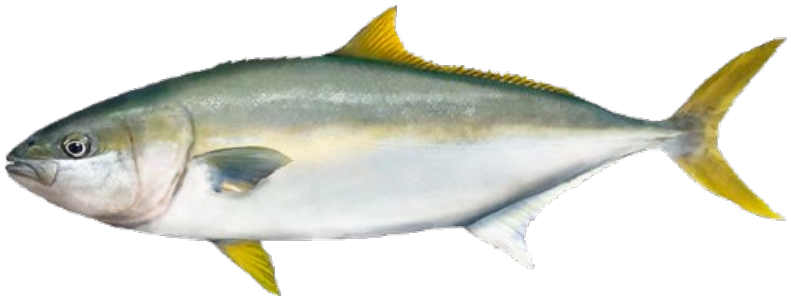
Optimizing dietary specifications for yellowtail kingfish, *Seriola lalandi*: requirements and interactions of sulfur amino acids and taurine



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Statement of the Contribution of Others

The following table describes the nature of contribution of co-contributors for this thesis.

Chapter	Title	Nature of contribution of co-contributors
1	General introduction	**Caroline L. Candebat:** Conceptualization, Writing - Original Draft **Igor Pirozzi**: Writing - Review & Editing, Supervision
2	Dietary methionine spares the requirement for taurine in juvenile yellowtail kingfish (*Seriola lalandi*)	**Caroline L. Candebat**: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing - Original Draft **Mark Booth:** Conceptualization, Resources, Writing - Review, Supervision, Funding acquisition **Mohamed Basseer Codabaccus**: Investigation, Writing – Review **Igor Pirozzi**: Conceptualization, Methodology, Formal analysis, Visualization, Resources, Writing - Review & Editing, Supervision, Funding acquisition
3	The sulfur amino acid requirements of juvenile yellowtail kingfish (*Seriola lalandi*)	**Caroline L. Candebat**: Conceptualization, Methodology, Investigation, Data processing and curation, Formal analysis, Visualization, Writing - Original Draft **Mark Booth**: Conceptualization, Resources, Supervision, Funding acquisition **Igor Pirozzi**: Conceptualization, Methodology, Resources, Writing - Review & Editing, Supervision, Funding acquisition
4	Chapter 4. Nutritional relevance of dietary methionine, cysteine, and taurine for hepatic, intestinal, and circulatory system in juvenile yellowtail kingfish (*Seriola lalandi*)	**Caroline L. Candebat**: Conceptualization, Methodology, Investigation, Data processing and curation, Formal analysis, Visualization, Writing - Original Draft, Funding acquisition **Frances Stephens**: Investigation, Formal evaluation, Writing - Review & Editing **Mark Booth**: Methodology, Funding acquisition **Fernando Fernando**: Resources, Data processing and curation, Writing - Review & Editing **Andreas Lopata:** Conceptualization, Review, Supervision **Igor Pirozzi**: Methodology, Resources, Writing - Review & Editing, Supervision, Funding acquisition

5	General discussion	**Caroline L. Candebat:** Conceptualization, Writing - Original Draft **Igor Pirozzi**: Writing - Review & Editing, Supervision **Andreas Lopata**: Review, Supervision

The following table shows the persons and organizations who have contributed to this thesis.

Nature of assistance	**Contribution**	**Name and Affiliation of co-contribution**
Intellectual support	Mentoring and peer support	**Dr. Leo Nankervis:** Senior lecturer, Aquaculture nutrition, James Cook University, Australia **Dr. Frances Stephens:** Consultant fish pathologist, previously employed by Department of Fisheries, Western Australia **Karen Reeks:** Lab manager, Veterinary Pathology Department, James Cook University, Australia **Walter Hautaniemi:** Lab technician, Veterinary Pathology Department, James Cook University, Australia **Dr. Michael Salini:** Ridley Agriproducts, Australia, provided ingredients for the manufacture of experimental diets **Dr. Angela Liu:** Lecturer, Sydney University, Australia **Julianna Kadar:** Shark biologist, Macquarie University, Sydney, Australia **Kunal Pratap**: PhD fellow, James Cook University **Steven Gamble:** Port Stephens Fisheries Institute, NSW **Fisheries Research and Development Corporation:** (1) Yellowtail kingfish Health Training Workshop, 10-11 September 2018 & (2) K4P Research Workshop 2018
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		bringing 'white' fish to the market" (RnD4Profit-14-01-027 7 & FRDC No. 2016-200.20).
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Seriola lalandi, Aquafeed, Sulfur amino acids requirements, Methionine, Cysteine, Taurine, Amino acid interactions, Non-linear regression modelling, Digestibility, Fish health, Histology

Australian and New Zealand Standard Research Classification – 2020		
Code	Fields of Research (FoR)	%
300303	Animal nutrition	50
300501	Aquaculture	30
300902	Veterinary anatomy and physiology	10
300910	Veterinary pathology	10

Abstract

The aquaculture production of the carnivorous yellowtail kingfish (*Seriola lalandi*; hereafter referred to as YTK) continues to expand, from commercial farming in Pacific waters off Australia, Japan, and the Americas to indoor recirculating aquaculture systems in Europe and the USA. Over the past decade, Australia's mariculture of YTK has become a growing contributor to global YTK production, reaching a yield of 3,000 metric tons and a value of $38 million in 2019. Its expansion is primarily driven by its meat quality, high market acceptance, and rapid growth. Nevertheless, production bottlenecks continue to hamper productions with Australian yields accounting for only a small proportion of the global YTK production.

Carnivorous fish species have a high dietary protein requirement compared to omnivores and herbivores, and, thus, rapidly growing juvenile YTK must be fed diets containing up to 50 to 60% crude protein. Fishmeal has always been considered the gold standard to satisfy the protein and amino acid requirements in carnivorous fish. However, the use of fishmeal from capture fisheries has become an unsustainable practice. Hence, aquafeeds are alternatively formulated with more sustainable proteins, including those derived from plants, and supplemented with crystalline amino acids to meet dietary requirements. Although plant proteins are an economical and arguably, a more environmentally viable source, the replacement of fishmeal protein by plant proteins in aquafeed can be challenging due to their differing properties. In contrast to fishmeal protein, plant proteins are often less digestible for carnivorous fish and are high in fiber, carbohydrates, and antinutrient constituents. Additionally, plant proteins often lack essential amino acids, such as methionine, required by carnivorous fish during the grow-out period. The

essential amino acid present in the lowest amount in feed limit productivity and the potential to grow in fish.

Methionine (Met), a sulfur amino acid (SAA), is often the first limiting amino acid in plant-proteins. Therefore, Met is routinely supplemented in aquafeed in its unbound and synthetic form. Presently, the industry is using the recommended level of Met for the closely related Japanese yellowtail (*S. quinqueradiata*) in YTK aquafeed, yet the dietary SAA requirement of YTK is unknown. The need to establish the dietary requirement for SAAs and taurine (Tau) may be especially important for YTK, as alternative protein sources and low-quality fishmeal may lack Met and Tau, relative to high-quality fishmeal. Thus, the sustainable and large-scale production of YTK requires the formulation of aquafeed that are tailored to species-specific nutrient requirements.

Met is an essential proteinogenic amino acid that needs to be obtained through the feed because farmed fish cannot sufficiently synthesize Met *de novo* to satisfy the minimum obligatory methionine (MOM) and total sulfur amino acids (TSAA) requirement. The MOM requirement can only be met by Met exclusively and not by any of its metabolites, such as S-adenosylmethionine or cysteine (Cys). Conversely, the TSAA requirement is defined as the total endogenous demand for Met and Met metabolites. This demand can be entirely met by dietary Met, provided that the MOM requirement is an integral part of the TSAA requirement, and the synthesis of metabolites from Met is unrestricted. A proportion of the TSAA requirement, that is completely satisfied by Met, is designated for the synthesis of Cys via transsulfuration. This proportion reflects the dietary Cys, which can spare dietary Met to satisfy the TSAA

requirement, also called cysteine sparing effect. Met is often the first limiting amino acid in formulated aquafeed with substantial plant protein inclusions. Therefore, the ability of Cys to spare a proportion of dietary Met to meet the TSAA requirement in YTK may be important in the efficient formulation of aquafeed. Both Met and Cys are metabolic precursors for the β-sulphonic amino acid Tau. The dietary requirements for Tau appear to depend on the species and life-stage capacity to produce enzymes that enable the synthesis Tau *de novo* from Cys. Thus, the intracellular availability of Met, Cys, and Tau is essential for the fitness and growth of farmed fish.

YTK's dietary requirements for the SAAs Met and Cys, and their amino-sulfonic acid derivative Tau, are unknown or have been investigated without consideration of the metabolic sparing concept within this nutritional group. Presently, dietary specifications for Met and Tau for the formulation of commercial YTK feed are based on published data of closely related *Seriola spp.* For Japanese yellowtail (*S. quinqueradiata*), the dietary Met specification is 11.1 g kg^{-1} diet, and for California yellowtail (*S. dorsalis*), the dietary Tau specification ranges from 2.6 to 10.2 g kg^{-1} diet. However, in the absence of species-specific research, it is unknown if the Japanese and Californian yellowtail dietary specifications for Met and Tau are suitable for YTK. Moreover, anecdotal reports from an Australian commercial YTK producer indicate that dietary Tau deficiencies may have caused green liver syndrome in YTK.

Thus, the subsequent chapters of this Ph.D. thesis were specifically designed to investigate these issues by determining the SAA and Tau requirements, interactions, physiological significance and effects on the intestinal and hepatic system. Specifically, the dietary requirement for Tau and

interactions with dietary Met, and the dietary requirement for Met and interactions with dietary Cys in juvenile YTK, were investigated. Further, tissue samples were collected for histological and biochemical examination. Quantification of the requirements and assessment of tissues provide optimum dietary SAA and Tau specifications for YTK aquafeed to promote good health and growth. This research is described in three chapters.

The first chapter provides an overview of fish nutrition, aquafeed, YTK aquaculture, sulfur amino acid and taurine requirements, and their role in maintaining intestinal and hepatic physiology. Three important knowledge gaps were identified and formed the following key aims of this thesis:

1. to determine the quantitative requirement for SAAs and Tau in juvenile YTK
2. to investigate the metabolic interactions of SAA and Tau and consequences for requirements in juvenile YTK
3. to understand the physiological significance of SAAs and Tau on the intestinal and hepatic status of YTK.

The second chapter of this thesis assesses the dietary sparing capacity of Met on the Tau requirement in juvenile YTK. Currently, Tau is utilized as a growth and health-promoting supplement in formulated aquafeed. Fourteen isonitrogenous (491.7 ± 1.6 g kg^{-1}) and isoenergetic (22.2 ± 0.1 MJ kg^{-1}) diets were formulated, applying an orthogonal dose-response design, and manufactured with raw ingredients and crystalline amino acids to meet dietary specifications. Each diet contained one of seven incremental levels of dietary Tau (1.6 to 20.4 g kg^{-1}) and one of two Met levels (low level: 10.9 ± 0.2 g Met kg^{-1} as per industry standard, or high level: 17.2 ± 0.6 g Met kg^{-1}). At the completion of the seven-week feeding trial, the

dietary Tau requirement in juvenile YTK fed dietary Met at 10.9 g kg^{-1} was found to be optimal for feed conversion ratio (FCR) and specific growth rate (SGR) at 6.2 and 7.7 g Tau kg^{-1} diet, respectively. The daily digestible Tau requirement at lower digestible Met (0.25 g $kgBW^{-1}$ d^{-1}) was found to be optimal for FCR and SGR 0.13 and 0.16 g $kgBW^{-1}$ d^{-1}, respectively. Yet, YTK with a digestible Met intake of 0.34 g $kgBW^{-1}$ d^{-1} and above the current industry formulation (17.2 g kg^{-1} diet) exhibited no need for dietary Tau supplementation and outperformed YTK fed the lower dietary Met series. Therefore, this study suggests that the dietary Met specification currently used by the YTK feed industry is insufficient to meet the SAA requirements of YTK. The study further suggests that dietary Tau is conditionally essential for juvenile YTK. Recommendations on the reassessment of current industry specifications for dietary Met based on these findings are made.

The need to reevaluate the recommended dietary Met in aquafeed for YTK led to the second feeding trial, which forms the third chapter of this thesis. This chapter aims to quantify the MOM requirement, TSAA requirement, and Cys's sparing capacity. Ten isonitrogenous (624.1 ± 8.2 g kg^{-1}) and isoenergetic (22.2 ± 0.1 MJ kg^{-1}) diets were formulated, again applying an orthogonal dose-response design, and manufactured with raw ingredients and crystalline amino acids to meet dietary specifications. Each diet contained one of five incremental levels of dietary Met (7.9 to 25.2 g kg^{-1}) and one of two levels of dietary Cys, where the lower dietary Cys series, at 5.6 g kg^{-1}, was representative of the TSAA requirement and the higher dietary Cys series, at 13.9 g kg^{-1}, was representative of the MOM requirement. At the completion of the eight-week feeding trial, the dietary Met requirement at low levels of dietary Cys (5.6 g kg^{-1}) in juvenile YTK for FCR, SGR, and protein retention efficiency (PRE) was met at 20.5, 18.4, and

17.8 g Met kg^{-1} diet, respectively. The daily digestible Met requirement at lower digestible Cys (0.14 g $kgBW^{-1}$ d^{-1}) was found to be optimal for FCR, SGR, and PRE at 0.60, 0.55, and 0.52 g $kgBW^{-1}$ d^{-1}, respectively. Hence, the average dietary and daily digestible TSAA requirements (Met + Cys) is 24.5 g kg^{-1} diet and 0.70 g $kgBW^{-1}$ d^{-1}, respectively. The dietary Met requirement at a higher level of dietary Cys (13.9 g kg^{-1}) in juvenile YTK was found to be optimum for FCR, SGR, and PRE at 13.9, 14.1, and 14.9 g kg^{-1}, respectively. The daily digestible Met requirement at a higher level digestible Cys intake (0.37 g $kgBW^{-1}$ d^{-1}) was found to be optimum for FCR, SGR, and PRE at 0.43, 0.41, and 0.43 g $kgBW^{-1}$ d^{-1}, respectively. Therefore, the daily average digestible MOM was 0.42 g Met $kgBW^{-1}$ d^{-1}, corresponding to a dietary MOM requirement of 14.3 g Met kg^{-1}. Measured requirements for both MOM and TSAA suggest that current Met and Cys inclusions for YTK commercial aquafeed do not satisfy requirements. The study recommended 18.9 g Met kg^{-1} diet and 5.6 g Cys kg^{-1} diet to meet the TSAA of 24.5 g kg^{-1} diet. Further, it was apparent that YTK fed diets with suboptimal and supraoptimal levels of SAAs exhibited inferior FCR and SGR. However, only YTK fed diets with suboptimal levels of SAAs developed clinical signs of cataracts.

The anecdotal reports on green liver syndrome and the occurrence of cataracts in YTK led to the fourth chapter of this thesis, the aim of which was to investigate the effects of different levels of Met, Cys, and Tau on the liver and posterior intestine. Hence, after completion of each feeding trial, the blood, liver, and posterior intestines of YTK were collected to investigate the effect of varying dietary SAAs and Tau on the plasma biochemistry, liver histology and surface color, and posterior intestine histomorphology and histochemistry. Observations of the homeostatic, metabolic, protective, digestive, and absorptive properties of the systems were used to evaluate

the health status of YTK and determine the extent to which suboptimal, optimal, and supraoptimal combinations of dietary Met, Cys and Tau may alter function. Results from the biochemical analysis of YTK blood plasma showed that:

1. YTK fed lower dietary Met at 10.9 g kg^{-1} diet had lower cholesterol and triglyceride levels, yet triglyceride levels increased with increasing dietary Tau
2. YTK fed higher dietary Met at 17.2 g kg^{-1} had elevated aspartate transaminase and lactate dehydrogenase activities, indicating their potential role in energetics and altered amino acid metabolism.

