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Crowe, B. H., Harris, J. O., Bansemer, M. S., & Stone, D. A. J. (2021). Restricted feeding and dietary energy levels affect liver structure in cultured Yellowtail Kingfish (Seriola lalandi , Valenciennes) at summer water temperatures. Aquaculture Research.

which has been published in final form at https://doi.org/10.1111/are.15469

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Restricted feeding and dietary energy levels affect liver structure in cultured Yellowtail Kingfish (*Seriola lalandi*, Valenciennes) at summer water temperatures

Short running title: Restricted feeding and dietary energy

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Keywords

Yellowtail kingfish; energy level; fatty liver; histology; restricted feeding

Abstract

Excess dietary lipids may be stored as lipid droplets in liver hepatocytes where they cannot be transported from the cells. Yellowtail kingfish (*Seriola lalandi*, Valenciennes) are cultured using formulated feeds, putting them at risk of fatty livers. This study investigated the effect of restricted feeding (apparent satiation [100%] vs. apparent sub-satiation [80%]) and three different dietary energy levels (Diet 1 [19.0 MJ kg^{-1} gross energy], Diet 2 [20.2 MJ kg^{-1} gross energy] and Diet 3 [18.4 MJ kg^{-1} gross energy]) on liver structure and function of sub-adult Yellowtail kingfish (1.87 ± 0.01, mean kg ± SE) over 84 days. Fish fed to 100% satiation had significantly ($P < 0.05$) greater weight gain than fish fed to 80% satiation. Both hepatosomatic index (HSI; %) and vacuolisation levels of liver hepatocytes were unaffected by restricted feeding, but significantly ($P = 0.016$ and $P = 0.039$, respectively) increased with an increase of dietary energy. Fish fed high energy diets showed extensive large hepatocyte vacuolisation. Despite these adverse changes, results reported elsewhere showed that increased dietary energy level improved the overall growth performance and feed utilisation of sub-adult Yellowtail kingfish compared to fish fed lower energy/lipid diets. Therefore, high energy diets have the potential to be used in the production of Yellowtail kingfish.

1. Introduction

The amount of food delivered to cultured species is an area widely studied. Many are fed to satiation, but restriction feeding, or sub-satiation feeding is often used. This is the intentional reduction of the amount of feed or the frequency of feeding to a cultured species (Reigh, Williams & Jacob, 2006). Feed costs can be as high as 45% of total operational cost, and consequently sub-satiation feeding has the potential to reduce production costs and increase profitability if growth rates are maintained (Reigh et al., 2006). The ability for a species to grow to a harvest size at a sub-satiation feeding level could lead to reduced feed, feeding and production costs thereby increasing industry sustainability. So far we have seen a variety of responses to this approach. Channel catfish (*Ictalurus punctatus*, Rafinesque) exhibited the highest growth rate when fed at 100% satiation compared to fish fed 74% sub-satiation (Reigh et al., 2006). However, Atlantic salmon (*Salmo salar*, Linnaeus) fed a high energy diet (22.1 MJ kg^{-1}) at ~ 63% satiation had an increased final weight compared with fish fed at 100% satiation (Johnsen et al., 2011). Successfully applying this strategy will require species-specific satiation trials.

In periods of sub-satiation feeding when nutrient availability is reduced, stored lipids and amino acids from muscle fibres can be used for metabolic energy, potentially reducing final weight (Valente, Bower, & Johnston, 2012). In commercial fish production, high lipid diets may be used to ensure sufficient dietary energy is available so that dietary protein is only used for muscle growth (Nanton et al., 2007). This can produce problems when dietary lipid levels are too high, as adverse effects on liver structure and function can occur. The liver is responsible for lipid metabolism and storage, and an excess of dietary lipids can cause a condition referred to as fatty liver (Lu et al., 2013). Fatty liver is the result of fatty acids being modified by acyl-CoA synthetases and stored as lipid droplets in liver hepatocytes where they cannot be transported from the cells (Her et al., 2011). Severe fatty liver disease is referred to as steatosis. This is associated with irregularly shaped hepatocytes in fish fed high energy diets and it occurs when

