1.3	93.7±0.5	95.7±2.0	93.9±2.7	0.330	0.919	0.963
Glycogen	0.1±0.0	0.2±0.2	0.2±0.1	0.2±0.1	0.1±0.0	0.3±0.2	0.582	0.640	0.557

[1] Values are mean ± SE; $n = 3$.

[*] A significance level of $\alpha = 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.

[†] Vacuole volume as a proportion of the liver cell volume (Crowe et al. 2021); Regression results level were significantly affected by crude protein inclusion (40% CP > 44% CP > 48% CP).

6.4.5 Biochemical analyses

6.4.5.1 Total bile acid concentration

No interaction was observed between CP level and CL level in total bile acid concentrations for liver (P = 0.746), gallbladder content (P = 0.288), foregut (P = 0.192), midgut (P = 0.764) or hindgut (P = 0.464; Table 6.4). Crude protein level did not significantly affect total bile acid concentrations for liver (P = 0.459), gallbladder content (P = 0.478), foregut (P = 0.428), midgut (P = 0.894) or hindgut (P = 0.655; Table 6.4). Similarly, CL level did not significantly affect total bile acid concentrations for liver (P = 0.426), foregut (P = 0.790), midgut (P = 0.572) or hindgut (P = 0.472; Table 6.4). However, fish fed diets containing 30% CL had significantly decreased total bile acid concentration of gallbladder content compared to that of fish fed diets with 25% CL level (P = 0.031; Table 6.4).

Table 6.4. Total bile acid and taurine concentrations from sampling points within the enterohepatic bile acid cycling and excretory system of Yellowtail Kingfish, *Seriola lalandi*, fed experimental diets with graded protein and lipid levels for 84 days.[1]

Diet	1	2	3	4	5	6			
Nominal crude protein level (%)	40	44	48	40	44	48	2 factor ANOVA (*P value*)[*]		
Nominal crude lipid level (%)	25	25	25	30	30	30	Protein level (A)	Lipid level (B)	A × B
Total bile acid concentration (µmoles g^{-1})									
Liver	15.04±1.2	18.22±2.7	17.92±2.7	15.13±1.0	15.47±0.7	16.84±1.9	0.459	0.426	0.746
Gallbladder content[†]	18.37±1.2	20.68±1.3	20.11±1.4	17.83±0.5	16.94±0.4	18.54±0.5	0.478	**0.031**	0.288
Foregut	13.92±0.1	16.63±2.8	13.89±0.2	12.92±0.8	13.59±0.5	16.88±2.5	0.428	0.790	0.192
Midgut	15.55±0.6	15.67±0.5	16.24±0.6	15.48±0.7	15.51±0.9	15.45±0.9	0.894	0.572	0.864
Hindgut	19.22±2.4	17.54±1.4	16.11±0.9	16.67±0.2	16.37±1.4	17.21±1.3	0.655	0.472	0.464
Taurine concentration (µmoles g^{-1})									
Liver	0.07±0.00	0.10±0.01	0.10±0.02	0.07±0.01	0.07±0.00	0.07±0.00	0.376	**0.013**	0.478

[1] Values are mean ± SE; $n = 3$.
[*] A significance level of $\alpha = 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.
[†] Gallbladder content two factor-ANOVA results level were significantly affected by crude lipid inclusion (20% CL > 30% CL).
[#] Taurine two factor-ANOVA results level were significantly affected by crude lipid inclusion (20% CL > 30% CL).

6.4.5.2 Taurine concentration

No interaction was observed between CP level and CL level in liver taurine concentrations (P = 0.478; Table 6.4). Crude protein levels did not significantly affect taurine concentrations in the liver of fish fed different diets (P = 0.376; Table 6.4). Taurine concentrations in the liver were significantly higher in fish fed diets containing 25% CL compared to that of fish fed diets containing 30% CL (P = 0.013; Table 6.4).