Results from the liver assessment showed that:

1. YTK fed diets containing adequate dietary Tau and Met had thinner intrahepatic bile duct walls
2. YTK had no segmental or diffuse green liver discoloration that was previously linked to green liver syndrome
3. YTK fed dietary taurine at 8.5 and 11.9 g kg^{-1}, satisfying the Tau requirement at a dietary Met content of 10.9 g kg^{-1} had 21% redder liver surface colors.
4. The reddest and brightest livers were from YTK fed dietary Met, Cys, and Tau at 18.8 g kg^{-1}, 5.2 g kg^{-1}, 11.7 g kg^{-1}, respectively, which were closest of matching all three requirements for MOM, TSAA, and Tau.

Assuming that redder livers are necessarily healthier, then satisfying SAA and Tau requirements may contribute to hepatic health.

Results from the posterior intestine assessment showed that:

1. YTK fed complete levels of dietary Met, Cys, or Tau had less acidic goblet cell mucus, more mixed and neutral goblet cell mucus, and less total goblet cell mucus production. This change in cell mucus profile indicates a shift in digestive and absorptive properties of the posterior intestine.
2. YTK fed more dietary Tau and Met had more posterior intestinal absorptive surface area.
3. YTK fed dietary Cys at 13.9 g kg^{-1} had reduced absorptive surface area, which marks an upper tolerated Cys threshold.
4. YTK fed the least dietary Tau (1.6 g kg^{-1}) and Met (12.0 g kg^{-1}) had more supranuclear vacuolization in villi tissue.
5. Increased supranuclear vacuolization, combined with the presence of low plasma triglyceride levels in YTK fed insufficient Tau and Met, indicate a decrease in lipid clearance from the intestinal enterocytes and, thereby, decreased energy available for metabolism.

Overall, YTK fed adequate dietary Met, Cys, and Tau that approximated the dietary MOM, TSAA, and Tau requirements exhibited good hepatic and posterior intestinal health, and a shift of intestinal functions toward improved nutrient digestion and absorption.

In conclusion, it was found that the intracellular availability of Met, Cys and Tau is critically important for maintaining good health, growth, and physiological homeostasis in juvenile YTK.

Additionally, it was found that the metabolic interactions and the sparing capacity of amino acids affect SAA requirements and alter the extent to which they must be supplied exogenously (i.e., sparing the requirement for Tau by Met and the sparing of TSAA requirement by Cys). Further, YTK appear to have the enzymatic ability to synthesize Tau *de novo* from Met and Cys, removing the need for Tau supplementation if adequate dietary SAAs are provided. These studies also suggest that adequate levels of dietary Met, Cys, and Tau shift functions toward improved nutrient absorption and the maintenance of physiological homeostasis in juvenile YTK. As individual elements of a cohesive whole, the research presented in this thesis provides data on the species-specific requirements for SAAs and Tau in juvenile YTK and has direct implications for the bespoke tailoring of aquafeed formulations to optimize growth, feeding efficiency, and health of farmed YTK. Finally, the nutrient requirement data may assist in the formulation of aquafeed using sustainable and economically viable source of proteins.

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List of Abbreviations and Symbols

#	Number of
%	Percent
~	Approximately
°C	Degrees Celsius
‰	Promille
AB+	AB+ goblet cell mucus
AB+PAS+	AB+PAS+ goblet cell mucus
ADC	Apparent digestibility coefficient
ADO	Cysteamine dioxygenase
AICc	Akaike's information criterion
Ala	Alanine
ALP	Alkaline phosphatase
AM	Ante meridiem
ANOVA	Analysis of Variance
Arg	Arginine
AST	Aspartate transaminase
Asx	Aspartate and Asparagine
AUD	Australian dollar
BW	Body weight
C	Cysteine
CAA	Crystalline amino acids
CDO	Cysteine dioxygenase
CEAA	Conditional essential amino acid
CHO	Cholesterol
CP	Crude protein
CSD	Cysteine sulfinic acid decarboxylase
CSIRO	Commonwealth Scientific and Industrial Research Organisation
Cys	Cysteine
D	Percent nutrient in diet
d	Days
DM	Dry matter
$\mathbf{D}_{\mathbf{marker}}$	Percent yttrium marker in diet
DNA	Deoxyribonucleic acid
DP	Digestible protein
NSW DPI	Department of Primary Industries, New South Wales, Australia

EAA	Essential amino acid
F	Percent nutrient in feces
FAO	Food and Agriculture Organization of the United Nations
FBW	Final body weight
FCR	Feed conversion ratio
FI	Feed intake
FM	Fishmeal
$\mathbf{F_{marker}}$	Percent yttrium marker in feces
g	Grams
g fish^{-1}	Gram per fish
g kg^{-1}	Grams per kilogram
GE	Gross energy
Glx	Glutamine and glutamate
Gly	Glycine
His	Histidine
HSI	Hepatosomatic index
IBW	Initial body weight
IFR	Intraperitoneal fat ratio
Ile	Isoleucine
IPFW	Intraperitoneal fat weight
K	Condition factor
kg^{-1} diet	Kilogram per diet
kgBW^{-1}	Per kilogram bodyweight
L	Litre
LD	Lactate dehydrogenase
LEA	Lamina epithelial area
LED	Light-emitting diode
Leu	Leucine
LPA	Lamina propria area
LW	Liver weight
Lys	Lysine
Met	Methionine
MI	Muscularis interna thickness
Min	Minimum
MJ kg^{-1}	Megajoules per kilogram
MR	Muscle ratio
MRE	Muscle retention efficiency

MxC	Interaction of methionine and cysteine
n	Sampling size
NA	Not Assessed
NaH_2PO_4	Monosodium phosphate
$NaHCO_3$	Sodium bicarbonate
NEAA	Non-essential amino acid
NFE	Nitrogen free extract
NS	Non-Significant
NSW	New South Wales, Australia
PAS+	PAS+ goblet cell mucus
PCoA	Principal coordinate analysis
pH	Scale used to specify the acidity or basicity
Phe	Phenylalanine
PIC	Posterior intestine circumference
PM	Post meridiem
Ppm	Parts per million
PRE	Protein retention efficiency
Pro	Proline
PSFI	Port Stephens Fisheries Institute, New South Wales, Australia
r^2	Coefficient of correlation
RAS	Recirculating aquaculture system
RFI	Relative feed intake
RNA	Ribonucleic acid
S	Submucosa including stratum compactum and stratum granulosum
SAA	Sulfur amino acids
SAM	S-Adenosylmethionine
SBM	Soybean meal
SD	Standard deviation
SEM	Standard error of the mean
Ser	Serine
S-PAS+	Small PAS+ bullet-shaped bodies
SPC	Soy protein concentrate
SV	Supranuclear vacuoles
TAN	Total ammonia nitrogen
Tau	Taurine
TGC	Total goblet cell mucus
Thr	Threonine

TIW	Total intestinal wall thickness
TL	Total length
TSAA	Total sulfur amino acids
TSAA (Met)	Total sulfur amino acid met by methionine
TSAA (Met+Cys)	Total sulfur amino acid met by methionine and cysteine
TVC	Total villi
TVH	Total villus height
TW	Total fish weight
TxM	Interaction of taurine and methionine
Tyr	Tyrosine
VA	Villus area
Val	Valine
Vit	Vitamin
VL	Villus length
VSI	Viscerosomatic index
VT	Villus tips
VW	Viscera weight
WG	Weight gain
Y_2O_3	Yttrium oxide

Glossary

Ad libitum	Until satiation
Amino acid	Organic compound that contains an amino, carboxyl, and a side chain group.
Aquafeed	Compound feed for farmed fish
Biomarker	Response variable that is used to measure optimal or suboptimal condition at given dietary specification
Broodstock	Fish raised for breeding purposes
Carbohydrate	Organic compound that includes sugars, starches, cellulose etc. often of plant origin
Cataract	Clouding of the lens that may lead to vision lost if untreated
Compound feed	Mixture of processed or unprocessed macronutrients from plant and animal sources that may contain additives. Often pellet-shaped for suitable fish consumption
Conditional essential	Substrate or metabolite that not necessarily needs to be obtained exogenously through the diet, yet might become limiting if metabolic precursors are not readily available to meet endogenous requirements
Conditionally essential amino acid	Proteinogenic amino acid that can be sufficiently synthesized *de novo* to meet endogenous requirements if precursors are sufficiently available
Cysteine	Conditionally essential sulfur amino acid that is required for the protein synthesis and is an intermediate metabolite for the taurine and glutathione synthesis
Cysteine sparing	The maximum proportion of the total sulfur amino acid (Met) requirement that can be substituted by cysteine instead of methionine. The designated cysteine proportion for the *de novo* synthesis of cysteine by dietary methionine
***De novo* synthesis**	Simple molecules are used for the synthesis of more complex molecules
Essential amino acid	Proteinogenic amino acid that cannot be synthesized *de novo* by the organism and has therefore be exogenously obtained through the diet to meet the endogenous requirements
Feed conversion ratio	The amount of feed that is required to grow a kg of fish
Formulated feed	A mixture of processed and unprocessed macronutrients and micronutrients that derive from plant, animal, and synthetic

	sources. Formulated feed were designed according to the animals dietary requirements and specifications
Homeostasis	Physiological equilibrium between interdependent elements for the maintenance of function and processes
MetCys study & M+C diet	Terminology to identify that diets and tissue that were assessed in chapter 5 originate from chapter 3
Methionine	Essential sulfur amino acid that is required in feed for normal growth and development of organisms
Minimum obligatory methionine requirement	The minimum quantity of methionine that needs to be exogenously provided to satisfy the methionine specific metabolic demands. Methodology: The dietary methionine intake at excess dietary cysteine, methyl donor etc. that satisfies the response criteria of the requirement study such as growth and feed conversion ratio. Assumption: None of methionine metabolites can spare or reduce the minimum obligatory methionine requirement if supplemented
Morphometric indices	Include HSI, VSI, MR, IFR and indicate tissue size proportional to whole body size
Physiology	Chemical and physical functions and mechanism that enable the survival, maintenance, repair, growth, and maturation of an organism
Protein	Complex structure made from proteinogenic amino acids
Salinity	Concentration of dissolved salts in seawater
Specific growth rate	The percent increase of fish biomass per day
Suboptimal	Below optimum
Sulfur amino acid requirement	Generic terminology referring neither to the TSAA requirement by solely methionine or by a combination of methionine and cysteine
Sulfur amino acids	Methionine and cysteine
Supraoptimal	Above optimum
Synthetic and crystalline amino acid	Free or non-bound amino acids
TauMet study & T+M diet	Terminology to identify that diets and tissue that were assessed in chapter 5 originate from chapter 2
Taurine	β-sulphonic amino acid and metabolic of the trans-sulfuration pathway of methionine and cysteine
Taurine requirement	The endogenous requirement for dietary taurine due to the species inability to synthesize it *de novo* from methionine and cysteine.

Total sulfur amino acid (Met) requirement	The requirement for dietary sulfur amino acids that can be completely met by dietary methionine. Methodologically: The dietary methionine intake in the absence of dietary cysteine that satisfies the response criteria. Assumption: Methionine can be converted to cysteine as needed to meet the endogenous cysteine requirement
Total sulfur amino acid (Met+Cys) requirement	The requirement for dietary sulfur amino acids that can be met by dietary methionine and cysteine. Methodology: The dietary methionine and cysteine intake that satisfies the response criteria. Assumption: Methionine and cysteine are metabolically connected
Viscera	All organs in fish cavities, including heart, spleen, gallbladder, liver, intestine, stomach, fat

Chapter 1. General introduction

The global population is growing toward 10 billion people by mid-century, 27% more than today's figure (United Nations, 2019). The unimpeded growth raises the question how the food-producing sectors will meet the growing demand for protein foods from animals of almost 70%, while reducing the ecological impact (Searchinger et al., 2018). Fish forms an essential source of high-quality proteins and surpasses the per-capita protein consumption of any other animal origin (Tacon, 2020; Tacon et al., 2020). About half of the seafood consumed is now derived from aquaculture, which continues to be the fastest-growing food-producing sector, reflecting annual growth of 5.3% and tripling the fish production over the past 20 years (FAO, 2020; Lynch et al., 2017). To sustain this rapidly growing industry and ensure the future of food security, it is critical to continue developing knowledge and implementing innovative technologies. One of the most important implementations in aquaculture is to optimize fish nutrition to exploit the full potential and productivity of farmed fish.

In 2018, 70% of farmed fish relied on intensive feeding practices, whereas 80 to 100% of salmon, trout, and marine fish relied on formulated aquafeed (FAO, 2020; Tacon, 2020). Aquafeed is the most costly and sensitive variable in the aquaculture sector, accounting for up to 70% of operating costs (Losordo & Westerman, 1994; Rubino, 2008; Tacon, 2005). Presently, knowledge gaps in fish nutrition pose production bottlenecks that require research to meet species- and life-stage-specific feed and nutrient requirements. Innovations and developments in fish nutrition research directly assist in meeting the protein demands of the growing population, increase the commercialization of aquaculture species, and reduce environmental impacts (Jones et al., 2015).

Traditionally, fishmeal has been considered the most suitable protein ingredient for aquafeeds, primarily because of its suitable amino acid profile, good acceptance and high digestibility, particularly for carnivorous species (Glencross et al., 2007). However, fluctuating prices, limited supply, and continuous impact on the environment have led fish nutrition researchers to study viable alternative protein and amino acid sources to meet the requirements of target species (FAO, 2020; Glencross et al., 2007). The perception that aquafeeds with high fishmeal content are the only means to increase yields and profits in carnivorous fish has changed, and there is now an appreciation that formulated aquafeeds with proteins designed to meet species- and life-stage-specific amino acid requirements may suffice (Nunes et al., 2014).

Of the myriad of identified amino acids, only 20 amino acids form the standard building blocks of proteins. However, not all proteins contain these 20 amino acids in equal amounts; certain amino acids may be lacking. Sustainable proteins and even low-quality fishmeal make aquafeeds susceptible to essential amino acid deficiencies for farmed, carnivorous fish species and life-stages for which quantitative requirements have not yet been established. Essential amino acids need to be provided exogenously in feed as fish are unable to satisfy the endogenous requirement through *de novo* synthesis. In aquafeed, the most common limiting amino acid is methionine, a precursor of the *de novo* synthesis of cysteine. The intracellular availability of both sulfur amino acids is important for the fish's productivity. It enables the synthesis of other functional metabolites such as the β-amino sulfonic acid taurine, which is abundant in fishmeal, but is often lacking in alternative proteins. The formulation of aquafeed using synthetic amino acids and organic supplements such as crystalline methionine and taurine to address deficiencies or promote

growth has become common practice. However, the industry relies on quantitative requirement data to tailor formulated aquafeeds that meet the dietary requirements of target species, to improve feed conversion efficiency, and ultimately to reduce operating costs. Presently, quantified nutrient requirements of farmed fish are limited to domesticated freshwater fish or salmonids. Additionally, dietary requirements are often adapted from closely related species or species with apparently similar feeding preferences (Candebat et al., 2021, 2020). Therefore, there is a need to expand our understanding of the nutritional requirements of farmed target species. Such understanding allows optimal matching between the nutritional profiles of formulated aquafeeds and target species, while minimizing their impact on the environment (Rust et al., 2011; Tacon et al., 2021).