the storage capacity of the liver hepatocytes is exceeded by the dietary availability of lipids (Spisni, Tugoli, Ponticelli, Mordenti, & Tomasi, 1998; Kowalska et al., 2011). Steatosis can cause oxidative damage to DNA, leading to reduced liver cell regeneration preventing the absorption of essential nutrients for metabolic processes and growth (Her et al., 2011; Jensen-Urstad & Semenkovich, 2012). Cultured marine fish commonly have fatty livers when fed high lipid diets (Deng et al., 2010). Cultured Atlantic cod (*Gadus morhua*, Linnaeus) have impaired growth performance when fed high lipid diets resulting from excessive storage of dietary lipids in liver hepatocytes (Hansen et al., 2008). Likewise, high lipid diets fed to cultured large yellow croaker (*Pseudosciaena crocea*, Richardson) led to excess lipid storage and hepatic steatosis (Wang, Li, Hou, Gao, & Wang, 2015).

Many carnivorous cultured species are traditionally fed formulated feeds containing fish oil (FO) and fish meal (FM) as the main sources of dietary lipid and protein, respectively. However, as a result of the increased demand for food supply from aquaculture, terrestrial animal industry competition for FO and FM, and the inclusion of FO and FM in human foods, the prices of these marine derived ingredients are increasing (FAO, 2016) and substitutes are being sought. Fish oil contains high concentrations of the long-chain omega-3 polyunsaturated fatty acids (LC n-3 PUFAs) eicosapentaenoic acid (20:5n-3, EPA), docosapentaenoic acid (22:5n3, DPA) and docosahexaenoic acid (22:6n-3, DHA). These LC n-3 PUFAs are necessary for carnivorous marine finfish to sustain optimum health and growth as they are needed for numerous biological functions including cell membrane construction and are the precursors for eicosanoids; the hormone-like molecules that bind to proteins that regulate lipid and cholesterol metabolism (Tocher, Bendisken, Campbell, & Bell 2008; Martins et al., 2012). The LC n-3 PUFAs are also sources of metabolic energy used for growth, reproduction, immune defence and movement (Tocher, 2003; Trushenski & Lochmann, 2009). In diets where FO is replaced with an alternative oil source, traces of LC n-3 PUFAs are present in the diet from the oil component of FM. However, EPA and DHA are not found in terrestrial plant oils or animal fats unless supplemented

or derived from genetically modified stock (Bowyer, Qin, Smullen, & Stone, 2012). Thus, replacement of FO with terrestrial oils and fats in aquafeeds has the potential to reduce dietary LC n-3 PUFA levels. A more common approach is to ensure that the species' requirements for LC n-3 PUFAs are met, then to provide additional lipids required for energy from other sources.

The level of dietary FO supplementation or replacement is species-dependent, as incorrect levels of replacement with terrestrial plant or animal oils could also have negative influences on fish health. When FO is replaced by terrestrially-sourced lipids and fats, dietary cholesterol and subsequently plasma cholesterol are reduced (Bowyer et al., 2012; Liland et al., 2013). In addition, anti-nutritional factors in some terrestrially-sourced diet ingredients which in either their consumed state or by their metabolic by-products adversely influence food digestion and animal health (Francis, Makkar, & Becker, 2001). Anti-nutritional factors include protease inhibitors, tannins, lectins, certain oligosaccharides and non-starch polysaccharides all of which have the potential to negatively impact the intestinal morphology of fish (Chen et al., 2011; Lim et al., 2011). Furthermore, anti-nutritional factors include undesirable bitter tastes, reducing palatability and therefore, feed intake and fish growth (Collins et al., 2012). In red seabream (*Pagrus auratus*) reduced lipid metabolism has been observed as a result of FO replacement with canola oil (Glencross, Hawkins, & Curnow, 2004). Reduced growth rates were reported in Yellowtail kingfish (*Seriola lalandi*, Valenciennes) fed diets containing moderate to high levels of canola oil (Bowyer et al., 2012). Fewer anti-nutritional factors in terrestrial animal oils compared with vegetable oil are advantageous in diet formulation for carnivorous finfish making them more likely to be commercially useful as a lipid source.