6.4.5.3 HMGCR and CYP7A1 enzymes

No interaction was observed between CP and CL levels and the concentration of HMGCR (P = 0.300) or CYP7A1 (P = 0.354) in liver tissue of fish (Table 6.5). Crude lipid level did not significantly affect either the HMGCR (P = 0.257) or CYP7A1 (P = 0.196) concentration in the liver tissue. Crude protein level had no significant effect on liver CYP7A1 concentration (P = 0.123) although a significant difference in HMGCR concentration occurred (P = 0.024). The HMGCR concentration in liver tissue of fish fed diets containing 40% CP level (Diet 1 and 4) were significantly less (P = 0.024) compared to the HMGCR concentration in liver tissue of fish fed diets containing 48% CP levels (Diet 3 and 6; Table 6.5). The HMGCR concentration in liver tissue of fish fed diets containing 44% CP (Diet 2 and 4) was not significantly different from the HMGCR concentration in liver tissue of fish fed diets containing either 40% CP level (Diet 1 and 4) or 48% CP levels (Diet 3 and 6; Table 6.5). Fish fed diets with 25% CL level had a numerical increase in HMGCR concentration with increasing dietary CP (Table 6.5). Similarly, fish fed diets with 30% CL levels had a numerical increase in HMGCR concentration with increasing dietary CP (Table 6.5).

Table 6.5. HMGCR and CYP7A1 concentrations of liver tissue from Yellowtail Kingfish, *Seriola lalandi*, fed experimental diets with graded protein and lipid levels for 84 days[1].

Diet	1	2	3	4	5	6			
Nominal crude protein level (%)	40	44	48	40	44	48	2 factor ANOVA (*P value*)[*]		
Nominal crude lipid level (%)	25	25	25	30	30	30	Protein level (A)	Lipid level (B)	A×B
HMGCR (µmoles g^{-1})[#]	7.72±0.66	9.87±1.81	15.57±2.85	7.11±1.37	10.32±2.05	10.6±0.83	**0.024**	0.257	0.300
CYP7A1 (µmoles g^{-1})[†]	3.49±0.00	3.58±0.07	3.58±0.04	3.46±0.06	3.56±0.07	3.43±0.02	0.123	0.196	0.354

[1] Values are mean ± SE; $n = 3$.
[*] A significance level of $\alpha = 0.05$ was used. Where significant differences were observed post-hoc tests were used (Tukey's Honestly Significant Difference test) to detect differences between treatments.
[†] Log transformed.
[#] HMGCR two factor-ANOVA results level showed a significant effect of crude protein inclusion (40% CP^{a}, 44% CP^{ab}, 48% CP^{bc}).

6.4.6 Blood analyses

Total bilirubin was significantly affected by CP inclusion (P = 0.046; Table 6.6). The total bilirubin of fish fed 48% CP was significantly less than that of fish fed 44% CP but not fish fed 40% CP, which were significantly similar to that of fish fed both 44% CP and 48% CP (Table 6.6). None of the other blood biochemical parameters was significantly affected by dietary CP or CL level, nor was there any interaction between dietary CP and CL levels (P > 0.05; Table 6.6). The alkaline phosphatase (ALP) concentration numerically increased with increased dietary CP levels in the blood of fish fed diets containing 30% CL (Table 6.6). Cholesterol concentrations were consistent across all diets. The triglycerides and bile acid concentrations numerically increased with increased dietary CP levels in the blood of fish fed diets containing 25% CL and 30% CL (Table 6.6).

Table 6.6. Blood biochemistry from Yellowtail Kingfish, *Seriola lalandi*, fed experimental diets with graded protein and lipid levels for 84 days.[1]

Diet	1	2	3	4	5	6			
Nominal crude protein level (%)	40	44	48	40	44	48	2 factor ANOVA (*P value*)[2]		
Nominal crude lipid level (%)[1]	25	25	25	30	30	30	Lipid level (A)	Protein level (B)	A × B
Biochemistry									
Protein (g L^{-1})	39±1	39±1	36±1	36±1	38±0	37±1	0.384	0.309	0.306
Albumin (g L^{-1})	12±1	12±1	11±0	11±1	11±0	12±0	0.138	1.000	0.272
Globulin (g L^{-1})	27±1	27±1	25±1	25±1	26±0	26±0	0.385	0.116	0.414
Total Bilirubin (mmol L^{-1})[4]	2±0	2±0	1±0	1±0	1±0	1±0	0.605	**0.046**	0.783
ALT (IU L^{-1})[3]	7±2	9±0	8±1	11±2	10±2	9±2	0.083	0.821	0.616
ALP (IU L^{-1})[3]	19±1	19±1	22±2	19±1	22±5	24±2	0.356	0.161	0.800
Cholesterol (mmol L^{-1})	3.5±0.3	3.6±0.2	3.4±0.1	3.9±0.2	3.9±0.1	3.5±0.3	0.122	0.370	0.673
Triglyceride (mmol L^{-1})	1.40±0.04	1.49±0.07	1.85±0.12	1.50±0.07	1.62±0.40	1.86±0.05	0.573	0.063	0.944
Bile Acids (mmol L^{-1})	2.6±0.6	8.1±2.4	10.3±3.7	9.1±1.6	10.0±5.0	17.4±6.5	0.132	0.126	0.768