1.1 Yellowtail kingfish

1.1.1 Biology

Seriola lalandi (Valenciennes 1833) is hereafter referred to by the common Australian name "yellowtail kingfish", also known as kingfish, hiramasa, gold-striped amberjack, and yellowtail amberjack. Yellowtail kingfish (YTK) of the *Seriola spp.* Genus and *Carangidae* family are pelagic schooling fish that inhabit subtropical to temperate waters of the southern Pacific (**Figure 1.1**; Craig et al., 2015; Premachandra et al., 2017). In Australian waters, two genetically distinct stocks inhabit kelp beds and rocky areas of eastern to southern coastal waters and western coastal waters (Miller et al., 2011). The optimal temperature for YTK is 22.8°C (Pirozzi and Booth, 2009), yet YTK grow well between 18 and 24 °C (Booth et al., 2010; Gommon et al., 2008; Pirozzi et al., 2019). YTK reach maturity at a minimum length of 83 cm in females and 55 cm in males (Stewart et al., 2008). YTK are carnivorous and feed off smaller fish, squid, and crustaceans (Baldwin, 2003). Similar to other species from the *Seriola spp.* Genus, YTK have a high metabolic rate, protein demand and high energy expenditure (Donohue et al., 2021; Pirozzi & Booth, 2009).

1.1.2 Taxonomy

Presently, proposed genetic and taxonomic differences between the California yellowtail and yellowtail kingfish, and consequently nutrient requirements, have led to some ambiguity. The California yellowtail, found in the northeast Pacific and known as *Seriola dorsalis* and *Seriola lalandi*, and yellowtail kingfish, which is found in the southern hemisphere of the Pacific and is also known as *Seriola lalandi*, have identified genetic differences. However, it has been suggested

that the magnitude of these genetic differences only indicates a population shift of the same species (Premachandra et al., 2017) rather than a genetic species shift (Craig et al., 2015). It remains unknown if the genetic differences were sufficient to cause the differences in nutrient requirements observed in this thesis between the California yellowtail and yellowtail kingfish.

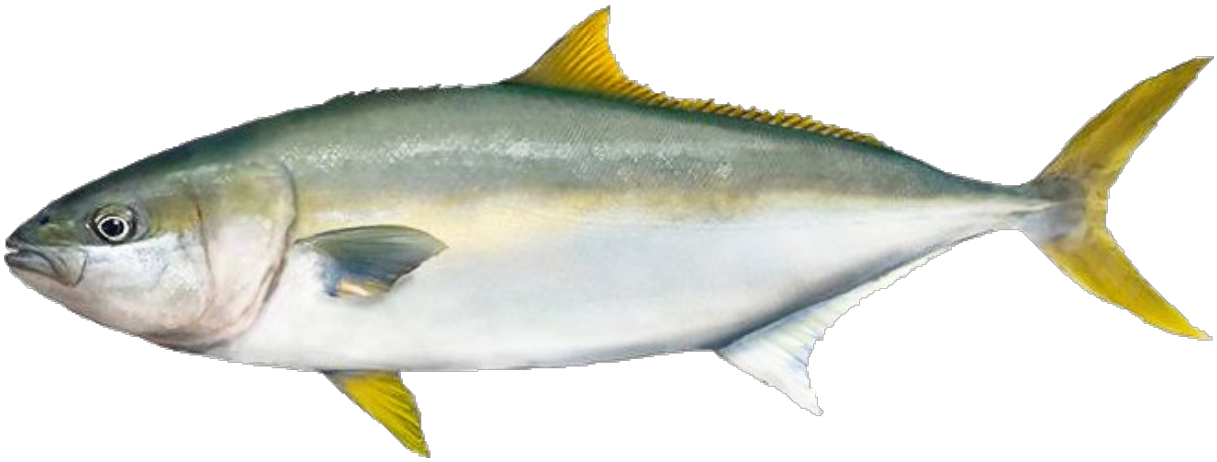

Figure 1.1 Yellowtail kingfish, *Seriola lalandi* (Valenciennes, 1833).

1.1.3 Aquaculture production

The aquaculture production of Japanese yellowtail (*S. quinqueradiata*) began in 1950 in Japan, which collection of primary data on culture and feeding laid the foundation for the aquaculture industry (Sapra, 2020). In Australia, the commercial production of YTK has been motivated by the species' rapid growth rates, meat quality, market acceptance and exclusiveness. Commercial developments over the past two decades have paralleled that of other *Seriola* species (Clarke et al., 2019; Roo et al., 2014; Sicuro & Luzzana, 2016). Presently, California yellowtail (*S. dorsalis*), greater amberjack (*S. dumerili*), Japanese yellowtail, and longfin yellowtail (*S. rivoliana*) are commercially farmed in sea cages in the Mediterranean Sea and in the Pacific Ocean along the

coastal waters of the Americas, Australia, East and Southeast Asia and South Africa. However, in Europe, South America and more recently in the USA, YTK are reared indoors in recirculated aquaculture systems (RAS) facilities (Bever, 2021; Sicuro & Luzzana, 2016).

The YTK aquaculture industry in Japan, has reached 4,600 ton of YTK in 2014, which in Japan form only a fraction of the 107,059 tons yield of Japanese yellowtail (Sicuro & Luzzana, 2016). The Australian YTK production has reached 3,000 tons in 2019 (Clarke et al., 2019; Savage and Hobsbawn, 2015). However, during the fourth quarter of the COVID-19 pandemic, the sales of Australian farmed YTK increased globally by 31% in both domestic and international markets, which spurred the Australian YTK aquaculture industry to expand its target production to 10,000 tons (Gezelius, 2021). Presently, the exclusive white flesh of YTK forms the counterpart to the highly available red flesh of salmon in the Australian sushi and sashimi food market (Clarke et al., 2019; Jirsa et al., 2011). Whole YTK is available for mainstream consumers at about AUD 28 kg^{-1}, whereas whole salmon and barramundi are already available at about AUD 20–21 kg^{-1} (Seafood and More, 2021).

Over the past 15 years, the nutritional requirements of YTK have been extensively studied, and the use of alternative proteins to fishmeal has been shown to be well tolerated by YTK. However, most essential nutrient requirements of YTK are still unknown, which complicates the nutrient-based formulation of aquafeed using proteins other than from fishmeal to meet YTK dietary requirements (Clarke et al., 2019). These factors limit the mariculture of YTK in Australia.

1.1.4 Nutritional requirements of farmed yellowtail kingfish

YTK are highly pelagic and active, with an increased metabolic rate in comparison to more sedentary fish species such as Mulloway (Pirozzi & Booth, 2009). Thus, YTK require feed that can sustain high metabolic rates and meet consequentially high nutrient requirements. Since the start of YTK farming in Australia, several studies have been conducted examining the nutrient requirements needed to improve feed conversions and to maximize productivity (e.g., Booth et al., 2010; Booth & Allan, 2013; Dam et al., 2019; Donohue et al., 2021; Bowyer, 2012). However, compared to other carnivorous species such as salmon, there are still profound gaps in the nutrient requirement profile of YTK that must be addressed for the nutrient-based feed formulation for YTK.

Cultured YTK are fed compound feed that are mostly designed to meet the generic nutrient requirements of the *Seriola* genus. However, the specific macronutrient and energy requirements for YTK have been assessed. The different life-stages of grow-out YTK require different levels of dietary proteins and energy (Booth et al., 2010). Thus, YTK of 50 g and 2 kg require a daily digestible intake of 31.1 and 452.0 kJ $fish^{-1}$ d^{-1}, and 1.35 and 11.01 g CP $fish^{-1}$ d^{-1}, respectively. Thus, it was recommended that YTK of ≤ 200 g, 200 g to 1kg, and ≥1 kg should be provided a digestible energy content of 12, 15, and 18 MJ DE kg^{-1} diet and digestible protein content of 456, 465, 432 g DP kg^{-1} diet for good growth (Booth et al., 2010). Results from the latter study highlight the importance of high-quality and suitable protein sources for YTK aquafeed to ensure the well-being of the animal as well as optimizing the productivity of farms. As previously reported, YTK are carnivorous and have a low tolerance for dietary carbohydrates. The inclusion

of carbohydrates in YTK aquafeed can induce hyperglycemia. However, pregelatinized wheat starches are tolerated (Booth & Allan, 2013). At present, and excluding the publications from this thesis, there are no requirement studies on the essential amino acids in YTK except published data from this thesis.

1.2 Proteins and amino acids in fish nutrition

Farmed fish, like any other organism, rely on the general principle that substances must be exogenously ingested and assimilated to provide energy and substrates for a variety of functions, the simplest being the maintenance of life and growth. Fish and other vertebrates depend on a variety of essential dietary nutrients that include amino acids, fatty acids, sugars, minerals, vitamins, and water to meet vital requirements (Rust et al., 2011). However, the macronutrient composition and ingredient origin of aquafeeds often place different and specialized demands on the digestive and absorptive capacities of fish that are dictated by the anatomical and physiological traits of the tissue. These traits often correlate with the food source that the natural habitat offers (Rønnestad et al., 2013; Sundell & Rønnestad, 2011), although the continued domestication of farmed fish in recent decades, including salmon, has extended the acceptance of commonly not tolerated proteins (Lorenzen et al., 2012).

The diverse aquatic habitats of fish result in a variety of trophic specialists and generalists that feed of plant material (herbivorous), plankton (planktivorous), detritus (detrivorous), other animals (carnivorous) and a mixture of plant and animal food sources (omnivorous) (Olsson, 2011). Therefore, there is no universal food that is suitable for all fish species. Fish that feed on

plant material or a combination of plant and animal material normally have longer intestines, extending processing times, and can digest plant material to utilize carbohydrates as an energy source. However, carnivorous species have shorter intestines and access energy more easily from proteins of animal origin (Kamalam et al., 2017; Kaushik & Seiliez, 2010; Olsson, 2011). However, this distinction between herbivorous and carnivorous fishes is subject to variation, as other factors such as stomach size, the presence of a pyloric caeca and large stomach, and enzyme capacity may affect the ability to digest certain foods (Buddington et al., 1997; Olsson, 2011). Regardless of species-specific food preferences, the general consensus is that fish require a richer crude protein diet (Kaushik & Seiliez, 2010) than terrestrial livestock such as pigs and chickens (Miller, 2005) to meet their dietary requirement for essential amino acids.

Organisms have a diverse range of internal proteins, comprised of proteinogenic amino acids in different combinations and sequences (Halver, 2002; National Research Council, 2011a). Endogenously, proteins perform a wide variety of functional roles, such as repairing and forming tissue, storing, and transporting molecules, providing energy, catalyzing biochemical reactions, controlling cell signaling and cycles (National Research Council, 2011a). Yet, despite numerous protein requirement studies, fish do not have a dietary protein requirement *per se* but rather have a requirement for its constituent amino acid building blocks.

1.2.1 Protein digestibility and amino acid bioavailability

The constitution, origin, and processing of proteins can impact individual amino acid bioavailability (Gaylord & Gatlin, 1996; Glencross et al., 2007). Presently, digestibility data for macronutrients and micronutrients form a critical source for the nutrient-based formulation of

aquafeed to ensure adequate nutrient levels, efficient feed conversion ratios and to reduce nitrogenous and phosphorous waste (Dam et al., 2019; Gaylord & Gatlin, 1996). For example, soybean protein concentrate can match the crude protein content of fishmeal, but the species-specific ability to digest soybean protein may limit the bioavailability of its full nutritional profile (De Silva & Anderson, 1995).

Generally, plant-based proteins are often difficult to digest for carnivorous fish (Halver & Hardy, 2002). However, carnivorous fish such as rainbow trout (*Oncorhynchus mykiss*) and cobia (*Rachycentron canadum*) have been shown to digest corn gluten well with apparent digestibility coefficients (ADC) of e.g. 95% and 94% respectively (Yamamoto et al., 1998; Zhou et al., 2004). Japanese flounder (*Paralichthys olivaceus)*, YTK, and Japanese yellowtail have ADCs of 79% (Kim et al., 2010), 31% (Dam et al., 2019) and 50% (Masumoto et al., 1996) for corn gluten protein.

In addition to potentially low digestibility, methionine and lysine are often the first limiting amino acids in plant-derived proteins and ADCs low for carnivorous species. The ADCs for soybean protein concentrate derived methionine for Japanese flounder, YTK and Japanese yellowtail are 61%, 67% and 87%, respectively, whereas the ADCs for lysine are 83%, 58% and 91%, respectively. This stands in contrast to the ADCs for methionine (92%, 80%, 92%) and lysine (94%, 63%, 93%) from fishmeal in Japanese flounder (Deng et al., 2010), YTK (Dam et al., 2019) and Japanese yellowtail (Yamamoto et al., 1998), respectively.

Nutrient requirements are often assessed by correcting the nutrient specifications proportional to its digestibility (Booth et al., 2010; Lupatsch et al., 1998). This is done by assessing the ADC of the experimental diets, which is then used to correct the dietary intake of the nutrient of interest. A common method to determine the ADC is to measure the difference in concentration of nutrients in ingested feed and then in fecal material (Moyano et al., 2015).

1.2.2 Protein sources for aquafeeds

1.2.2.1 Fishmeal

For the culture of carnivorous and omnivorous fish, fishmeal is still considered the most nutritious and digestible protein option yet is the most expensive and volatile aquafeed ingredient (FAO, 2020; Glencross et al., 2007; Tacon et al., 2009). Seafood from capture fisheries has been virtually static at 96 million tons since the early 2000s, of which 33% of fish stocks are currently overfished, and 67% are fully exploited (FAO, 2020). Since 2000, fishmeal prices have tripled from USD 444 to USD 1,367 per ton in 2019 (FAO, 2021; IndexMundi, 2021). Subsequently, within the 18 months following the start of the global COVID-19 pandemic, fishmeal prices have increased by an additional 10% (IndexMundi, 2021).

From 2000 to 2010, the global expansion of the aquaculture industry has also increased the demand for aquafeed and, therefore, fishmeal. As a result, the demand for fishmeal increased by 40%, despite intentions to move away from capture fishery resources for aquafeeds. From 2010 to 2018, the use of fishmeal in aquafeeds only increased by 2%, which may reflect the 20-year transition and change in the aquaculture supply chain toward increased production of omnivorous

species, overall improved feed conversion, diversification of feed with a variety of protein sources from plant and animal by-products, and the integration of fishmeal from trimmings (Hall et al., 2011; Naylor et al., 2021; Shannon & Waller, 2021; Shepherd & Jackson, 2013).

Major investments in fish nutrition research on carnivorous fish species such as Atlantic salmon (*Salmo salar*), rainbow trout, and European seabass (*Dicentrarchus labrax*) led to the modification of feed from an animal-protein-based feed to a plant protein-based feed (Teletchea & Fontaine, 2014). Eventually, in 2016, fishmeal only contributed 10% of the protein in salmon feed (FAO, 2020) and 0% in Skretting's Premium FLX feed (Nutreco, 2016), demonstrating that aquafeeds are moving away from their reliance of capture fisheries proteins.

1.2.2.2 Plant, terrestrial livestock, insect, and algal proteins

The discovery and utilization of novel protein sources are growing, yet scalable and sustainable implementation of these novel proteins are still in progress. Plant protein sources such as soybean meal have become an integral part of aquafeed formulation due to consistent price and supply. However, plant proteins such as soybean meal may pose a risk to fish welfare and production. Even small inclusions of plant proteins can induce detrimental effects on fish liver and gut health by altering the intestinal microbiome and morphology, immune and endocrine system and maturation (Raskovic et al., 2011; Simó-Mirabet et al., 2018). These changes were found to be due to high levels of dietary fiber and carbohydrates (Gatlin et al., 2007), indigestible antinutrients (Francis et al., 2001), variable digestibility (Dam et al., 2019), poor palatability (Davis et al., 1995), and unsuitable amino acid profiles (Kaushik & Seiliez, 2010).