Yellowtail kingfish is a carnivorous pelagic finfish species with a high metabolic rate, commercially farmed in Australasia, New Zealand, Japan, Taiwan, North America and South America (Hilton, Poortenaar, Sewell, 2008; Booth, Allan, & Pirozzi, 2010; Bowyer et al., 2013). Yellowtail kingfish exhibits fast growth rate and excellent meat quality, reaching a harvest size of 3.0 – 3.5 kg in 18 – 24 months (Fernandes and Tanner, 2008; Miegel, Pain, van Wettere,

Howarth, & Stone, 2010; Bowyer et al., 2012). The Australian Yellowtail kingfish industry is expanding. In 2010 Australia's largest producer, Clean Seas Seafood Ltd, South Australia, reported ~3,000 tonne production levels with recent plans to expand this to 4,250 tonnes (Stone, D'Antignana, & Bansemer, 2016; CSS, 2020). It is imperative that efficient feeding practices and optimised feeds underpin the development of this species in aquaculture. The aim of this study was to investigate the effect of feeding diets of different dietary energy levels on the liver structure and function of sub-adult Yellowtail kingfish fed at 100% apparent satiation and 80% apparent satiation during optimal summer water temperatures.

2. Materials and methods

2.1. Experimental design

In this study, a factorial design was utilised, with two feeding strategies (100% apparent satiation and 80% satiation) and three dietary energy levels (Table 1) resulting in six treatment combinations. The effect on liver structure, liver enzymes, haematology, feed utilisation and health of Yellowtail kingfish were investigated. Growth was reported in Stone et al. (2016) with a summary of results included here for further context. The experiment was conducted over an 84 day period during the southern hemisphere spring/summer (September to February) of 2014/2015 at the South Australian Research and Development Institute (SARDI) South Australian Aquatic Sciences Centre (SAASC) at West Beach, Adelaide, Australia. Experimental protocols followed the guidelines approved for AS-CRC Project No. 2013/730: Refining Yellowtail kingfish feeds and feed management, prepared by SARDI SAASC, Adelaide, Australia. At the commencement of the experiment, fish were anaesthetised in seawater using AQUI-S® (AQUI-S® New Zealand Ltd.) at a concentration of 0.01 mL L^{-1}, and individual fish were weighed (kg) and the fork length measured (mm). A total of 342 fish (mean body weight ± SD, 1.87 ± 0.01 kg; mean fork length ± SE, 496 ± 1 mm; n = 342) were randomly distributed between eighteen 5000 L fibreglass tanks with 19 fish per tank. Each of the six treatments was randomly assigned to three replicate tanks.

151 The feeding methods were as follows. For the apparent 100% satiation treatments,
152 Treatments 1 (Diet 1), 3 (Diet 2) and 5 (Diet 3), fish were fed for 4 minutes $tank^{-1}$ daily at 0830 h.
153 Yellowtail kingfish fed to 100% apparent satiation were provided feed in excess of their
154 maintenance requirements during spring/summer water temperatures (Stone et al. 2016;
155 Bansemer et al. 2018). Fish in 80% sub-satiation treatments, Treatments 2 (Diet 1), 4 (Diet 2) and
156 6 (Diet 3), were fed to apparent satiation every Saturday (4 minutes $tank^{-1}$), followed by a day of
157 no feed on Sunday, and then fed from Monday to Friday to the targeted 80% sub-satiation level
158 calculated from 80% of the apparent satiation feed intake on Saturday until all feed was
159 consumed. Yellowtail kingfish fed to 80% apparent satiation were provided feed below their
160 maintenance requirements for spring/summer water temperatures (Stone et al. 2016; Bansemer et
161 al. 2018). This feeding cycle was repeated over the duration of the experiment, and sub-satiation
162 levels were calculated weekly.
163
164 ***2.2. Experimental system***
165 The recirculating aquaculture system (RAS) was undercover in ambient conditions with
166 supplemental fluorescent lighting provided during the natural light period (12L: 12D). Ambient
167 temperature sea water was treated by settlement and sand filtration and circulated through the
168 RAS. The sea water was then returned through filter-screen baffle-boards, pumped through a
169 drum filter with 70 μm^2 filter pores (HDF1603 Hydrotech, Saint-Maurice, France), returned to the
170 bio filter, and finally treated with UV light sterilisation (D-32051 Wedeco, Herford, Germany) at
171 a rate of 35,000 L h^{-1} by two electric centrifugal pumps (Grundfos, Regency Park, Australia)
172 before being returned to the tanks. One hundred percent of the RAS water volume was replaced
173 daily.
174
175 ***2.3. Fish source and acclimation***

Sub-adult Yellowtail kingfish (*Seriola lalandi*) were obtained from Cleanseas Tuna Ltd. sea cage facilities at Port Lincoln, Australia. The fish were acclimated for four weeks in 5000 L fibreglass tanks supplied by the RAS at SARDI SAASC and fed a commercial diet (Ridley Aquafeeds Ltd. Pelagica diet; gross energy 19.3 MJ kg^{-1}, crude lipid 24%, crude protein (CP) 46%, before the commencement of the experiment.