[1] Values are mean ± SE; $n = 3$. SE less than 0.01 are reported as "0.00".
[2] A significance level of $\alpha = 0.05$ was used for all statistical tests, where significant differences were observed post-hoc tests were used (Student-Newman-Keuls test) to detect differences between treatments.
[3] ALT = alanine aminotransferase; ALP = alkaline phosphatase.
[4] Bilirubin two factor-ANOVA results level were significantly affected by crude protein inclusion (44% CP^{a}, 40% CP^{ab}, 48% CP^{b}).

6.5 Discussion

The growth performance for this trial is reported by Stone and Booth (2018). In short, fish fed the 25% CL diets generally grew better than those fed the 30% CL diets. The SGR of fish was significantly affected by the interaction between CP and CL level, primarily driven by the significant growth increase for fish fed Diets 1 and 2.

6.5.1 Liver histology and somatic indices

Fish fed increased dietary CP presented decreased VPLC%, typically a sign of decreased nutrient storage with hepatocytes. The decreased VPLC% is also not simply due to less lipid being available for storage, as the gross energy was similar for 25% CL diets (Diet 1, 2, & 3; mean of 3 diets ~19.83 MJ kg^{-1}) and for the 30% CL diets (Diet 4, 5 & 6; mean of 3 diets ~21.03 MJ kg^{-1}; Stone and Booth, 2018). As no significant effect on VPLC% by dietary CL level and no interaction between dietary CL level and CP level occurred, the CP levels can be assessed independent of the CL level. Both the protein:energy ratio (g CP MJ^{-1} GE) and calculated digestible protein:digestible energy ratio (g DP MJ^{-1} DE) increased with increasing nominal CP level within the 25% CL diets (20.3 – 23.23 g DP MJ^{-1} DE) and within the 30% CL diets (18.2 – 21.1 g DP MJ^{-1} DE; Stone and Booth, 2018). Therefore, a greater ratio of protein to lipid within the diets is associated with a reduction in the deposition of nutrients within hepatocytes. Hepatosomatic index was not significantly different between any of the diets but did numerically decrease with increased protein inclusion within each CL level. Although not significant, a whole-body apparent energy deposition reduction in fish fed diets with increasing dietary CP level was also noted (Stone and Booth, 2018).

Typically, high energy diets allow preferential catabolism of lipids, sparing the protein component of diet for more effective deposition of protein into tissue. Conversely, excess protein can also be utilised for energy production through deamination of amino acids (Francis

and Turchini, 2017). Ekmann et al. (2013) demonstrated that dietary protein contributed significantly to *de novo* lipid synthesis and total lipid deposition in gilthead sea bream, *Sparus aurata*. It is also known that excess dietary lipids are stored in the liver increasing the hepatocyte volume of Yellowtail Kingfish (Crowe et al., 2021). Here we suggest that a greater protein to energy ratio resulted in less dietary lipids being stored in hepatocytes. This was a result of energy provision by deamination of amino acids, providing sufficient energy for growth and reducing their capacity to store energy.

6.5.2 Biochemical analyses

6.5.2.1 Total bile acid and taurine concentration

The present study showed that the total bile acid concentration of the gallbladder was greater in fish fed the 25% CL diets compared to the 30% CL diets. Bile acids are essential for facilitating the absorption of dietary lipids and fat-soluble vitamins (Lefebvre et al., 2009; Bowyer et al., 2012). Primary bile acids are steroids synthesised from cholesterol in liver hepatocytes (Mohapatra and Mishra, 2011; Nguyen et al, 2011; Holm et al., 2013). Once synthesised, they are secreted into the canaliculi, drained into bile ducts and finally after being conjugated with taurine or glycine are stored in the gallbladder as bile salts (Mohapatra and Mishra, 2011; Nguyen et al, 2011; Holm et al., 2013; El-Sayed, 2014; Guerra-Olvera and Viana, 2015). Bile acid conjugation in mammals occurs at the terminal side-chain carboxylic acid with either glycine or taurine (Monte et al., 2009). However, studies on several species have indicated that bile acids may only conjugate with taurine in the liver of teleosts (Vessey et al., 1990; Kim et al., 2008). Further supporting this point, significant elevation in liver taurine concentration was observed in fish fed the 25% CL diets compared to fish fed the 30% CL diets. A numerical increase within the 25% CL diets was also observed with the two increased protein levels (44% CP and 48% CP). Before bile is stored in gallbladder, teleost bile acids