The underlying mechanisms of these diet-induced pathological conditions are not well understood, and information on how and to what extent each specific plant protein component or noxious agent induces or modifies pathological conditions is limited. Although European seabass supported 93% of proteins from plant sources based on gross biometrics (Kaushik et al., 2004), the pathological evaluations using 86% proteins from plant sources demonstrated intestinal alterations in mucus production, submucosal layers and lamina propria, indicating inflammatory responses (Torrecillas et al., 2017) possibly inducing long-term clinical symptoms. The etiology of these pathological conditions due to plant protein inclusions is not yet fully understood. However, data on the relationship between amino acid dose and fish health using quantitative histology may clarify how the tissue functionality is altered.

Concentrated plant proteins, such as soy protein concentrate, have been shown to be nutritionally more suitable yet are more expensive than fishmeal (Rust et al., 2011). Further, soybean is currently the subject of debate because its increasing utilization is associated with ecotoxicity, increased pressure on the carbon footprint, freshwater and land resources, and biodiversity loss from forest clearings in Brazil (Naylor et al., 2021). As a result, plant-based ingredients that have been in demand over the past decades have become sources of unintended environmental and social stress. Aquafeed producers are, therefore, looking for new, more sustainable protein sources (Naylor 2021).

Algae, such as spirulina and chlorella, are a novel plant protein source with promising nutritional value that could replace terrestrial plant and animal proteins (Shields & Lupatsch, 2012). However, algal production requires further research to overcome limitations in supply and ensure

competitiveness and efficacy across taxa and life-stages (Shields & Lupatsch, 2012). Proteins from terrestrial livestock, including meat, blood, feathers, and other by-products, form another integral part of current aquafeeds. The utilization of by-products and waste material from processing industries demonstrate several advantages over plant proteins, including the absence of antinutritional factors and a comparable nutritional value to resources from capture fisheries. However, disadvantages of this resource include high contents of less bioavailable collagen and ash content and limited use due to safety and public concerns (Bureau et al., 1999; Tacon et al., 2021).

More recently, insect proteins derived from mealworm, black soldier fly, silkworm pupae and housefly, were found to be of interest for the aquaculture industry due to their quick life-stage turnover, easy reproduction, low feed conversion ratio, low production costs, and low environmental footprint (Basto et al., 2020; Bosch et al., 2019; Henry et al., 2015). However, high chitin levels, dietary supplementation of feed, amino acid deficiencies of meals, and bioaccumulation of insecticides and other toxins in meals are of concern and require further assessment (Henry et al., 2015; Rust et al., 2011).

1.2.3 Synthetic and crystalline amino acids in aquafeed

Proteins from sources other than fisheries have become an integral part of aquafeed. However, calibrating for limiting amino acids may lead to an overload of crude protein, non-essential amino acids, and harmful substrates such as antinutrients (Nunes et al., 2014; Selle et al., 2020). The cost-effective production and isolation of amino acids now enable the use of synthetic and crystalline amino acids to fine-tune the nutrient-based formulation of aquafeeds to mitigate

against the effects of incomplete proteins (Leuchtenberger et al., 2005; Nunes et al., 2014; Selle et al., 2020). Crystalline and synthetic amino acids are increasingly included as nutraceuticals in animal feeds, and aquafeeds (Alagawany et al., 2020; Varghese et al., 2021), which concept appears to overlap that of functional amino acids (Aronson, 2017; Wu, 2013). Unbound amino acids are produced either via extraction from protein hydrolysates, such as crystalline L-cysteine from feathers, or via chemical synthesis to produce racemic mixtures, such as synthetic DL-methionine (Selle et al., 2020). Seemingly, crystalline amino acids, such as lysine, methionine, threonine, and tryptophan, prevent diseases, increase immunomodulatory potential, health benefits and can reduce the use of antibiotics (Alagawany et al., 2020; Varghese et al., 2021). Methionine and lysine are often the first limiting amino acids in incomplete proteins. Consequently, they are among the most utilized crystalline amino acids for nutrient-based formulations (Nunes et al., 2014).

1.2.4 Essentiality and significance of amino acids

To meet the amino acid requirements of farmed fish, the proportion of protein in aquafeed can range from 30 to 60%. Of the myriad amino acids, only 20 form the standard primary structure of proteins (Brosnan & Brosnan, 2006). Nutritionally, these 20 proteinogenic amino acids are divided into the following categories:

1. Essential amino acids, which must be obtained exogenously through the feed due to the fishes inability to synthesize the amino acid *de novo* or at a speed that cannot satisfy the requirement for growth.

2. Semi-essential amino acids, which may need to be obtained exogenously through feed as their endogenous *de novo* synthesis strongly depends on the availability of their metabolic precursors.
3. Non-essential amino acids, which can be sufficiently synthesized *de novo* to meet requirements (Reeds, 2000).

The continuous supply of ten of these 20 amino acids is essential in aquafeed to ensure the production of semi-essential and non-essential amino acids and metabolites (**Table 1.1**). Knowledge of the nutritional essentiality of these 20 proteinogenic amino acids has remained relatively consistent since 1986 (Li et al., 2009b; Wilson & Halver, 1986). However, the classification of amino acids by the fish's metabolic ability and nutritional value is constantly under revision. Numerous studies have demonstrated that the significance of proteinogenic amino acids exceeds that of buildings blocks for growth. Therefore, the concepts of functional and nutraceutical amino acids were introduced, highlighting the pivotal role amino acids play in maintaining and regulating systemic functions, metabolism, reproduction, and immune responses such as those of the liver and intestine (Andersen et al., 2016; Wu, 2020, 2013, 2010, 2009).

Table 1.1 Proteinogenic amino acid classification by metabolic and nutritional essentiality in fish. Adapted from (Li et al., 2009b).

Essential amino acid	Semi-essential amino acid	Non-essential amino acid
Arginine	Cysteine	Alanine
Histidine	Glutamine	Asparagine
Isoleucine	Proline	Aspartate
Leucine		Glutamate
Lysine		Glycine
Methionine		Serine
Phenylalanine		Tyrosine
Threonine		
Tryptophan		
Valine		

Note. The essentiality of proteinogenic amino acids can fluctuate according to the species, environment, and life-stage.

In fish, 56 to 88% of assimilated and endogenously degraded proteinogenic amino acids are used for protein synthesis, forming a protein pool that provides for systemic net growth and maintenance (Houlihan et al., 1995). To date, amino acid requirements are quantified primarily measured through growth and feeding efficiency of fish fed different levels of nutrients, while responses of systemic function such as of liver and blood are used as supplementary metrics (Cowey, 1994). The limited use of response variables that reflect amino acid requirements for the maintenance of physiological and metabolic homeostasis may be due to the myriad amino acid functions and limited knowledge of amino acid fluxes (Reeds, 2000). Yet, systemic maintenance can account for a large proportion of the protein budget (Andersen et al., 2016). Amino acids and metabolites have shown to be of physiological significance in the preservation of functions for a variety of tissue systems in different species, including the digestive, absorptive, protective, metabolic, cleansing and defensive functions of the intestine and liver (Li et al., 2007; Wu, 2020).

For example, in pigs, 20% or more of the available amino acid pool is retained from the feed in the intestinal enterocytes and does not enter the animal's circulation. This amino acid store assists in developing, fueling and maintaining enterocyte function and shaping and regulating the intestinal mucosa (Kong et al., 2018; Sundell & Rønnestad, 2011; Wu, 2010).

Data on the effects of dietary amino acids at suboptimal and supraoptimal levels on systemic fish function and pathology are limited. Yet, the widespread and large-scale use of alternative proteins and crystalline amino acids may lead to over- or underestimation of dietary specifications due to the lack of single or multiple amino acids and limited information on their bioavailability (D'Mello, 2003; Oliva-Teles, 2012; Peres & Oliva-Teles, 2005). In salmonids and carp, dietary lysine, methionine and tryptophan deficiencies led to increased mortality, caudal fin erosions, bilateral cataract and scoliosis, whereas excess dietary levels of leucine in rainbow trout induced scoliosis, deformed opercula, scale deformities, scale loss, and spongiosis (Tacon & FAO, 1992). It is important for farmers to supply an aquafeed to YTK that can maintain and positively affect the functioning of the gastrointestinal and hepatic systems to maintain good health and exploit the full potential of productivity in YTK.

1.3 Sulfur amino acids and taurine

1.3.1 In proteins

Fishmeal as the main protein source for aquafeed formulations has been steadily replaced, even in aquafeed formulated for carnivorous fish (Oliva-Teles et al., 2015). Proteins from sources other than fish are more sustainable and price stable, but the lack of one or more amino acids limit

their implementation in aquafeed. One of the first limiting essential amino acids in proteins from plants, terrestrial livestock and insects is methionine, which requirement is reflected in the sum of the sulfur amino acids, methionine, and cysteine (**Table 1.2**). Thus, correct proportion and total sum of methionine and cysteine are required to support optimal growth in fish. Taurine is another critical nutrient for several marine carnivorous species and is often lacking in proteins from non-animal sources. The methionine, cysteine and taurine moieties of these proteins are often substantially lower relative to fishmeal (**Table 1.2**). It should be noted that the composition and amino acid profile of proteins depends on various factors, such as the ingredient species, place of cultivation, season, and processing method, and may be subject to variation even within the same product (Glencross et al., 2007).

Table 1.2 Dry matter contents (%) of crude protein, methionine, cysteine, and taurine in various proteinogenic ingredients that are commonly used for the formulation of aquafeeds and proportional contents to the average contents of fishmeal (average Met content= 1.9 g kg^{-1}; average Met+Cys contents =2.5g kg^{-1}).

Aquafeed protein ingredients		CP[1]	Met[2]	Cys[3]	M+C[4]	M % FM[5]	M + C % FM[6]	Tau[7]	Ref.[8]
Terrestrial plant sources	Corn gluten	58–75	1.10	0.30	1.40	58	57	-	Gorissen et al. (2018)
	Corn gluten	65	1.27	0.93	2.20	67	90	0.00	Unpublished 2021
	Corn gluten	71	1.52	0.94	2.46	80	101	0.00	Dam et al. (2019)
	Lupin	61	0.20	0.20	0.40	11	16	-	Unpublished 2021
	Lupin kernel (dehulled)	39	0.31	0.71	1.02	16	42	0.01	Dam et al. (2019)
	Soybean meal	47	1.03	0.93	1.96	54	80	0.00	Unpublished 2021
	Soybean meal	61–91	0.30	0.20	0.50	16	20	-	Gorissen et al. (2018)
	Soybean (dehulled)	54	0.66	0.73	1.39	35	57	0.00	Li et al. (2011)
	Soy protein concentrate	67	0.88	0.79	1.67	46	68	0.00	Dam et al. (2019)
	Wheat	22	0.32	0.38	0.70	17	29	0.04	Dam et al. (2019)
	Wheat	74–88	0.70	0.70	1.40	37	57	-	Gorissen et al. (2018)
Animal sources	Blood meal	98	1.26	2.09	3.36	66	137	0.15	Li et al. (2011)
	Blood meal	93	1.24	0.42	1.66	65	68	0.00	Unpublished 2021
	Blood meal	98	1.21	0.88	2.09	64	85	0.00	Dam et al. (2019)
	Casein	96	2.88	0.47	3.35	151	137	0.03	Li et al. (2011)
	Casein	67–78	1.60	0.10	1.70	84	70	-	Gorissen et al. (2018)
	Feather meal	86	0.79	0.44	1.23	41	50	0.03	Li et al. (2011)
	Fishmeal, tuna trims	**72**	**1.85**	**0.49**	**2.34**	**97**	**96**	**0.37**	Dam et al. (2019)
	Fishmeal	**68**	**2.10**	**0.50**	**2.60**	**110**	**106**	**0.72**	Dam et al. (2019)
	Fishmeal	**62**	**1.46**	**0.45**	**1.91**	**77**	**78**	**0.21**	Unpublished 2021

Aquafeed protein ingredients		CP[1]	Met[2]	Cys[3]	M+C[4]	M % FM[5]	M + C % FM[6]	Tau[7]	Ref.[8]
	Fishmeal	**69**	**2.20**	**0.73**	**2.93**	**116**	**120**	**0.02**	Li et al. (2011)
	Krill meal	52	1.20	0.32	1.52	63	62	0.34	Unpublished 2021
	Krill meal	60	1.45	0.43	1.88	76	77	0.24	Dam et al. (2019)
	Meat meal	48	0.58	0.26	0.84	30	34	0.06	Dam et al. (2019)
	Meat meal	52	0.64	0.34	0.98	34	40	0.05	Unpublished 2021
	Meat and bone meal	54	1.14	0.51	1.65	60	68	0.02	Li et al. (2011)
	Poultry by-product	66	1.44	1.09	2.53	76	103	0.02	Li et al. (2011)
	Poultry by-product	67	1.06	0.98	2.04	56	83	0.17	Unpublished 2021
	Poultry by-product	85	2.06	1.22	3.28	108	134	0.24	Dam et al. (2019)
Insect larvae meal	*Hermetia illucens*	46	0.88	0.16	1.04	46	43	<0.1	Basto et al. (2020)
	H. illucens (defatted)	52	1.37	0.23	1.60	72	65	0.12	Basto et al. (2020)
	Tenebrio molitor	54	0.74	0.36	1.10	39	45	0.16	Basto et al. (2020)
	T. molitor (defatted)	66	0.96	0.15	1.11	50	45	<0.1	Basto et al. (2020)
Micro-algae	*Athrospira maxima*	60-71	1.40	0.4	1.80	74	74	-	Becker (2007)
	Chlorella vulgaris	51-58	2.20	1.4	3.60	116	147	-	Becker (2007)
	Scenedesmus obliquus	50-56	1.50	0.6	2.10	79	86	-	Becker (2007)
	Spirulina platensis	46-63	2.50	0.9	3.40	131	139	-	Becker (2007)

[1] Crude protein content [2] Methionine [3] Cysteine [4] Methionine+cysteine [5] Met proportional to the average Met content of fishmeal [6] M+C proportional to the average M+C content of fishmeal [7] Tau, Taurine [8]

1.3.2 Metabolism

Of the twenty proteinogenic amino acids that make up the primary structure of proteins, two (methionine and cysteine) contain a sulfur atom (Brosnan & Brosnan, 2006). Due to their connection through the unidirectional transsulfuration pathway, both are classified together as sulfur amino acids and this enables methionine to provide sulfur for the synthesis of cysteine (Stipanuk, 2004). Taurine is methionine's and cysteine's amino-sulfonic acid derivative that is often mistakenly classified as a sulfur amino acid. Taurine only carries a sulfur atom and amino group and lacks the characteristic carboxyl group of an amino acid (**Figure 1.2**). Major sites for sulfur amino acid and taurine metabolism in fish are the liver and pyloric ceca (Haga et al., 2015).