2.4. Experimental diets

In this study, three experimental diets (9 mm pellets), referred to as Diet 1, Diet 2 and Diet 3 were tested using two different feeding strategies. The biochemical composition of the three diets is displayed in Table 1. The diets were formulated to contain 9% FO plus 9% PO (Diet 1), 7% FO plus 19% PO (Diet 2) and commercial-in-confidence (Diet 3). Diet 1 and Diet 2 were formulated by SARDI, Clean Seas Pty. Ltd. and Ridley Corporation Ltd. staff, and manufactured using cooking extrusion technology by Ridley Corporation Ltd. Diet 3 (Hiramasa Japanese Yellowtail diet) was formulated and manufactured using cooking extrusion technology by Hayashikane Sangyo Co. Ltd. Diet 1 contained 19.0 MJ kg^{-1} gross energy with 9% FO and 9% PO as the added lipid source and 2660 mg $100g^{-1}$ combined EPA and DHA; Diet 2 contained 20.2 MJ kg^{-1} gross energy with 7% FO and 19% PO as the added lipid source and 2470 mg 100 g^{-1} combined EPA and DHA; Diet 3 contained 18.4 MJ kg^{-1} gross energy with a confidential formulation, however, Clean Seas Pty. Ltd. has indicated that FO was the added lipid source based on the presence of 4880 mg 100 g^{-1} combined EPA and DHA (Table 1). Feed was stored at 4 °C until used. Feed was stored at 4 °C until used. The proximate composition, fatty acid profiles and amino acid profiles for each diet are shown in Table 1.

2.5. Water quality

Water quality parameters were monitored daily, unless otherwise indicated. Temperature (21.5 ± 1.1 °C, mean ± SD) and dissolved oxygen (93.5 ± 7.0 % saturation, mean ± SD) were monitored using an OxyGuard Handy Polaris temperature and dissolved oxygen probe (OxyGuard International A/S, Farum, Denmark). The pH (7.82 ± 0.12, mean ± SD) was monitored using a Eutech pH Testr 30 multiparameter handheld probe (Eutech Intruments Pty. Ltd., Singapore, Singapore). Salinity (35.6 ± 0.9 g L^{-1}, mean ± SD) was monitored using an ISSCO UR-2 hand-held refractometer (model RF20; Extech Instruments Corporation, Nashua, USA). The total ammonia concentration (< 0.25 mg L^{-1}) was determined weekly using a commercial water testing kit (Aquarium Pharmaceuticals, Pennsylvania, USA).

2.6. Sample collection

At the completion of the experiment, feeding was stopped 24 h before harvest. Three fish from each tank were euthanized and measured for their body weight (nearest 0.1 g) and fork length (nearest 0.1 mm). For each fish, the visceral cavity was cut open and the liver was removed and weighed (nearest 0.01 g) to determine the hepatosomatic index (%) (HSI) = (liver wt / body wt) x 100. A one cm^3 section of the left lobe of the liver was dissected and immediately placed into a histology cassette and fixed with 10% seawater-buffered formalin (pH of 7.2) for histological evaluation. Blood samples were obtained from the caudal vasculature using 21 gauge needles and 5 mL syringes for analysis of digestion and nutrient utilisation.

2.7. Liver histology

Liver samples were fixed in formalin for 24 hours 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. Sections were haematoxylin and eosin (H&E) stained and examined at 200-fold

magnification using a light microscope (Olympus BX40). Three 182,109 μm^2 microphotographs were taken per stained slide using a digital camera (Moticam 2 MP 2000, Motic, Kowloon, Hong Kong). Quantitative assessments of the vacuole volume as a proportion of the liver cell volume (VPLC) was determined by applying a H&E stain sensitive colour threshold using Fiji Image J processing software (National Institutes of Health, USA).