require conjugation with taurine, an essential nutrient required for osmoregulation and membrane stabilisation (Takagi et al. 2005; Nguyen et al., 2011). In mammals, taurine is synthesised in the liver and brain from methionine and cysteine via series of enzymatic processes. Cysteine sulfinate decarboxylase is the rate-limiting enzyme responsible for the production of taurine from cysteine, ultimately aiding in the enhancement of bile acid production through activating CYP7A1 (Chatzifotis et al., 2008; Takagi et al. 2011; El-Sayed et al., 2014). In fish however, the ability to synthesise taurine differs among species and the ability to do so is particularly lower, or negligible, in marine carnivorous species due to reduced levels or absence of CSD compared with freshwater species, thus requiring a dietary intake (Takagi et al. 2011; El-Sayed et al., 2014). Diets in the present study were formulated to contain suitable taurine levels for growth and animal health (Stone and Booth, 2018). We hypothesise that taurine was sequestered by the liver for greater bile acid synthesis to maximise the potential for nutrient uptake when a reduced dietary amount of lipids was available.

6.5.2.2 HMGCR and CYP7A1 enzymes

Cholesterol synthesised in the liver is regulated by the rate limiting enzyme HMGCR. Diets that replace fish meal with plant proteins or simply reduce the overall dietary protein component typically lead to increased HMGCR in the liver via *de novo* cholesterol synthesis (Deng et al., 2014). Within the present study HMGCR concentrations increased numerically with increasing dietary protein for each CL% level. Elevation of HMGCR gene expression is also associated with increased dietary carbohydrate in European sea bass, *Dicentrarchus labrax*, juveniles (Castro et al, 2015). The opposite was seen within the present study where a decrease in carbohydrate resulting from diet formulation corresponded with increased HMGCR concentration in the liver. An explanation for the increased HMGCR concentration is the provision of readily available amino acids providing precursors, such as methionine, for

cholesterol synthesis that would be required for conjugation with taurine for bile acid synthesis. However, liver CYP7A1 concentration of fish fed different CL% levels was not significantly different indicating that the synthesis of bile was not elevated in either of the CL% levels at the time of sampling. Although, fish were sampled during an interprandial period (i.e., in the fasting state) after having feed with-held for 24 h and may have already stored required volumes of bile acids within the gallbladder (Wang et al., 2012).

6.5.3 Blood analyses

When aged red blood cells are phagocytised, bilirubin, a derivative of heme, is released into the blood stream. Total bilirubin commonly is used as an indicator of liver function with elevated levels indicating hepatic insult (Lee et al., 2009; Wang et al., 2012; Higgins, 2013). A greater level of total bilirubin in fish fed 44% CP is an indication that the liver was not functioning as well as that of fish fed diets containing 48% CP and 40% CP. A healthy mammalian reference for total bilirubin is <21 µmol L^{-1} (Higgins, 2013), while cultured yellowtail, *S. quinqueradiata*, fed a commercial diet with 52.2% CP and 14.9% CL, have bilirubin concentrations of 4.00 ± 1.00 g L^{-1} (mean ± SD; Kawanago et al., 2014). Despite the increase in total bilirubin for fish fed the 44% CP diets, all treatment group total bilirubin levels were lower than reference levels indicating that the fish livers were functioning adequately with changes of dietary CP levels between 40 and 48%.

Alkaline phosphatase (ALP) is required for normal biliary tract function with elevated blood concentrations an indicator of cell injury (Higgins, 2013; Kawanago et al., 2014; Nakanuma et al., 2017). Although the ALP blood concentrations were greater for the 30% CL diets, the concentration range of all groups was less than that observed in observed in wild Yellowtail Kingfish (31.15 ± 10.53 IU L^{-1}; mean ± SD) and cultured yellowtail, *S. quinqueradiata*, 28.2 ± 4.3 – 41.6 ± 6.3 IU L^{-1} (mean ± SD) and several studies on Yellowtail Kingfish (Chapter 2,

3 and 4; Kawanago et al., 2014). The ALP blood concentrations indicate that the biliary tract function of fish fed diets up to 30% CL are within adequate functioning limits for Yellowtail Kingfish. However, fish fed diets containing 20% CL were less likely to develop adverse biliary tract effects than those fed at a greater dietary CL.