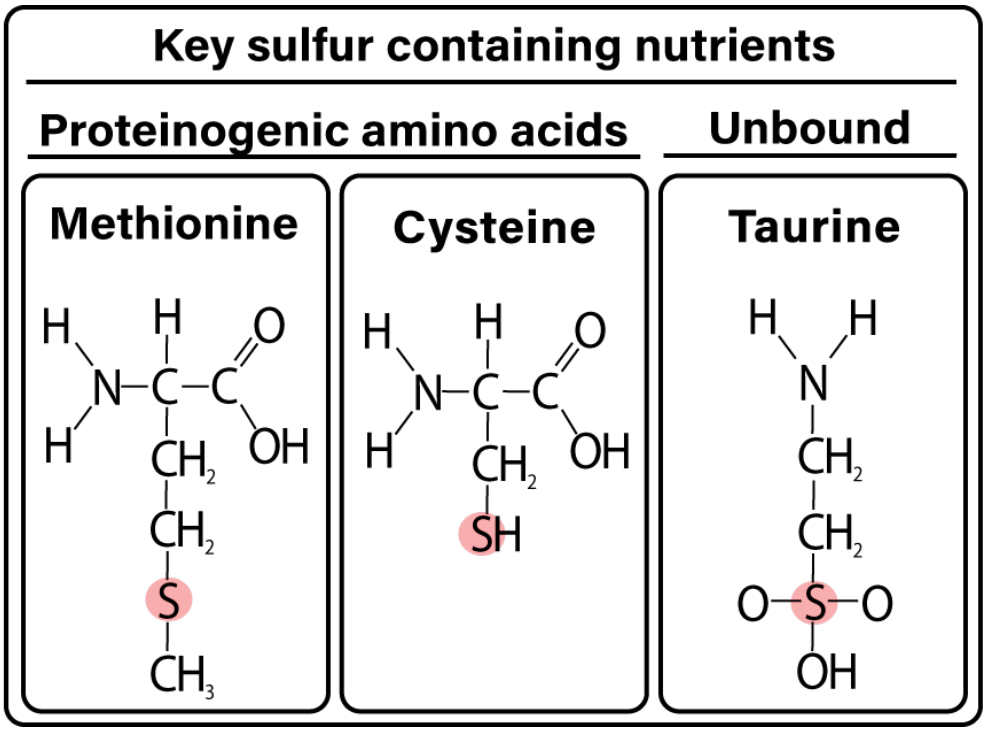

Figure 1.2 Chemical structure of the sulfur amino acids methionine and cysteine, and the amino-sulfonic acid taurine. Adapted from Brosnan and Brosnan (2006).

In fish that are fed optimal amounts of methionine to meet physiological requirements, methionine metabolism is regulated toward anabolism. However, fish fed methionine at suboptimal and supraoptimal levels undergo a metabolic shift toward protein catabolism and reduced turn-over (Rolland et al., 2015). Methionine is an essential amino acid that must be obtained exogenously by farmed fish since only small quantities of homocysteine are re-methylated to methionine at levels that cannot satisfy the sulfur amino acid requirement **(Figure 1.3**; Li et al., 2009b). Yet homocysteine's re-methylation is still an important process that preserves the recycling of methyltetrahydrofolate and catabolism of betaine (Taylor et al., 2018). Metabolically, methionine is the precursor of a variety of functional substrates, such as S-adenosylmethionine (SAM), homocysteine (HCy), cysteine, and taurine, and is an anchor point for several metabolic pathways, such as betaine and folate (**Figure 1.3**). In animals, the methionine flux via transmethylation, transsulfuration, and re-methylation is regulated by concentrations of SAM and HCy, wherein high cellular levels of HCy increase transsulfuration and high levels of SAM inhibit the re-methylation of HCy (Stipanuk, 2004). Thus, in fish fed adequate methionine, the methionine flux supports protein synthesis and the removal of ingested methionine from the cycle for the synthesis of SAM as the methyl donor S-adenosylhomocysteine as the amine donor, and homocysteine as the sulfur donor for cystathionine (Courtney-Martin et al., 2012).

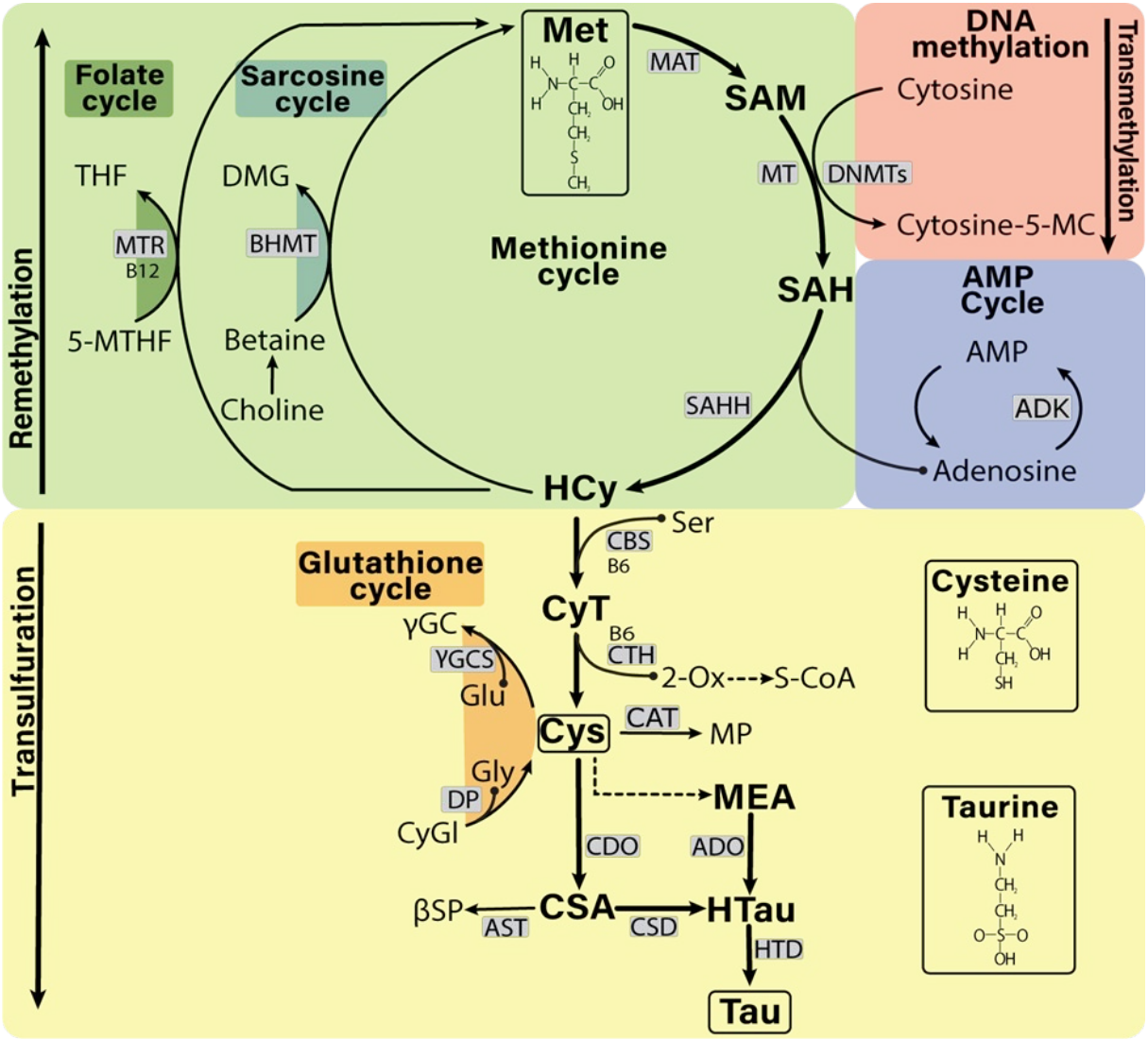

Figure 1.3 Illustration of a simplified methionine-cysteine-taurine metabolism and adjunct cycles. Methionine-cysteine-taurine pathway (bold arrows), enzymes (grey boxes). Abbreviations: Methionine, Met; homocysteine, HCy; methionine adenosyltransferase, MAT; S-adenosylmethionine, SAM; methyltransferase, MT; S-adenosylhomocysteine, SAH; S-adenosylhomocysteine hydrolase, SAHH; vitamin B6, B6; cystathionine β-synthase, CBS; serine, Ser; cystathionine, CyT; cysteine, Cys; 2-oxobutanoic acid, 2-Ox; cystathionine γ-lyase, CTH; Succinyl-CoA, S-CoA; taurine, Tau; mercaptopyruvate, MP; cysteine aminotransferase, CAT; γ-glutamyl cysteine, γGC; glutamate, Glu; glutamate-cysteine ligase, γGCS; Cysteinyl glycine, CyGl; dipeptidase, DP; cysteine dioxygenase, CDO; cysteine sulfinic acid, CSA; cysteine sulfinic decarboxylase, CSD; hypotaurine, HTau; β-sulfinyl pyruvate, βSP; glutamic oxaloacetic transaminase, AST; cysteamine, MEA; cysteamine dioxygenase, ADO; adenylate kinase, ADK; adenosine monophosphate, AMP; betaine homocysteine methyltransferase, BHMT;

dimethylglycine, DMG; 5-methyl tetrahydrofolate, 5-MTHF; methyl tetrahydrofolate-homocysteine methyltransferase, MTR; tetrahydrofolate, THF. Figure adapted from Ball et al. (2006b), Salze & Davis (2015), Stipanuk (2004), and Taylor et al. (2018)., Gropper & Smith (2012), Bjursell et al. (2011), Cook et al. (2006), Sumizu (1962), Liang et al. (2011), Kageyama et al. (2018), Nunes & Serpa (2018).

Cysteine is formed through the condensation of the methionine-derived homocysteine, which carries its sulfur atom, and serine (Stipanuk, 2004). In fish, the cysteine flux toward glutathione (GSH) or taurine formation is not well understood. However, in rats, low cysteine levels increased GSH production, whereas high cysteine levels increased sulfate and taurine production (Stipanuk et al., 1992). Cysteine is a semi-essential amino acid that must be exogenously supplied when there are inadequate levels of methionine to support cysteine synthesis. Yet cysteine's endogenous presence forms an integral part of the glutathione cycle and provides the substrate for mercaptopyruvate production (Brosnan & Brosnan, 2006). In addition, cysteine is the metabolic precursor of taurine (Brosnan & Brosnan, 2006).

The synthesis of taurine from cysteine appears to occur via several pathways (Jacobsen & Smith, 1968). The cysteamine and cysteine sulfinic acid pathways have been repeatedly confirmed, while the significance of the cysteate pathway is unclear. Assessments of its importance range from it being a major contributor to the taurine synthesis in fish to being the spontaneous cysteine oxidation from analytical processing (Cook et al., 2006; Salze & Davis, 2015). The most commonly described pathway for taurine production in mammals and fish is the cysteine sulfinic acid pathway, which involves the oxidation of cysteine to cysteine sulfinic acid through the enzyme cysteine dioxygenase (CDO), followed by the decarboxylation to hypotaurine by cysteine sulfinic acid decarboxylase (CSD). The essentiality of taurine in aquafeed depends on enzymatic activity and how different pathways contribute at different rates to the total taurine pool (Salze & Davis, 2015).

1.3.3 Dietary sulfur amino acid and taurine requirements

Upon review of the literature on sulfur amino acid requirements in fish, livestock, and humans, definitions and experimental designs for sulfur amino acid requirements and metabolic relationships are particularly inconsistent in fish nutrition research. The most pronounced methodological inconsistencies among studies are (1) the consideration or (2) the neglect of cysteine's relationship to methionine and thus, may significantly affect estimates, interpretation, and presentation of the sulfur amino acid requirements. The former type of study often presents the sulfur amino acid requirements as methionine + cysteine (Met+Cys), total sulfur amino acid or minimum obligatory methionine requirement, whereas latter studies often describe sulfur amino acid requirements as 'methionine requirement'. The division of methionine and cysteine into essential and conditionally essential, although both may contribute to the total sulfur amino acid requirement, and the inconsistent use of terminology may have contributed to the wide range of interpretations (Ball et al., 2006).

In livestock and human nutrition there is the common consensus that cysteine can spare significant amounts of methionine in the feed of organisms and thus requirements are classed into two to three distinct sulfur amino acid requirements that dictate distinct experimental approaches (Ball et al., 2006; National Research Council, 2011a, 2011b). There are two variants of the total sulfur amino acid requirement and the minimum obligatory methionine requirement. The total sulfur amino acid (TSAA) requirement describes the amount of dietary methionine that is needed to satisfy the sulfur amino acid requirement in the absence of dietary cysteine (TSAA [Met]). However, in fish and livestock nutrition research the TSAA requirement may also be describe as the amount of dietary methionine needed to meet the requirement at low dietary

cysteine inclusions (TSAA [Met+Cys]; Twibell et al., 2000). The minimum obligatory methionine requirement can only be met by dietary methionine and constitutes a portion of the TSAA (Met or Met + Cys) requirement. The remaining TSAA requirement is the portion that can be met by dietary cysteine, which is called the cysteine sparing effect. Thus, hypothetically, the addition of cysteine to a sulfur amino acid-free diet would reduce the rate of transsulfuration of methionine and redirect it in favor of transmethylation, sparing the methionine flux toward the transsulfuration pathway to produce cysteine (Courtney-Martin et al., 2012).

In carnivorous marine fish, the TSAA requirements have been estimated primarily through growth studies in which fish were fed incremental levels of methionine at zero or consistently low cysteine concentrations in purified, semi-purified, and practical diets (Halver, 2002). The dietary TSAA (Met + Cys) specification to meet requirements in carnivorous marine fish vary greatly between species (**Table 1.3**; Poppi et al., 2017; X. Wang et al., 2016). The TSAA (Met+Cys) requirement for black seabream is 17.1 to 17.2 g kg^{-1}, of which 15.0 to 15.3% were met by cysteine (15.0–15.3% Cys; *Sparus macrocephalus*; Zhou et al., 2011), golden pompano 14.6 g kg^{-1} (11.6% Cys; *Trachinotus ovatus*; Niu et al., 2013), Japanese yellowtail 14.2 g kg^{-1} (21.8% Cys; Ruchimat et al., 1997), European seabass 13.1 g kg^{-1} (8.4% Cys; Tulli et al., 2010), grouper 15.7 g kg^{-1} (16.6% Cys; *Epinephelus coioides*; Luo et al., 2005), barramundi 17.7 to 20.2 g kg^{-1} (32.7-38.6% Cys; *Lates calcarifer*; Poppi et al., 2017), Japanese flounder 15 to15.9 g kg^{-1} (4% Cys; Alam et al., 2000), hybrid grouper 21.9 g kg^{-1} (33.8% Cys; Xiaojun Li et al., 2020), silver pompano 16.9 to 17.1 g kg^{-1} (31.0–31.4% Cys; Ebeneezar et al., 2020). The TSAA (Met + Cys) for YTK is unknown.

Table 1.3 Total sulfur amino acid (Met+Cys) requirement in fish (g kg^{-1}).

Generic name	Scientific name	Met (g kg^{-1})	Cys (g kg^{-1})	TSAA (Met+Cys)	Cys (% of TSAA)	Reference
Black seabream	*Sparus macrocephalus*	14.5	2.6	17.1	15.0-15.3	Zhou et al. (2011)
Golden pompano	*Trachinotus ovatus*	12.9	1.7	14.6	11.6	Niu et al. (2013)
Japanese yellowtail	*Seriola quinqueradiata*	11.1	3.1	14.2	21.8	Ruchimat et al. (1997)
European seabass	*Dicentrarchus labrax*	12.0	1.1	13.1	8.4	Tulli et al. (2010)
Grouper	*Epinephelus coioides*	13.1	2.6	15.7	16.6	Luo et al. (2005)
Barramundi	*Lates calcarifer*	10.9- 11.9	5.8-6.8	17.7	32.7-38.6	Poppi et al. (2017)
Japanese founder	*Paralichthys olivaceus*	14.4	0.6	15	4	Alam et al. (2000)
Hybrid grouper	*Epinephelus fuscoguttatus x Epinephelus lanceolatus*	14.5	7.4	21.9	33.8	Li et al. (2020)
Silver pompano	*Trachinotus blochii*	11.6-11.7	5.2-5.3	16.9	31.0-31.4	Ebeneezar et al. (2020)

In fish cysteine can substitute 40 to 60% of the TSAA requirement (Abidi & Khan, 2011; Goff & Gatlin, 2004; Harding et al., 1977; Kim et al., 1992; Moon & Gatlin, 1991; Nguyen & Davis, 2009a; Poppi et al., 2017; Twibell et al., 2000; Zehra & Khan, 2016). However, care needs to be taken when formulating for methionine and cysteine, as both were found to be toxic when in surplus (National Research Council, 2011a; Regina et al., 1993). A methionine surplus in rats and rainbow trout led to inferior growth and feed efficiency (Harper, 1958; Poppi et al., 2011; Sauberlich, 1961), whereas a cysteine surplus led to inferior growth and increased mortality (Osman et al., 1997).