2.8. Blood analyses

Two mL of blood was placed into EDTA-containing vacutainers and stored on ice until same-day analysis of blood biochemistry and haematology parameters by IDEXX Laboratories (Unley, Australia). Haematocrit values were obtained by centrifuging (Clements Orbital 160, Lidcombe, NSW, Australia) blood in pre-heparinised capillary tubes at 1100 *g* for 1 min at SARDI SAASC.

2.9. Statistical analyses

IBM SPSS Version 20 for Windows (IBM SPSS Inc., Chicago, IL, USA) software was used for all statistical analyses. 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. A two-factor ANOVA was used to assess the effects of diet type (Diet 1, 2 and 3) and feeding strategy (100% satiation vs. 80% sub-satiation). Where significant interactions were observed, the data were analysed using Tukey's Honestly Significant Difference (HSD) multiple range test. 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.

3. Results

3.1. Diet proximate composition

253 The proximate composition and fatty acid profile of the diets is shown in Table 1. The
254 gross energy of Diet 2 was 5.90% greater than Diet 1 and 10.90% greater than Diet 3 (Table 1).
255 Diet 2 contained more cholesterol than the other diets; 4.00% more than Diet 1 and 17.50% more
256 than Diet 3 (Table 1).
257 Total EPA, DPA and DHA concentrations of Diet 2 was approximately the same as Diet 1
258 and both were approximately half that of Diet 3 (Table 1). The linoleic acid content of Diet 2
259 (3410 mg 100 g^{-1}) was 25.80% greater than that of Diet 1 (2530 mg 100 g^{-1}) and 75.95% greater
260 than that of Diet 3 (820 mg 100 g^{-1}) (Table 1). Likewise, the α-linolenic acid content of Diet 2
261 (450 mg 100 g^{-1}) was 22.2% greater than that of Diet 1 (350 mg 100 g^{-1}) and 42.2% greater than
262 that of Diet 3 (230 mg 100 g^{-1}) (Table 1).
263
264 ***3.2. Growth performance and feed utilisation***
265 For growth performance and feed utilisation of Yellowtail kingfish in this experiment, refer
266 to Stone et al. (2016). In short, growth performance and feed utilisation indices were not
267 significantly affected by the interaction between diet type and feeding strategy. At the completion
268 of the trial fish fed Diets 1 and 2 weighed significantly more, had greater specific growth rates
269 (SGRs) and improved condition factor when compared to fish fed Diet 3. Fish fed Diets 1 and 2
270 consumed significantly more feed than fish fed Diet 3. Fish fed Diet 3 had significantly greater
271 feed conversion ratio (FCR) than those fed Diet 2, however, fish fed Diet 1 were not significant
272 from either. Fish fed at 100% satiation weighed significantly more, were significantly longer, and
273 had significantly greater biomass, SGRs and condition factor than fish fed at 80% satiation. Fish
274 fed at 100% satiation consumed more feed and had a greater feed intake rate (FIR) and FCR than
275 fish fed at 80% satiation.
276
277 ***3.4. Hepatosomatic index***

278 The HSI was not affected by any interaction between diet type and feeding strategy (P =
279 0.858; Table 2). The HSI was significantly affected by diet type (P = 0.016; Table 2). Fish fed
280 Diet 3 (0.73 ± 0.04%) produced significantly lower HSI than fish fed Diet 2 (0.91 ± 0.04%),
281 however fish fed Diet 1 (0.79 ± 0.04%) were not significantly different than either of the other
282 diet types (Table 2). The HSI was not significantly affected by feeding strategy (P = 0.063; Table
283 2).
284
285 ***3.5. Liver histology***
286 The mean VPLC was not significantly affected by an interaction between diet type and
287 feeding strategy (P = 0.935; Table 2). The mean VPLC was significantly affected by diet type (P
288 = 0.039; Table 2). Fish fed Diet 3 (0.55 ± 0.04%) had significantly lower mean VPLC than fish
289 fed Diet 2 (0.71 ± 0.04%), while fish fed Diet 1 (0.64 ± 0.04%) were not significantly different to
290 either (Table 2). Fish fed Diet 2 showed extensive large vacuolisation with hepatocytes
291 containing optically empty content identified as intracytoplasmic lipid droplets (Figure 1a). Fish
292 fed Diet 3 showed a decreased vacuolisation and an absence of intracytoplasmic lipid droplets
293 (Figure 1b). The mean VPLC was not significantly affected by feeding strategy (P = 0.427; Table
294 2).
295
296 ***3.6. Blood biochemistry and haematology***
297 Blood biochemistry or haematology parameters were not significantly affected by any
298 interactions between diet type and feeding strategy (P > 0.05; Table 3). Neither diet type (P=
299 0.805) nor feeding strategy (P = 0.387) had a significant effect on HCT (Table 3).
300 Diet significantly affected blood urea (P = 0.002) and cholesterol (P = 0.023; Table 3).
301 Blood urea was significantly higher in fish fed Diet 1 (7.54 ± 0.25 mM) compared to those fed
302 Diet 2 (6.13 ± 0.25 mM). However, blood urea for fish fed Diet 3 (6.86 ± 0.25 mM) was not
303 significantly different to fish fed the other two diet types (Table 3). The blood cholesterol level of