Cholesterol concentrations, a combination of *de novo* cholesterol and dietary cholesterol, were consistent across all diets (Maita et al., 2006). These were less than that what we have previously observed in wild Yellowtail Kingfish (9.03 ± 0.59 mmol L^{-1}; mean ± SD;) and more in line with what we have observed in cultured Yellowtail Kingfish (Chapter 2; Crowe et al., 2021). The triglycerides concentrations numerically increased with increased dietary CP levels in the blood of fish fed both the 25% CL diets and 30% CL diets. Mammalian references indicate that if plasma triglyceride levels are >1.8 mmol L^{-1} the species is considered to be hypertriglyceridemic (Higgins, 2013). The triglyceride level were slightly above this reference in fish fed diets containing 48% CP. Until normal ranges for this species are better understood, care must be taken when interpreting potential hypo- and hyper- triglyceridemic effects of diets as all concentrations were below those observed in wild Yellowtail Kingfish (3.37 ± 1.18 mmol L^{-1}; mean ± SD; Chapter 2).

Elevated total bile acid concentrations in the blood can be an indication of hepatic dysfunction or simply portosystemic shunting of total bile acids to the blood after efflux by enterocytes (Morita et al., 1978; Kullak-Ublick et al., 2004; York, 2017). In Atlantic salmon, reduced blood total bile acids levels are associated with impairment to intestinal reabsorption by dietary ingredients which reduce the enterohepatic recovery of already synthesised bile acids (Kortner et al., 2013). Total bile acid concentrations of fish in the current trial numerically increased with increased dietary CP levels in the blood of fish fed both the 25% CL diets and 30% CL diets. All total bile acid concentrations were within the range observed in wild Yellowtail

Kingfish (0.9 – 22.7 mmol L^{-1}; Chapter 2). In combination with no elevation in CYP7A1 concentration (liver bile acid synthesis) we suggest that in all fish the already synthesised bile acids were readily transferred to the blood after efflux by enterocytes. It does appear though, that the enterohepatic pool of bile acids was being maintained at a greater volume in diets with increased CP. This is most likely due to an increased availability of precursors for cholesterol synthesis required for the synthesis of bile acids, as indicated by the increased HMGCR concentration.

6.6 Conclusion

At the water temperatures (21.7 ± 1.2 °C) recorded in this study, Yellowtail Kingfish may be able to be fed diets with up to 30% CL and 48% CP without significant negative impact on hepatocyte vacuolisation, lipid and glycogen storage. However, a negative relationship between increased CP and hepatocyte vacuolisation exists and should be considered when formulating feeds so that liver structure and function do no become impaired with long term feeding. Liver HMGCR concentration of fish fed diets significantly increased above 40% CP levels. Increasing the CL% in diets significantly decreases the bile acid content in the gallbladder and decreased liver taurine concentrations, but no other digestive tissues were affected up to 30% CL inclusion levels. The duration of feeding of the experiment diets and the production age of Yellowtail Kingfish they are fed to should be closely monitored. The fish have plasticity enabling them to maintain enterohepatic function up to 30% CL and 48% CP. The data presented here assists with the interpretation of the growth data and feed intake data reported elsewhere for formulation of feeds. Altering the CL and CP levels in production diets may have desired effects beyond those examined in this experiment and should be investigated.

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Chapter 7: General discussion

7.1 Introduction

Addressing the specific dietary requirements of a commercially cultured species is essential to the animals health and the businesses' success (Lucas and Southgate, 2012; Alvarez-Lajonchère and Ibarra-Castro, 2013). The costs of fish meal (FM) and fish oil (FO) traditionally used as the main protein and lipid source, respectively, are being driven up by demand by competition for their use (El-Sayed, 2014; FAO, 2020b). At the commencement of this research the largest Australian Yellowtail Kingfish producer Clean Seas Seafood Ltd (CSS), South Australia, produced 3,500 tonnes with reports of increased growth in domestic and international markets and plans to pursue an additional 4,250 tonne capacity lease (CSS, 2020). At present, CSS has inshore licences allowing up to 10,000 tonnes of production (CSS, 2022). The substitutions or alternatives to FM and FO must be established to make diets more cost-effective and environmentally sustainable (Gatlin et al., 2007; Stone and Bellgrove 2013; Stone et al., 2016).