Taurine deficiencies in Japanese yellowtail and red seabream led to the green liver syndrome, inferior growth and increased susceptibility to diseases, whereas the addition of taurine ameliorated these effects (Li et al., 2007; Takagi et al., 2010, 2008). The discovery that some fish are unable to synthesize taurine from cysteine and that the green liver syndrome can be associated with taurine deficiencies in low-fishmeal diets has led to an increasing number of studies examining the taurine requirements of marine carnivorous fish species in recent years (Salze and Davis, 2015). The taurine requirement for YTK is unknown and must, therefore, be determined.

To date, the current recommended methionine and taurine specifications for YTK feed are based on the work by Ruchimat et al. (1997) for the closely related Japanese Yellowtail at 11.1 g kg^{-1} diet and by Salze et al. (2018) for the closely related California yellowtail at 2.6 and 10.2 g kg^{-1} diet using zero-fishmeal and a 29.1% soy protein diet and a methionine content of 11 g kg^{-1}. However, the degree of interactions of sulfur amino acids and taurine is not known for any of the

Seriola species. The TSAA, MOM, cysteine sparing, sulfur amino acid toxicity, and taurine requirement in YTK are unknown and must be determined to improve YTK aquaculture.

1.3.4 Metabolic and physiological functions

The biological significance of methionine is undisputed and exceeds that of just being a building block and start-codon for the protein synthesis is well recognized (Finkelstein et al., 1988; Wu, 2013, 2010). Cellular methionine has shown to signal and regulate glucose, lipid, amino acid metabolism and protein synthesis and turnover in fish (Skiba-Cassy et al., 2016). The catabolism of methionine ensures the production of SAM and S-adenosylhomocysteine.

The intermediate metabolite SAM is regarded as the essential biological methyl donor and is involved in the production of small molecules such as creatine, phosphatidylcholine, carnitine, polyamine and epinephrine, and in the modification of DNA, RNA, proteins and inactivation of neurotransmitters (Brosnan et al., 2007). S-adenosyl homocysteine is an anti-inflammatory agent and assists with cellular energy transfer. The transsulfuration of methionine produces cysteine, which is a structural component of proteins and builds disulfide linkages. Cysteine is also the precursor for glutathione and taurine (Salze & Davis, 2015; Wu, 2009).

Taurine is a highly versatile substrate that is involved in multiple biological functions in cells, including osmoregulation, maintenance of membrane integrity, antioxidation, redox state regulation, calcium transport, and cytoprotection of the central nervous, retinal and muscular system (Huxtable, 1992; Omura & Inagaki, 2000; Wijayasinghe et al., 2017; Wu & Prentice, 2010). Within the liver, taurine conjugates with bilirubin forming ditaurobilirubin, which enables

the mobilization and removal of haemolytic waste through the biliary system (**Figure 1.5**; Goto, 2001; Sakai et al., 1987). Additionally, taurine conjugates with bile acids in the fish liver, thereby enabling the emulsification of fats in the intestine (Kim et al., 2015).

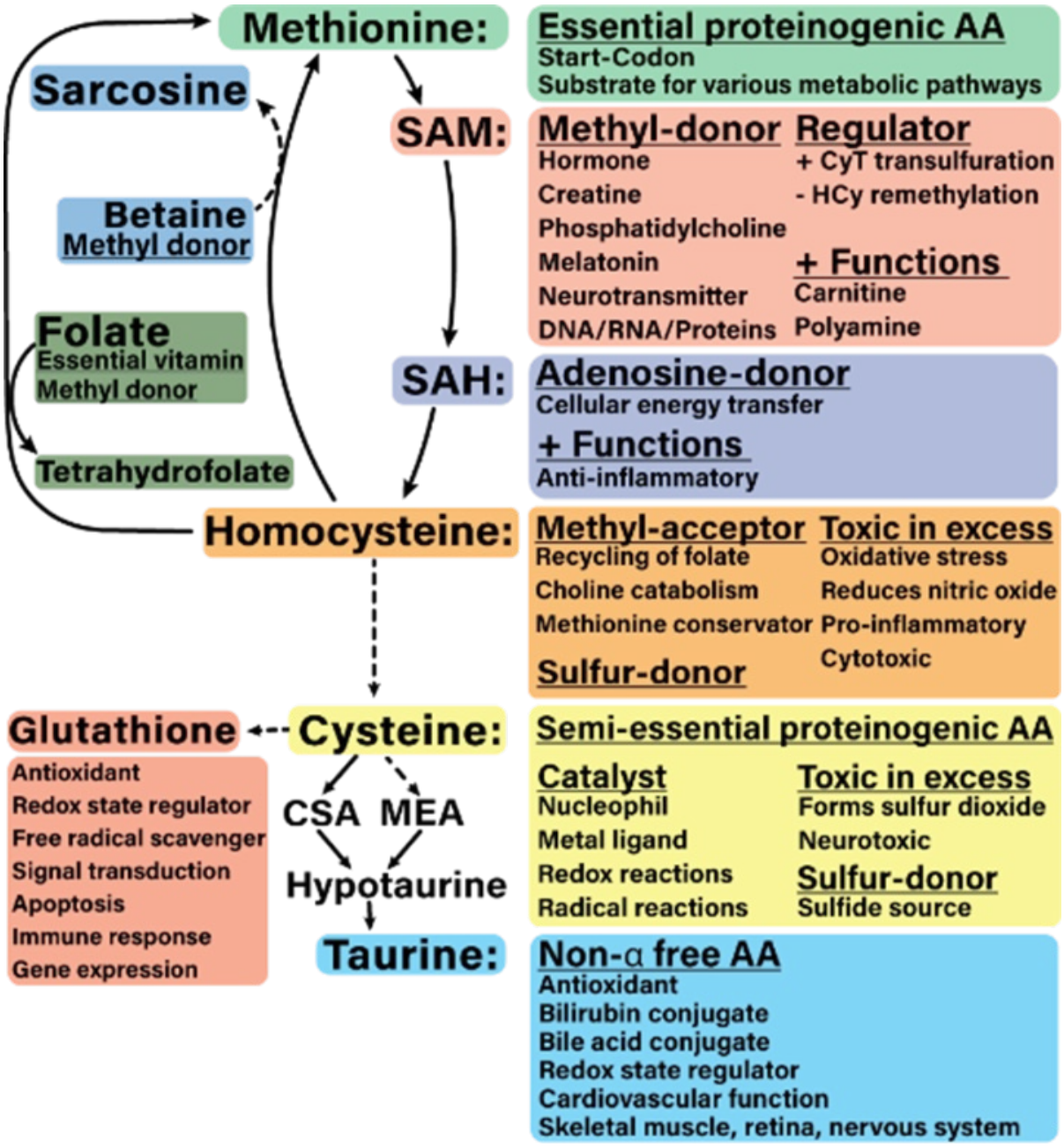

Figure 1.4 Key metabolic and physiological functions of substrates involved in the methionine, cysteine, and taurine metabolism, beyond protein retention in fish, livestock, and humans. Abbreviations: AA, amino acid; SAM, S-adenosylmethionine; SAH, S-adenosyl homocysteine; CyT, cystathionine; HCy, homocysteine; CSA, cysteine sulfonic acid; MEA, cysteamine.

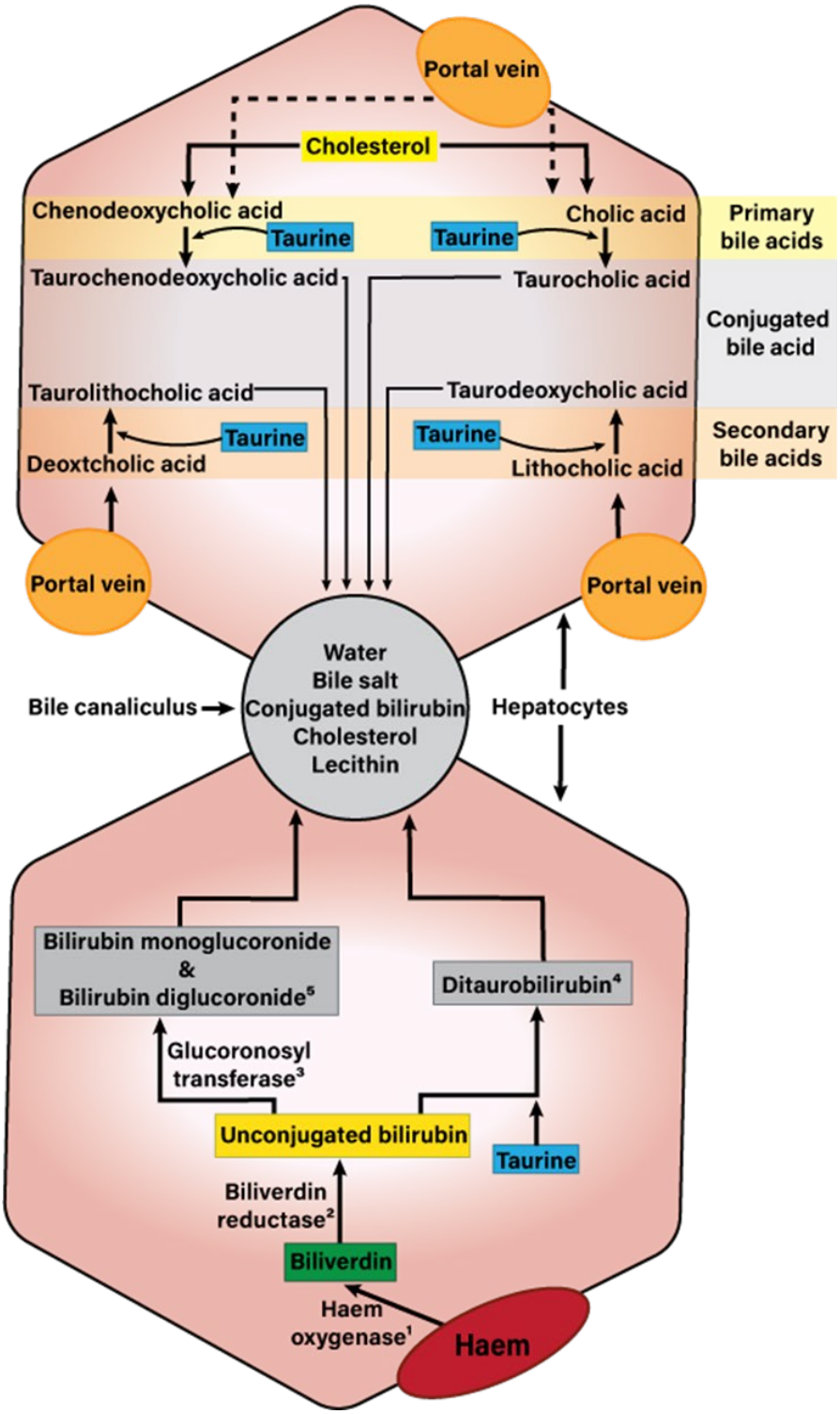

Figure 1.5 Biological functions of taurine in bile formation in fish's intrahepatic biliary system, facilitating the intestinal digestion of lipids by conjugating with bile acid and waste excretion of bilirubin by forming ditaurobilirubin. Concepts adapted from: [1]Wei et al. (2003), [2]van den Hurk (2006), [3]Leaver et al. (2007), [4]Goto et al. (2001) & Sakai et al. (1987), [5]Goto et al. (2001).

1.4 Research aims and objectives

Presently, alternative sources of protein, including plant protein meals and animal by-products, are routinely utilized to formulate and manufacture aquafeeds. These are often nutritionally incomplete and require the supplementation of essential amino acids such as methionine to satisfy the metabolic and nutritional requirements of carnivorous fish. However, specifications on the supplementation of essential amino acids are frequently derived from other species. Inadequate supply may negatively affect fish physiology and overall growth performance. Therefore, it is necessary to estimate the requirements of the target species to formulate nutritionally balanced diets.

The sulfur amino acids methionine and cysteine, and their amino-sulfonic acid derivative taurine, are all metabolically active molecules interlinked with the nutritional requirements of the animal. Dietary sulfur amino acid and taurine are vitally essential for many marine carnivorous fish, including for those species that are closely related to YTK. However, a comprehensive understanding of the quantitative requirements for SAA and taurine in YTK is missing. In addition, there have been reports of the occurrence of green liver syndrome in maricultured YTK, raising concerns about the completeness of the nutrient content of aquafeed for YTK in aquaculture (D'Antignana, 2012; D'Antignana and Bubner, 2008).

Although substantial work has been completed on the significance of taurine on hepatic health and maintaining the overall homeostasis of the organism, little is known on its effects in YTK. To optimize the growth and health of fish, it is important to understand their obligatory dietary

requirements and the capacity for bioconversion of nutrients to meet the physiological demand for metabolites. Sulfur amino acids constitute proteins and play myriad physiological roles that can affect the metabolism and health of cultured organisms. Excess sulfur amino acids can be further broken down to form the taurine, which further provides antioxidant, anti-inflammatory and osmolarity roles. Taurine also conjugates with cholesterol derivatives to form bile salts for fat metabolism and facilitates the removal of the by-products of iron metabolism. Although deficiencies in this group of nutrients have been linked to poor growth and health, the effects of these deficiencies on organ structure and function in fish are largely unknown. A more comprehensive understanding of quantitative and qualitative nutritional requirements and effects is required to efficiently and sustainably grow the global and Australian YTK industry.

Measurements on ADCs of experimental diets and the health status of YTK after completion of the feeding trials will allow the calibration of requirements for more accurate estimates and a better understanding of sulfur amino acid's and taurine's contributions for healthy fish. Additionally, the assessment of sparing capacities of methionine and cysteine on the taurine and total sulfur amino acid requirements, respectively, may be valuable at times where feed are formulated based on nutrient specifications when nutrients are limiting. Finally, the histological assessment of the intestinal and hepatic condition in YTK will deliver a more comprehensive understanding of sulfur amino acid and taurine induced alterations on the hepatic and intestinal properties and functions in YTK.

Chapter 2 focuses on quantifying the dietary requirement for taurine and captured the sparing capacity of methionine on the taurine requirement in YTK. The orthogonal, factorial design

investigated the significance of dietary taurine for YTK and whether this species can synthesize taurine *de novo* from methionine sufficiently to meet requirements. This identifies if dietary taurine supplementation to low-taurine diets is required to sustain optimized growth and feed efficiency. This study is published in the peer- reviewed journal *Aquaculture* (Candebat et al., 2020).

Findings of the previous chapter indicated that the current dietary methionine level used by industry for YTK aquafeed does not meet the requirement of YTK and has led to the investigation of the sulfur amino acid requirements of YTK in Chapter 3. Chapter 3 focuses on quantifying the total sulfur amino acid requirements for methionine at low dietary cysteine contents, the minimum obligatory methionine requirements at excess of dietary cysteine, and determines the sparing of cysteine on the total sulfur amino acid requirements in juvenile YTK. Therefore, the main objective of this study is to establish a comprehensive quantitative database of sulfur amino acid requirements in juvenile YTK that can be used to formulate YTK aquafeeds that satisfy species-specific requirements. The applied orthogonal and factorial design for the experimental diets improve our understanding of the relationship of sulfur amino acids and the effects of dietary excess and deficiency in the fish. These results may improve dietary methionine to cysteine ratios, put economic and sustainable ingredients in perspective and reduce the risk of over- or under-supplementing methionine in nutrient-based formulations. This study was published in the peer-reviewed journal *Aquaculture* (Candebat et al., 2021).