304 fish fed Diet 1 (8.00 ± 0.22 mM) and Diet 3 (7.98 ± 0.22 mM) was significantly higher than fish
305 fed Diet 2 (7.18 ± 0.22 mM) (Table 3).
306 Triglycerides (P = 0.013) were significantly affected by feeding strategy (Table 3). Fish fed
307 at 80% satiation had greater triglycerides than fish fed at 100% satiation (Table 3). Other blood
308 parameters were not significantly affected by diet or feeding strategy.
309
310 **4. Discussion**
311 Results from the current study shows that high energy diets have the potential to be used in the
312 production of Yellowtail kingfish with little need to consider feeding strategy. Feeding strategy
313 has important production impacts, as Stone et al. (2016) reported Yellowtail kingfish fed higher
314 energy diets (Diets 1 and Diet 2) weighed significantly more, had greater SGRs and improved
315 condition factor when compared to fish fed lower energy diets (Diet 3). Also, fish fed at 100%
316 satiation weighed significantly more, were significantly longer, and had significantly greater
317 biomass than fish fed at 80% satiation (Stone et al. 2016). Similarly, juvenile Japanese yellowtail
318 (*Seriola quinqueradiata*, Temminck & Schlegel) also exhibited higher growth rate when fed at
319 100% satiation compared to fish fed 80% sub-satiation (Watanabe et al., 2000). All diets in the
320 present trial provided adequate nutrients for growth and health of the Yellowtail kingfish.
321 However, higher energy diets and 100% satiation delivered the greatest growth (Stone et al.
322 2016). Management of optimum feeding levels (FIR and FCR) based on the dietary energy
323 requirements of a species, should be considered if feeding at sub-satiation levels so that the most
324 efficient commercial outcome is achieved (Watanabe et al, 2000).
325 ***4.1. Histology and hepatosomatic index***
326 Although the livers in the present study were not observed to have steatosis, highest
327 amounts of lipids were stored in the livers of fish fed Diet 2, which contained high energy/lipid
328 levels. In the present trial, no other adverse health effects were observed in fish fed the high
329 energy Diet 2. Furthermore, growth performance and condition factor improved for fish fed the

high energy Diet 2 when compared to fish fed the lower energy Diets 1 and 3 (Stone et al. 2016). This indicates that early-stage grow out production of Yellowtail kingfish using a high energy diet may be suitable over an 84 day period. However, further research should be undertaken to observe what impact fatty livers, caused by high energy diets, may have on growth performance or health of Yellowtail kingfish over a longer period of time during different stages of commercial production.

The HSI was significantly greater in the fish fed the high energy diet, Diet 2, than in the fish fed the lower energy Diets 1 and 3. In the complementary study, fish fed Diet 2 had increased final weight when compared to fish fed Diets 1 and 3 (Stone et al., 2016). With both liver size relative to body size, and body size highest in response to the high energy diet, we see evidence of lipid availability exceeding the dietary requirement for energy use and growth, resulting in storage of lipids in the liver. This is similar to the response of pikeperch (*Sander lucioperca*, Linnaeus) when fed diets with excessive dietary lipid inclusion (Kowalska et al., 2011). Fish fed high energy diets in the current study performed at commercially acceptable growth rates (Stone et al., 2016). Therefore high energy diets may have positive implications on commercial production of Yellowtail kingfish. Given the relatively short duration of the growth trial compared to 2 years production cycles and the short duration of production cycles compared to the potential life span of Yellowtail kingfish , the finding of varying degrees of fatty livers may be of little consequence to fish harvested before any adverse effects of this condition influence growth performance. The implications of increased storage of lipids and subsequent increased HSI seen in the livers of sub-adult Yellowtail kingfish in the present study need consideration if high energy diets are to be successfully applied. The effects of feeding high lipid diets throughout the entire production cycle are currently unknown. With extended periods of exposure to high energy diets, the livers of marine fish have been reported to develop tissue degradation (Her et al., 2011). Future research should focus on periods of extended feeding from juvenile life stages