Research into cost-effective feeds with optimal growth results is under way in Australia assisting in the production and profitability of a developing Yellowtail Kingfish industry (Stone and Booth, 2018). Previous nutrition requirement have been largely based upon formulated feeds for Atlantic salmon and barramundi and closely related *Seriola* spp. (Abbink et al., 2011; Bowyer et al., 2012a and 2012b; Booth et al., 2013; Stone and Bowyer, 2013; Stone et al., 2016). These reviews were extensive although did not consider the impact diet manipulation may have on liver structure and function in Yellowtail Kingfish (Stone and Bellgrove, 2013). Currently, the only information available for comparison is from studies with Atlantic salmon and other *Seriola* spp. (Takagi et al., 2005 and 2008; Nguyen et al., 2011; Kortner et al., 2013; Stone et al., 2016).

The focus of this PhD research was improving knowledge of Yellowtail Kingfish nutrition through understanding liver function. The research objective was to investigate how the enterohepatic system may be impacted by diet manipulation through partial or complete substitution of FM and/or FO with alternative ingredients. The specific research aims were to obtain information that would contribute to a baseline data of wild Yellowtail Kingfish and investigate any changes in cultured Yellowtail Kingfish that diet manipulation may have on: (i) liver structure and morphology; (ii) blood biochemistry; (iii) liver cholesterol metabolism; (iv) liver bile acid metabolism; (v) total bile acid concentrations of the enterohepatic and excretory system tissues; and (vi) total bile acid concentrations of faeces. Major findings and outcomes are outlined below, and future directions are recommended.

7.2 Summary of major findings

The data presented here assists with understanding the effect that diet manipulation can have on the enterohepatic system of Yellowtail Kingfish. Understanding these effects on bile and cholesterol metabolism, as well as liver structure and health, will assist in future development of formulated feeds by providing understanding of the variation between wild and cultured Yellowtail Kingfish. This data will also aid in a more thorough understanding of influences that diet manipulation may have on the health and growth of Yellowtail Kingfish. The parameters investigated within this study may have desired effects beyond those examined in this experiment and their use should be investigated further. The cultured Yellowtail Kingfish within this study showed signs of morphometric liver changes. However, have the fish have plasticity enabling them to metabolically adjust and grow sufficiently within the parameters investigated within this study despite the appearance of adverse effects. It is recommended that sea-cage pilot scale trials are conducted before these diets used are put into commercial production. The main concern for this is the potential for the impact long-term feeding may

have resistance to stressors and survival, particularly if an adverse environmental or biological event occurs. The findings by Chapter are presented for reporting on specific responses at given water temperatures and diet formulations.

1. Chapter 2

This is the first reporting of gross and somatic indices, liver histology, bile acid and taurine concentrations, liver enzyme concentration and blood biochemistry for wild caught Yellowtail Kingfish from South Australian waters. Little is known about the diet of wild Yellowtail Kingfish with the basis of formulated feed knowledge originating from the Atlantic salmon industry or closely related *Seriola* spp. (Stone and Bellgrove, 2013; Stone and Booth, 2018). The geographical genetic distinction between *Seriola* spp. represents local adaptions to regional environmental conditions and highlights the need for the understanding of local wild populations, particularly if cultured animals are the seed of wild populations (Wegner 2018). In recent years, nutritional requirements of cultured Yellowtail Kingfish have been investigated in detail (Stone and Booth, 2018). However, understanding the condition of wild Yellowtail Kingfish enterohepatic system along with their prey selection, particularly if reared in different geographical differences, should be investigated to fully understand the difference between wild and cultured counterparts. This data will assist in future development of formulated feeds by providing understanding of the variation between wild and cultured Yellowtail Kingfish for the parameters investigated. A greater collection of fish would have improved the comparison and analyses of fish collected within this study. However, this work contributes to the establishment of baseline data.

2. Chapter 3

At winter water temperatures (15.8 ± 1.4 °C), increasing dietary crude lipid (CL) from 20% to 30% negatively impacted the health of fish livers. The livers of fish fed 30% CL presented