Chapter 4 focuses on investigating the impacts of varying dietary sulfur amino acids and taurine levels that are suboptimal, optimal, and supraoptimal of requirements and to infer functional

changes that relate to these nutritional imbalances. This study uses histology, morphology, and biochemistry to describe changes in inflammatory, immune, digestive, homeostatic stability, compositional and metabolic properties in the liver and posterior intestine tissue of juvenile YTK. In addition, the study aims to establish a comprehensive quantitative histological and biochemical database that may provide reference values and an improved understanding of the clinical symptoms in juvenile YTK with a focus on nutritional imbalances. This study is currently under review in the British Journal of Nutrition.

Chapter 5 forms the general thesis discussion, clarifying in a condensed list the findings of each chapter and discussing their significance and implications for industry and fish nutrition research.

Chapter 2. Dietary methionine spares the requirement for taurine in juvenile yellowtail kingfish (*Seriola lalandi*)[1]

2.1 Abstract

Taurine, a β-sulphonic amino acid, is a growth and health promoting dietary supplement in commercial finfish aquaculture. Reported recommendations for taurine supplementation in Seriola spp. feeds broadly range from 2.6 to 10.2 g kg^{-1} diet. Methionine is an essential amino acid and substrate for various metabolic compounds and acts as a methyl and sulfur donor, potentially sparing taurine. Dietary methionine requirements are currently unknown for yellowtail kingfish (*Seriola lalandi*); however, recommendations for the closely related Japanese Yellowtail (*Seriola quinqueradiata*) indicate that 11.1 g kg^{-1} diet is adequate. The taurine requirement and sparing effect of methionine of juvenile yellowtail kingfish was quantified by conducting a feeding experiment and applying a factorial, orthogonal dose-response design. Fourteen isonitrogenous and isoenergetic diets were prepared using practical raw ingredients with either one of two levels of methionine (10.9 ± 0.2 g kg^{-1} or 17.2 ± 0.6 g kg^{-1}) and either one of seven levels of taurine, increasing from 1.6 to 20.4 g kg^{-1}, respectively. Triplicate groups of 14 fish (53.3 ± 0.4 g $fish^{-1}$) were fed one of the 14 diets over seven weeks.

1 Data from this chapter were published as: Candebat, C. L., Booth, M. A., & Pirozzi, I. (2021). The sulfur amino acid requirements of juvenile yellowtail kingfish (*Seriola lalandi*). Aquaculture, 534, 736234.

Based on growth and feeding results, juvenile yellowtail kingfish do not require dietary taurine supplementations when the basal taurine diets content is at least 1.6 g taurine kg^{-1} at a dietary methionine content of 17.2 ± 0.6 g kg^{-1} diet. This demonstrates that dietary methionine has a sparing effect on taurine supplementation. Yellowtail kingfish fed dietary methionine exceeding the current minimum industry standard (11.1 g kg^{-1}), grew more rapidly than those fed high dietary taurine contents at dietary methionine levels approximating that of current industry practice, indicating the indispensability of adequate methionine supply. Breakpoint analysis on the specific growth rate in yellowtail kingfish fed a methionine level of current industry practice, estimated a digestible taurine requirement of 0.16 g $kgBW^{-1}$ d^{-1} at an average digestible methionine intake of 0.25 g $kgBW^{-1}$ d^{-1} This equates to a dietary taurine content of 7.7 g kg^{-1} diet at a dietary methionine content of 10.9 g kg^{-1} diet. Our results indicate that in juvenile yellowtail kingfish: adequate dietary methionine spares dietary taurine supplementation; insufficient dietary methionine provokes a taurine requirement; and current industry specifications for dietary methionine for yellowtail kingfish aquafeed require reassessment.

2.2 Introduction

Taurine, a β-sulphonic amino acid, has many functional roles in the physiology of animals. Taurine is an intracellular organic osmolyte, regulating cell volume (Wijayasinghe et al., 2017). Taurine conjugates with bile acids and forms bile salt, which is essential for lipid utilization and digestion (Bellentani et al., 1987) and is also a substrate for the development, functionality and cell protection of the central nervous, retinal and muscular system (Wu and Prentice, 2010). In juvenile Japanese Yellowtail (*Seriola quinqueradiata*) deficiencies in dietary taurine are associated with green liver syndrome, inferior growth performance (Takagi et al., 2010) and increased susceptibility to diseases (Li et al., 2007). Methionine is an essential sulfur amino acid and cannot be synthesized *de novo* in sufficient quantities to meet requirement; therefore, adequate amounts of dietary methionine must be provided in aquafeed. Taurine is derived from the transmethylation and sulfuration pathway of methionine and cysteine, providing the substrates for taurine synthesis (Brosnan and Brosnan, 2006; National Research Council, 2011a; Wu, 2009). The relatedness, interactions, and sparing effects of SAA imply that quantifying the requirement of a species for one SAA must be done within the context of the concentration of precursors and derivates present in the diet.

Yellowtail kingfish (*Seriola lalandi*; hereafter referred to as YTK), is a high value aquaculture species often consumed as sashimi. In Australia, YTK are now farmed in South Australia and Western Australia and commercial scale production is being investigated in New South Wales. So far, the methionine requirement and interactions with taurine and other derivates have not been studied in YTK. The current recommended level of dietary methionine for YTK is based

on the work by Ruchimat et al. (1997) for the closely related Japanese Yellowtail at 11.1 g kg^{-1} diet; however, the degree of interaction of dietary SAA compounds is not known for either species. The dietary taurine requirement of California yellowtail (*S. lalandi*) was determined by Salze et al. (2018) to be between 2.6 and 10.2 g kg^{-1} diet using zero-fishmeal and 29.1% soy protein diets and a methionine content of 11 g kg^{-1} diet. Similarly, Martins et al. (2018), determined a taurine requirement of 4.7 to 5.0 g kg^{-1} diet in juvenile European sea bass (*Dicentrarchus labrax*), a marine carnivorous species, using low-fishmeal diets with 10 to 12 g methionine kg^{-1} diet, which is at the threshold of requirement for this species (Tulli et al., 2010).

Aquafeed manufacturers around the world are making a concerted effort to use low or zero-fishmeal inclusions in feed formulations. Increasing utilization of plant proteins and rendered animal products will mean that the total SAA content (methionine + cysteine) in diets may become limiting unless diets are formulated to deliver a balanced suite of essential nutrients. However, to achieve this goal, a comprehensive understanding of the quantitative nutrient requirements of the animal is required. The objective of this study was to determine the requirement for taurine and the sparing effect of methionine in juvenile YTK.

2.3 Methods

The experiment was performed under the NSW DPI Fisheries Animal Care & Ethics Research Authority known as 'Aquaculture Nutrition ACEC 93/5–Port Stephens' (ACEC, 2009).

2.3.1 Experimental design

A factorial dose-response approach was applied to quantify the dietary taurine requirement of juvenile YTK relative to dietary methionine content. The design used seven incremental levels of taurine ranging from 1.6 to 20.4 g kg^{-1} diet, crossed with two levels of dietary methionine (10.9 or 17.2 g kg^{-1} diet; **Table 2.1**). All diets were isonitrogenous (491.7 ± 1.6 g crude protein kg^{-1} diet) and iso-energetic (22.2 ± 0.1 MJ gross energy kg^{-1} diet;

Table 2.2), formulated to meet the protein and energy requirements of juvenile YTK (Booth et al., 2010; Pirozzi et al., 2019) using practical ingredients.

Triplicate groups of 14 YTK (53.3 ± 0.4 g $fish^{-1}$) were stocked into 200 L experiment tanks with water supplied via a RAS. YTK were randomly allocated to each experimental diet and hand fed to apparent satiation twice per day (10 AM and 4 PM) during weekdays and once per day (10 AM) on weekends for 45 days. The trial was terminated at week seven of the planned eight weeks, due to a pump failure, causing the loss of 29.4% of YTK. As the response time from pump failure to harvest was relatively short (< 1 h), all fish were sampled for final weight and subsequent feed intake analysis. However, we adopted a conservative, precautionary approach to ensure the biochemical integrity of the samples and chose to only sample the remaining 70.6% of live fish for final proximate and amino acid carcass analysis. Whole carcass of four fish per replicate tank were sampled from a minimum of n = 2 replicate tanks, except for diets 6 and 8 where n = 1, and analyzed for chemical compositional analysis. Fecal material of four to five fish fed diets 1, 4, 7, 8, 11, and 14 were collected from a minimum of n = 2 replicate tanks for chemical compositional and digestibility analyses.

Table 2.1 Measured proximate and amino acid composition of experimental diets (g kg^{-1} dry matter basis, unless indicated otherwise).

	Low methionine series							High methionine series						
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12	Diet 13	Diet 14
Proximate values														
Ash	117.0	115.8	119.3	118.6	117.4	116.2	115.4	116.1	116.6	117.2	114.9	113.5	111.0	110.0
Total lipid	144.5	149.0	158.4	154.1	152.8	151.5	157.7	178.3	161.3	155.8	160.6	139.9	144.6	154.3
Total protein	485.9	494.3	498.3	485.4	500.4	488.7	495.1	482.7	494.5	487.5	493.1	503.1	488.4	484.4
GE (MJ kg^{-1})[1]	21.9	22.0	22.2	22.2	22.1	21.9	22.9	21.8	22.0	22.1	22.3	22.2	22.2	22.7
NFE[2]	252.5	240.9	223.9	241.9	229.5	243.5	231.8	222.9	227.6	239.5	231.4	243.5	256.0	251.4
EAA[3]														
Arg	56.5	54.0	56.4	52.2	51.9	52.2	51.3	53.0	52.1	52.7	51.8	50.6	51.7	53.9
His	14.6	18.2	15.6	14.3	16.1	13.8	15.0	14.1	13.6	13.2	14.0	12.5	13.9	12.2
Ile	17.0	16.1	16.5	15.9	16.2	16.5	16.4	16.4	16.2	16.3	15.8	15.6	16.0	16.5
Leu	42.9	41.4	42.2	40.9	41.4	42.7	42.1	42.3	41.0	41.8	40.3	40.0	41.1	42.1
Lys	29.2	26.6	30.2	27.0	26.6	26.0	27.6	26.9	27.0	24.2	25.4	23.3	23.6	23.4
Met	12.0	10.5	11.1	10.8	10.9	10.1	11.1	16.7	19.3	16.4	18.8	16.0	17.9	15.0
Phe	23.8	23.4	23.3	23.2	23.5	24.2	24.0	24.6	23.6	24.7	23.0	23.0	23.8	24.2
Thr	20.7	20.0	20.6	19.4	19.4	19.5	19.0	19.9	19.3	19.9	18.7	18.7	19.2	20.4
Val	26.6	25.8	26.3	25.5	26.0	26.8	26.3	26.8	25.9	26.8	26.1	25.9	26.6	27.3
NEAA[4]														
Ala	30.0	29.3	29.3	28.0	28.3	28.9	28.0	29.4	28.3	29.0	27.6	27.9	28.6	30.2
Asx	43.4	42.1	43.2	40.6	40.2	40.8	40.1	41.6	40.6	40.6	39.0	38.8	40.1	40.3
Glx	70.5	67.1	68.0	64.5	62.6	64.0	62.4	64.2	62.4	61.5	60.0	59.7	61.4	61.9
Gly	27.6	27.2	27.1	25.7	26.4	26.2	26.4	30.1	26.1	27.8	26.0	25.6	26.7	28.4
Ser	25.0	25.2	25.1	22.9	23.9	22.8	23.1	23.4	22.6	25.5	23.2	22.5	23.5	28.4
Tyr	14.5	15.1	16.1	14.4	15.8	15.0	14.5	14.3	14.9	15.2	14.8	14.5	15.0	15.2
CEAA[5]														
Cys	6.8	6.1	5.6	5.4	5.6	5.8	5.7	5.9	5.4	5.5	5.2	5.6	5.6	5.8
Pro	25.9	25.2	24.9	23.9	24.0	24.5	24.4	25.4	24.1	24.0	23.5	23.2	24.1	24.3
Tau	1.6	4.8	8.5	11.9	15.0	17.3	20.4	1.6	5.1	8.1	11.7	13.9	18.3	20.0
SUM AA[6]	488.6	477.9	490.0	466.8	473.9	477.1	477.7	476.6	467.4	473.2	464.8	457.3	477.1	489.6

[1] GE, Gross energy, [2] NFE, Nitrogen-free extract= 1000- (ash+ crude protein+ fat), [3] EAA, essential amino acids, [4] NEAA, non-essential amino acids, [5] CEAA, conditional essential amino acids, [6] AA, amino acid.

Table 2.2 Formulation of experimental diets (n=14) containing low (10.9 g kg^{-1}) and high (17.2g kg^{-1}) methionine levels.

Ingredient (% DM)	Low methionine series							High methionine series						
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12	Diet 13	Diet 14
Wheat flour	17.9	16.2	15.4	15.4	15.4	15.4	15.4	15.4	15.4	15.4	15.4	15.4	15.4	15.4
Poultry meal	12	12	12	12	12	12	12	12	12	12	12	12	12	12
Fish oil	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0
Dehulled lupin	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Fishmeal Prime	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Fishmeal by-product	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Meat meal	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Corn gluten	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Blood meal	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Diatomaceous earth	1.7	3.1	3.56	3.23	2.91	2.59	2.27	3.29	2.97	2.65	2.32	2	1.68	1.36
NaH_2PO_4	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vit/min premix	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Rovimix Stay-C (35)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Choline chloride (70%)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Methionine	0.2	0.2	0.2	0.2	0.2	0.2	0.2	1.11	1.11	1.11	1.11	1.11	1.11	1.11
Taurine	0.0	0.3	0.64	0.97	1.29	1.61	1.93	0	0.32	0.64	0.97	1.29	1.61	1.93
Y_2O_3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

2.3.2 Fish handling and experimental system

All YTK were progeny of wild-caught broodstock held at the Port Stephens Fisheries Institute (PSFI), NSW, Australia. Prior to stocking, juvenile YTK were fed daily with commercial YTK 4 mm floating pellets (crude protein 50%, crude fat 14%, and crude fiber 4%) and held at water temperatures between 15 and 19 °C.

The experiment was performed under controlled conditions within a RAS. Effluent water was continuously exchanged with filtered and disinfected estuarine water at a rate of approximately 10% d^{-1}. YTK received a prophylactic hydrogen peroxide treatment (150 ppm for 30 min) against fluke at week three and week six. The 200 L tanks were covered with netting to prevent YTK from escaping and also partially covered with black plastic to ensure minimal disturbance during the experiment. The laboratory photoperiod was 12:12 and controlled using dimmed, overhead LED lighting. Dissolved oxygen was supplied at appropriate levels for YTK (Candebat et al., 2020) using industrial oxygen (BOC) and injected directly into the main supply manifolds of the RAS. The water quality of the RAS was monitored daily. Water quality throughout the study was (mean ± SD); water temperature (23.3 ± 0.6 °C), salinity (33.3 ± 4.6‰), dissolved oxygen (7.0 ± 1.0 mg L^{-1}), pH (8.3 ± 0.5), and TAN (0.7 ± 0.4 mg L^{1}).

2.3.3 Diet manufacture

Diets were formulated using a blend of prime fishmeal and fisheries by-product meal to reduce the residual taurine content of the basal formula as low as possible, while maintaining the palatability of the diets. Other protein sources, low or absent in methionine and/or taurine,

included blood, feather, poultry, corn gluten, and dehulled lupin meal. Diets were supplemented with crystalline methionine and taurine to achieve dietary specifications (**Table 2.1**). All diets were made at PSFI using laboratory scale equipment. Prior to pellet making, all raw materials were finely ground in a high-speed hammer mill (Raymond Laboratory Mill, Transfield Technologies, Rydalmere, NSW, Australia; 1.6 mm screen). Wheat flour was autoclaved for two min at 121°C prior to inclusion in the dry mash. Raw materials and supplements were then dry mixed in a Hobart mixer (Hobart Mixer; Troy Pty Ltd, Ohio, USA) before the addition of oil and fresh water to form a moist dough. The dough was then screw pressed into 6 mm pellets (Dolly, La Monferrina, Castell'Alfero, Italy). The moist pellets were then dried at 60 °C to a moisture content of $< 10\%$.