355 through to grow-out stages to ensure that high energy/lipid diets do not have a negative influence
356 on Yellowtail kingfish liver structure, and subsequently health and growth performance.
357 Marine fish species require MUFAs and PUFAs, particularly essential fatty acids EPA and
358 DHA, to sustain optimum health, growth, reproduction, immune defence and movement through
359 all life stages (Tocher, 2003; Trushenski & Lochmann, 2009; Geay et al., 2011). In a review of
360 literature, Stone & Bellgrove (2013) suggested that based on the known nutritional requirements
361 of Japanese yellowtail, Yellowtail kingfish post-juveniles require 2% dietary EPA and DHA to
362 meet their essential dietary requirements for growth. A recent study by Stone et al. (2020)
363 reported that the optimal dietary level of LC n-3 PUFA for large Yellowtail kingfish is between
364 2.12 and 2.26 g 100 g^{-1}. These levels were exceeded in this study for all test diets as shown in
365 Table 1. Using a grading system created by McFadzen et al. (1997), the accumulation of
366 hepatocytes containing optically empty content, identified as intracytoplasmic lipid droplets, was
367 greater in livers of fish fed Diet 2 than fish fed Diets 1 and 3. Fish fed Diet 3 showed a decreased
368 vacuolisation and an absence of intracytoplasmic lipid droplets indicating less lipid storage. In the
369 present study, the increased storage of lipid droplets in the liver of fish fed Diet 2 could be the
370 result of increased energy availability indicating an abundant supply of digestible lipids in the
371 diet. Future research should ensure that the essential dietary requirements for the n-3 PUFAs,
372 EPA and DHA, for Yellowtail kingfish are maintained when substituting or supplementing
373 dietary fish oil with alternative oils. Furthermore, future research should also focus on ensuring
374 that Yellowtail kingfish contains adequate levels of n-3 PUFAs for the consumer.
375
376 ***4.2. Blood biochemistry and haematology***
377 Haematology indices and biochemistry can provide information on fish condition, health,
378 nutrient status and digestive and metabolic function (Satheeshkumar, Ananthan, Senthilkumar,
379 Khan, & Jeevanantham, 2012). No significant difference was observed in the HCT of fish fed any
380 of the three experimental diets. Japanese yellowtail is known to be in an anaemic state when HCT

values are below 38% and in a healthy state when the HCT value is above 42% (Watanabe et al., 1998). The HCT values observed in all fish fed any of the three experimental diets were above 46% suggesting that, regardless of diet, all fish were in a good physical condition. Variations in lipid profiles between diets produced no discernible impact in this study. Similarly, rainbow trout (*Oncorhynchus mykiss*, Walbaum) fed diets containing a blend of fish oil and soy bean oil as a FO substitute saw no change in haemoglobin or HCT (Lu, Haga, & Satoh 2015). Likewise, no difference in haemoglobin or HCT was observed when coho salmon (*Oncorhynchus kisutch*, Walbaum) were fed a diet consisting of a blend of canola oil and flaxseed oil as a FO substitute (Twibell, Gannam, Hyde, Holmes, & Poole, 2012). Similarly, poultry oil, swine lard and beef tallow have been used to substitute FO in diets fed to rainbow trout with no effects on haemoglobin or HCT levels (Green and Selivonchick, 1990).