overall less homogenous hepatocyte distribution and less consistently positioned cuboidal cholangiocytes around bile ducts compared to those of fish fed 20% CL. Fish fed diets with 30% CL had increased VSI, HSI, liver hepatocyte vacuolisation and bile duct proliferation. This is a common response also seen in large yellow croaker, Atlantic salmon and European sea bass (Hansen et al., 2008; Hartviksen et al., 2014; Wang et al., 2015; Yan et al., 2015). The additional of emulsifier at 40 mg lipid kg^{-1} in diets with 30% CL and 20% CL has no benefit for the improvement in cholesterol and bile acid metabolism and the liver structure in sub-adult Yellowtail Kingfish culture. However, emulsifying agents at different concentrations or derived from different sources may have desired effects and their uses should be investigated, particularly a marine based emulsifier. The use of emulsifying agents at different water temperatures and life stage should also be investigated to determine efficacy before dismissal. Marine based emulsifying agents assisted in increased growth in juvenile cobia, *Rachycentron canadum*, (65 ± 1 g; Trushenski et al., 2013) and like other marine based ingredients may be more readily metabolised and utilised. Excess of dietary energy may accumulate as fatty deposits in different tissues or be stored as visceral fat (Turchini et al., 2009). The fish have plasticity in this respect, enabling them to grow and maintain enterohepatic function when diet formulations are manipulated. This has implications for producers as harvest yield of a whole fish weight is increased but the harvest yield of fillets may not increase accordingly, resulting in expenditure on unnecessarily high lipid feeds. Pilot scale commercial research trials are recommended before the use of emulsifiers or variations in CL levels are made to commercial production diets. establishment of baseline data.

3. Chapter 4

At the winter water temperatures recorded in this study (16.2 ± 2.1 °C), Yellowtail Kingfish may be able to be fed a formulated diet containing:

~25% dietary lipid level (2.12 g $100g^{-1}$ of ΣLC n-3 PUFA) with up to 100% of the FO component replaced by poultry oil (PO), and;

~25% dietary lipid level (2.12 g $100g^{-1}$ of ΣLC n-3 PUFA) with up 100% of the commercially used alternative lipid source (PO) with canola oil (CO);

without an apparent negative impact on VSI, HSI, liver lipid and glycogen storage, total bile acid concentration of the liver, gallbladder content, foregut, midgut, hindgut or faeces, nor liver taurine concentrations, liver 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) concentrations or any of the blood parameters measured in this study. Increasing dietary PO led to increased hepatocyte vacuolisation. Similarly, cholesterol-7α-hydroxylase (CYP7A1) concentration increased with increasing PO indicating a compensatory synthesis of bile acids for micellular solubilisation of dietary lipids or for increased cholesterol excretion. However, fish fed diets with lower PO and higher CO had decreased total bile acid concentrations in liver tissue which did not appear to be metabolically compensated for. For the conditions of this short-term tank trial, liver vacuolisation increased with increased dietary PO. Similarly, Campos et al. (2019) demonstrated the same affect in European sea bass (*Dicentrarchus labrax*) juveniles where fish fed with increasing levels of fish oil substitution by poultry oil resulted in increasing liver hepatocyte vacuolisation. These fish demonstrated no significant changes in final body weight or feed intake compared to the control diet (Campos et al., 2019). The liver lipid accumulation was suggested to be caused by an insufficiency of phospholipids as a result of higher poultry oil inclusion (Campos et al., 2019). The long-term feeding of diets containing high inclusion levels of PO to cultured Yellowtail Kingfish exposed to environmental factors and the effect this has on their liver function and structure should be closely monitored. In addition, adverse environmental or biological events may challenge the animals' health further, so the impacts this might also have on the liver or

whole animal growth and health is unknown and should be investigated by further research. Pilot scale commercial research trials are recommended before dietary FO is reduced and replaced with either PO, CO or a PO-CO blend at high inclusion levels within commercial production diets.

4. Chapter 5

At the winter water temperatures recorded in this study (16.7 ± 2.8 °C) Yellowtail Kingfish, fed a 30% wild derived FM (wd-FM) diet may have the FM replaced by up to 66.67% fish by-product meal (FBM), 33.3% poultry meal (PM) or 33.3% soy protein concentrate (SPC) without any significant impact on total bile acid synthesis, storage, or excretion. Likewise, hepatocyte vacuolisation, unsaturated neutral lipid storage and total lipid storage within hepatocytes are unaffected by dietary FM replaced with up to 66.67% FBM, 33.3% PM or 33.3% SPC. The level of FM replacement by FBM are consistent with that seen in Korean rockfish, *Sebastes schlegeli*, where up to 75% of dietary FM can be replaced with a tuna FBM (Kim et al., 2018). Recently, consistent reviews and studies have indicated the successful partial replacement of FM by PM and SPC across numerous species (Galkanda-Arachchige et al., 2020; Hong et al., 2020; Zhou et al., 2020; Fontinha et al., 2021; Rocker et al., 2021). The replacement of FM with alternative terrestrial ingredients is evidently species dependent (Galkanda-Arachchige et al., 2020). Of great importance is the recent reviews that highlight that different sources of FBM, PM and SPC will result in varying essential and non-essential amino acids profiles in the final feed delivered on farm (Plante et al., 2017; Lewis et al., 2019; Rocker et al., 2019). Fat deposition in the liver was similar between all treatment groups but severe bile duct proliferation and periductular fibrosis was observed in the livers of fish fed diets with wd-FM replacement with 10% PM or 10% SPC. The fish have a nutritional plasticity enabling them to grow and maintain enterohepatic function on a variety of diet formulations.