Cholesterol is essential for cell membrane growth and function (Liland et al., 2013; Norambuena et al., 2013). Plasma cholesterol levels were significantly greater in blood of fish fed Diets 1 and 3 when compared to fish fed Diet 2. Fish oil is known to contain high, although variable levels of cholesterol (Guerra-Olvera & Viana, 2015). Typical levels of cholesterol in FO range from 3.5 to 7.7 mg g^{-1} respectively (Turchini, Toretensen, & Ng, 2009; Norambuena et al., 2013; USDA, 2016). Diet 2 contained cholesterol levels greater than Diets 1 and 3 (Table 1), likely due to the higher total lipid level of Diet 2, compared to Diets 1 and 3. It is not possible to distinguish the source or contribution of cholesterol from the individual dietary ingredients used in this study. However, it is possible that the higher energy and cholesterol levels in Diet 2 resulted in a reduced synthesis of cholesterol *de novo* as a dietary excess was available (Norambuena et al., 2013). Plasma cholesterol can also be affected by the type of dietary lipids. When dietary lipid levels are held constant, an increase in plasma cholesterol levels may be observed when fish are fed FO as the main lipid source (Bowyer et al., 2012).

The greater growth of fish fed Diet 2 indicates that the cholesterol and energy available for metabolic processes, as well as growth, in this diet were sufficient for sub-adult Yellowtail

kingfish. The reduced gross energy levels, crude lipid level and dietary cholesterol in Diets 1 and 3 may have been contributing factors to the reduced growth observed in fish fed these diets when compared to Diet 2. Thus, reduced growth in the context of reduced dietary cholesterol may be explained by the metabolic expense required of these fish to synthesise cholesterol *in situ*. Similarly, rainbow trout fed diets containing FO with increasing levels of cholesterol inclusion showed positive increases in fish gross parameters when dietary cholesterol increased (Kaushik et al., 1995). Likewise, rainbow trout fed high cholesterol diets over 84 days showed no need for cholesterol synthesis as the dietary requirement was sufficient (Norambuena et al., 2013). However, in the same trial fish fed low cholesterol diets were capable of synthesising cholesterol *de novo* to a level where growth was not affected (Norambuena et al., 2013). Further trials should focus on the influence of dietary cholesterol at different concentrations to determine optimum inclusion levels for Yellowtail kingfish growth.

Conclusion

In the current study, higher dietary energy led to an excess amount of lipids being stored in the liver. This is evidence of increased energy availability, exceeding the dietary requirement for energy use. Results of liver histology indicate that despite fatty livers, using a high energy diet is suitable for the life stage of Yellowtail kingfish over the duration of the present trial. However, further research is required to understand the implications increased liver vacuolisation from high energy diets may have on the long term health and growth of Yellowtail kingfish over the extended production cycle. Complementary work indicated that increased dietary energy level (28% crude lipid) improved the overall growth performance and feed utilisation of sub-adult Yellowtail kingfish, compared to fish fed a 24% or 25% crude lipid diet. Likewise, fish fed at 100% satiation overall had improved growth performance and feed utilisation than fish fed at 80% satiation. Therefore, the use of high energy diets has the potential to be used in the production of Yellowtail kingfish. However, further research under commercial pilot scale

conditions is required to investigate liver structure and function to validate high dietary lipid diets prior to adopting this diet strategy to commercial production.

Acknowledgements

This research was supported by the Fisheries Research and Development Corporation and the Australian Seafood Cooperative Research Centre (AS-CRC) and seafood industry partner Clean Seas. The authors would like to acknowledge the support of the South Australian Research and Development Institute (SARDI) and Marine Innovation Southern Australia (MISA) for their financial support and the provision of the SARDI Aquatic Sciences, South Australian Aquatic Sciences Centre experimental facilities at West Beach. The authors would also like to thank the following people for their direct and valuable contribution: Dr Richard Smullen and Dr Simon Tabrett of Ridley Aquafeed (Ridley Aquafeed trading as Ridley Agriproducts Pty Ltd) for their input into experimental design, diet formulations and the manufacture of experimental diets. Yvette DeGraaf from Flinders Microscopy Biomedical Services for her assistance with histological processing. SARDI Staff, Wayne Hutchinson, Mark Guis, Paul Skordas, Krishna-Lee Currie, and Flinders University staff (Assoc. Prof. Kathy Schuller) and students including Jessica Buss, Duong Ngoc Duong, Thanh Haong Hai, Leigh Kuerschner and Arif Malik for their technical assistance. As the research in this manuscript drew on samples collected from a complementary study, this project was deemed by the Flinders University Animal Welfare Committee not to require ethical approval.

Data Availability Statement

The data obtained in this study are available from the corresponding author upon reasonable request.

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