However, fish fed PM and SPC have livers that presented overall less homogenous hepatocyte distribution and less consistently positioned cuboidal cholangiocytes around bile ducts compared to as fish fed wd-FM and FBM. The observation of severe bile duct proliferation and periductular fibrosis in the livers of fish fed diets with wd-FM replacement with 10% PM or 10% SPC should be considered before inclusion into commercial feeds and in long-term feeding of diets including these ingredients. The long-term feeding of diets containing PM and SPC to Yellowtail Kingfish and the effect this has on their liver function and structure should be closely monitored. Further investigation to the degree of bile duct proliferation and periductular fibrosis should occur. It is suggested that a quantitative analysis of the fractional area of these occurrences in multiple sections of the liver take place. As indicated previously, these connective tissue sheaths were greater in comparison to known examples of wild and cultured *Seriola* species and wild yellowtail flounder, *Laimanda ferruginea* and rainbow trout, *Salmo gairdneri* Richardson (Chapter 2; Hampton et al., 1989; Fahraes-Van and Spurell, 2003; Crowe et al., 2021). Should an adverse environmental or biological event occur challenging the animals' health further, the impacts this might also have on the liver or whole animal health and survival are unknown. Pilot scale commercial research trials are recommended before wd-FM inclusions levels are reduced in commercial production diets, particularly in diets that have wd-FM replaced at an inclusion level of of 10% or greater by PM or SPC.

5. Chapter 6

At the water temperatures recorded in this study (21.7 ± 1.2 °C) Yellowtail Kingfish may be able to be fed diets with up to 30% CL and 48% crude protein (CP) without significant negative impacts on hepatocyte vacuolisation, lipid and glycogen storage. A negative relationship between increased CP and hepatocyte vacuolisation exists and should be considered when formulating feeds so that liver structure and function do no become impaired with long term

feeding. Liver HMGCR concentration of fish fed diets significantly increased above 40% CP levels. Increasing the CL% in diets significantly decreases the bile acid content in the gallbladder and decreased liver taurine concentrations, but no other digestive tissues are affected up to 30% CL inclusion levels. It is suggested that a greater ratio of protein to lipid within the diets is associated with a reduction in the deposition within hepatocytes. The provision or energy by deamination of amino acids supplies sufficient energy for growth and reducing their capacity to store energy (Francis and Turchini, 2017). Detailed investigation of this was outside of the scope of this study and further research will be needed to elucidate the findings. The fish have the capacity to maintain normal enterohepatic function when fed diets containing up to 30% CL and 48% CP. The duration of feeding of the diets from this study and the production age of Yellowtail Kingfish they are fed to should be closely monitored to ensure the correct level of dietary CP is added. Pilot scale commercial research trials are recommended before current production diets with increased CL and CP before levels are altered.

7.3 Conclusion

This research contributes to the empirical information base needed to improve dietary formulations by quantifying how some alternative ingredients to FO and/or FM impact cultured Yellowtail Kingfish liver function and structure. The research provides the first report of gross and somatic indices, liver histology, bile acid and taurine concentrations, liver enzyme concentration and blood biochemistry for both wild caught and cultured Yellowtail Kingfish from South Australian waters. Furthermore, this research presents the important histological findings as to the negative impact that terrestrial ingredients have on the liver structure of Yellowtail Kingfish. However, Yellowtail Kingfish grew well in tank-cultured conditions despite reduced liver health indicating plasticity which will benefit a producer's capacity to use feeds of different formulations and from different suppliers. Currently, there is more known

about the dietary impact of alternative ingredients in other international *Seriola* spp. If researchers in this area are able to apply the results from both local and international research, then rapid advances in production are possible. The Australian Yellowtail Kingfish industry is still relatively new compared to other Australian aquaculture species, such as Atlantic salmon, and other international cultured *Seriola* spp. Further improvements in liver function and health are likely through dietary manipulations. We have contributed to the establishment of data that will assist in the future advance and development of formulated feeds for this commercially important species.

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