2.3.4 Performance variables and calculations

The following performance variables were used to measure the effects of taurine and methionine in rapidly growing juvenile YTK. Variables are based on a comparative slaughter assay approach. Livers were also visually assessed for indications of green liver syndrome as evidenced by the presence of green discoloration (Takagi et al., 2005).

$$Weight\ gain\ (WG)(\%) = (\frac{FBW-IBW}{IBW}) * 100 \qquad \text{(Equation 1)}$$

where FBW is final body weight (g) and IBW is initial body weight (g)

$$Specific\ growth\ rate\ (SGR)(\%\ day^{-1}) = \left(\frac{Ln\ FBW - Ln\ IBW}{days}\right) * 100 \qquad \text{(Equation 2)}$$

$$Feed\ conversion\ ratio\ (FCR) = \frac{feed\ intake\ (g\ fish^{-1})}{WG\ (g\ fish^{-1})} \quad \text{(Equation 3)}$$

$$Survival\ (\%) = \left(\left(\frac{100}{\#\ fish\ at\ stocking}\right) * \#fish\ at\ harvest\right) \quad \text{(Equation 4)}$$

$$Hepatosomatic\ index\ (HSI) = \left(\frac{liver\ weight\ (g)}{FBW}\right) * 100 \quad \text{(Equation 5)}$$

$$Viscerosomatic\ index\ (VSI) = \left(\frac{viscera\ weight\ (g)}{FBW}\right) * 100 \quad \text{(Equation 6)}$$

(where viscera include liver+ organs+ intraperitoneal fat)

$$Condition\ factor\ (K) = \left(\frac{FBW}{fork\ length\ (cm)^3}\right) *\ 100 \quad \text{(Equation 7)}$$

$$Muscle\ ratio\ (MR) = \left(\frac{\text{fillet weight}(g)}{FBW}\right) * 100 \quad \text{(Equation 8)}$$

$$Intraperitoneal\ fat\ ratio\ (IFR) = \left(\frac{visceral\ fat\ (g)}{FBW}\right) * 100 \quad \text{(Equation 9)}$$

2.3.5 Compositional analyses of whole carcass and diets

For the compositional analysis, whole carcass (n=4 fish per replicate tank) from each respective dietary treatment were homogenised, then subsamples of homogenate were dried, and finally milled. Processed whole carcass samples and diets were analyzed for dry matter, protein, nitrogen, gross energy, total lipid, ash, and amino acids according to routine methods outlined in AOAC,

(2005); additionally, diets were analyzed for yttrium (please see section below). The compositional analysis of whole body and diets was conducted by CSIRO (Agriculture and Food, Carmody Road, St Lucia, QLD 4067, Australia) and the Australian Proteome Analysis Facility (Macquarie University, Sydney Australia). Total protein content was determined by multiplying the nitrogen content of each sample by 6.25. Gross energy content was determined using an adiabatic bomb calorimeter (Parr Instrument Company, 6200 Calorimeter). Total lipid and ash content were gravimetrically determined after sample extraction using the Folch method (Folch et al., 1953) and by burning samples at 550°C in a muffle furnace, respectively. Amino acid profile analysis was performed according to the standard operating procedure, SOP QAAA-001. The amino acids profiles were determined by hydrolyzing samples, labelling amino acids using Waters AccQTag Ultra chemistry, and detecting amino acids via UPLC.

2.3.6 Apparent digestibility of dietary treatments

Following the conclusion of the growth trial the apparent digestibility of diets 1, 4, 7, 8, 11 and 14 were analyzed, these diets represented low, medium or high relative levels of dietary taurine for each methionine series. Digestibility was determined using stripping techniques (Glencross et al., 2007). Prior to stripping, YTK were anesthetized using 5 - 25 mg L^{-1} Aqui-S®. Feces were collected from the posterior intestine by applying gentle abdominal pressure. Contamination with urine or mucous was minimized and samples were immediately stored at -20°C. This procedure was repeated twice a week until approximately 3 g dry fecal matter was obtained. Fecal material from duplicate groups of three to four YTK were collected.

Each experimental diet and dried fecal material from fish fed respective diets were analyzed for dry matter, protein, nitrogen, gross energy, total lipid, ash, amino acids and yttrium. Samples for the yttrium quantification were digested, diluted and measured via inductively coupled plasma mass spectrometer (LCMS-8030, Shimadzu). ADC were calculated according to the equation described by Cho et al. (1982), with the exception that yttrium was used as the digestibility marker.

$$Apparent\ digestibility\ coefficient\ (ADC\ \%) = \ [1 - ((F/D) * (Dmarker/Fmarker)))] * 100$$

(Equation 10)

where F is % nutrient in feces; D is % nutrient in diet; Dmarker is % marker in diet; Fmarker is % marker in feces.

2.3.7 Data analyses

Raw data were processed via Microsoft Excel 2016 and further modelled via GraphPad Prism ver. 6 (La Jolla, CA, USA). The dose-response experiment approach was designed to evaluate the performance of YTK fed diets sub to supraoptimal taurine levels relative to requirement. Feed conversion ratio (FCR) and SGR were used as the response variables to measure requirements and Koops and Grossman (1993) multiphasic regression curves was selected to predict the requirement threshold:

$$y = A - b * \ s * \ln\left(1 + \exp\left(\frac{(c-x)}{s}\right)\right) \quad \text{(Equation 11)}$$

The model was visually assessed for quality of fit to the data and cross-validated via Akaike information criterion (AICc), absolute sum of squares, and r^2 (Gagné and Dayton, 2016).

2.3.8 Statistical analyses

Statistical analyses were performed using the R language and the R software environment for statistical computing (2.13.) using the packages car, carData, ggplot2, ggpubr, multcompView, and PMCMRplus. All response variables were validated for assumptions of normality and constant variance via Shapiro-Wilk normality test and Levene's test for homogeneity of variance, respectively. If assumptions were not met, data were sin, sqrt, or inverse transformed. All response variables were subject to a two-way analysis of variance (ANOVA) elucidating the effect of seven taurine levels, two methionine levels and their interactions. In the event of a significant interaction, all 14 treatment means were jointly compared via Tukey HSD posthoc test. In the event of no significant interaction, but significant main factor, the respective factor level means were compared using Tukey HSD posthoc test (Wei et al., 2011). Effects were considered significant at $p < .05$.

2.4 Results

2.4.1 Survival

Dietary treatments did not significantly affect the survival of YTK. All dietary treatments had a survival rate of 100% except for one mortality each for dietary treatments 5, 11 and 12.

2.4.2 Feed intake

The effect of dietary taurine level on feed intake (FI; DM g $fish^{-1}$ d^{-1}) of juvenile YTK was dependent on the level of dietary methionine ($P < 0.05$; **Table 2.3**). YTK fed the diet lowest in taurine and methionine had the lowest FI (3.41 ± 0.07 g $fish^{-1}$ d^{-1}) which then significantly increased with increasing taurine content. Increasing dietary taurine content had no further significant effect on FI at dietary taurine levels of 8.5 g kg^{-1} diet and above and an average methionine content of 10.9 g kg^{-1} diet. There was no significant difference among the FI of YTK fed diets 8 - 14 containing on average 17.2 g methionine kg^{-1}. The relative feed intake (RFI; g $kgBW^{-1}$) of juvenile YTK was similar across all dietary treatments except for diet 1, which was significantly lower.

Table 2.3 Biometric performance variables of juvenile YTK (mean ± SE; n=3. Taurine, methionine and cysteine digestible intake (g $kgBW^{-1}d^{-1}$) (mean ± SE; n=3). Apparent digestibility coefficients (%) of key measured proximate and amino acids (mean ± SE; n=2; dry matter basis). Different superscript letters indicate significant differences between all diets or among the diet means for that respective main factor. The significant effects were determined by two-way ANOVA and levels of significance are with respect to P< .05 (*), P< .01 (**), and P< .001 (***).

	Low Methionine diets (10.9 g kg^{-1})							High Methionine diets (17.2 g kg^{-1})							p- value		
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12	Diet 13	Diet 14	T[15]	M[16]	Tx M[17]
Growth and feed performance																	
IBW[1]	53.5±0.7	53.4±0.6	53.0±0.7	54.0±0.6	52.5±0.9	53.1±0.7	53.1±0.6	53.3±0.6	53.3±0.6	53.2±0.8	53.7±0.6	53.0±0.7	53.0±0.8	53.5±0.7	NS	NS	NS
FBW[2]	134.8±3.0^{a}	161.7±4.5^{b}	196.2±5.1^{cde}	187.9±4.5^{cd}	187.9±6.9^{cd}	191.1±4.3^{cde}	179.7±6.2^{bc}	207.8±4.5^{def}	228.8±5.6^{f}	212.5±6.3^{def}	211.2±5.1^{def}	205.4±5.4^{def}	214.5±5.2^{ef}	230.3±5.9^{f}	***	***	***
FI[3]	3.41±0.07^{a}	3.83±0.12^{ab}	4.58±0.16^{bcd}	4.45±0.19^{bcd}	4.54±0.10^{bcd}	4.44±0.04^{bcd}	4.20±0.28^{abc}	4.95±0.08^{cd}	4.99±0.10^{cd}	5.21±0.15^{d}	4.92±0.02^{cd}	5.07±0.09^{cd}	4.74±0.18^{cd}	5.12±0.13^{d}	**	***	*
WG[4]	151.8±1.2^{a}	202.8±7.8^{ab}	269.9±3.8^{bcd}	247.8±11.6^{bcd}	260.7±8.6^{bcd}	259.8±8.4^{bcd}	237.5±31.9^{abc}	289.6±9.0^{cd}	329.3±16.0^{d}	299.8±17.1^{cd}	303.0±6.0^{cd}	292.5±19.2^{cd}	304.3±11.2^{cd}	330.8±9.3^{d}	**	***	**
SGR[5]	1.92±0.01^{a}	2.31±0.05^{ab}	2.72±0.02^{cde}	2.59±0.07^{bc}	2.67±0.05^{bcd}	2.67±0.05^{bcd}	2.62±0.12^{bc}	2.83±0.05^{cde}	3.03±0.08^{de}	3.09±0.08^{e}	2.95±0.05^{cde}	3.099±0.07^{e}	2.91±0.06^{cde}	3.04±0.05^{de}	***	***	**
FCR[6]	2.01±0.05^{a}	1.70±0.02^{b}	1.53±0.03^{bc}	1.60±0.02^{bc}	1.55±0.03^{bc}	1.55±0.04^{bc}	1.57±0.08^{bc}	1.54±0.03^{bc}	1.37±0.05^{c}	1.47±0.04^{bc}	1.42±0.03^{c}	1.44±0.03^{c}	1.41±0.02^{c}	1.39±0.03^{c}	***	***	**
S[7]	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	97.6±2.4	100±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	97.6±2.4	97.6±2.4	100.0±0.0	100.0±0.0	NS	NS	NS
Morphometric indices																	
HSI[8]	0.80±0.03^{a}	NA[14]	0.86±0.02^{a}	0.84±0.01^{a}	NA	NA	0.80±0.03^{a}	0.83±0.02^{a}	NA	0.82±0.02^{a}	0.77±0.03^{a}	NA	NA	0.87±0.03^{a}	NS	NS	*
VSI[9]	6.35±0.17^{ab}	NA	5.78±0.10^{bc}	6.08±0.09^{ab}	NA	NA	6.07±0.16^{ab}	6.64±0.12^{a}	NA	5.98±0.16^{abc}	5.33±0.26^{c}	NA	NA	5.80±0.12^{bc}	***	NS	**
K[10]	1.28±0.02^{a}	NA	1.28±0.03^{a}	1.31±0.02^{a}	NA	NA	1.32±0.01^{a}	1.33±0.03^{b}	NA	1.38±0.01^{b}	1.50±0.07^{b}	NA	NA	1.37±0.02^{b}	NS	***	NS
MR[11]	32.9±1.2^{ab}	NA	36.5±1.0^{a}	36.4±1.3^{a}	NA	NA	37.5±0.4^{a}	35.4±1.6^{a}	NA	35.1±2.0^{a}	28.6±1.4^{b}	NA	NA	33.9±0.8^{ab}	***	NS	**
IFR[12]	0.45±0.04^{a}	NA	0.33±0.05^{a}	0.42±0.05^{a}	NA	NA	0.37±0.02^{a}	0.59±0.07^{b}	NA	0.65±0.07^{b}	0.51±0.09^{b}	NA	NA	0.57±0.04^{b}	NS	***	NS
Digestible intake (g $kgBW^{-1}$ d^{-1})																	
Tau	0.06±0.00^{a}	0.10±0.00^{b}	0.18±0.01^{c}	0.26±0.00^{d}	0.33±0.01^{e}	0.36±0.01^{f}	0.41±0.00^{g}	0.03±0.00^{a}	0.10±0.00^{b}	0.16±0.01^{c}	0.24±0.01^{d}	0.28±0.01^{e}	0.36±0.00^{f}	0.40±0.01^{g}	***	NS	NS
Met	0.42±0.02^{a}	0.22±0.00^{e}	0.22±0.01^{de}	0.23±0.00^{de}	0.23±0.01^{de}	0.20±0.00^{e}	0.22±0.00^{de}	0.34±0.00^{bc}	0.36±0.01^{ab}	0.32±0.01^{abc}	0.38±0.01^{ab}	0.31±0.01^{bc}	0.34±0.00^{bc}	0.29±0.01^{cd}	***	***	***
Cys	0.23±0.01^{a}	0.12±0.00^{b}	0.11±0.00^{b}	0.11±0.00^{b}	0.11±0.00^{b}	0.11±0.00^{b}	0.11±0.00^{b}	0.12±0.01^{b}	0.10±0.00^{b}	0.10±0.00^{b}	0.10±0.00^{b}	0.10±0.00^{b}	0.10±0.00^{b}	0.10±0.00^{b}	***	***	***
Apparent digestibility coefficient (%)																	
CP	82.3±0.4	NA	NA	80.0±5.3	NA	NA	80.0±1.5	82.4±0.2	NA	NA	77.7±0.3	NA	NA	80.6±4.5	NS	NS	NS
T. lipid	92.0±1.0	NA	NA	92.2±1.9	NA	NA	90.5±1.3	93.0±0.3	NA	NA	90.2±0.8	NA	NA	92.2±2.1	NS	NS	NS
GE[13]	70.3±4.1	NA	NA	67.6±8.3	NA	NA	65.4±2.6	71.9±0.0	NA	NA	67.1±0.3	NA	NA	66.9±3.2	NS	NS	NS
Tau	51.9±3.9^{a}	NA	NA	84.0±5.2^{b}	NA	NA	90.2±1.4^{b}	40.8±6.1^{a}	NA	NA	84.3±0.3^{b}	NA	NA	89.9±2.5^{b}	***	NS	NS
MeT	77.8±1.8^{a}	NA	NA	76.0±11.1^{a}	NA	NA	81.5±1.2^{a}	91.0±1.9^{b}	NA	NA	90.3±0.2^{b}	NA	NA	90.9±1.4^{b}	NS	*	NS
Cys	59.9±1.4	NA	NA	47.8±17.9	NA	NA	41.1±1.5	57.4±0.4	NA	NA	40.2±1.9	NA	NA	47.5±13.9	NS	NS	NS

[1]IBW, initial body weight (g); [2]FBW, final body weight (g); [3] FI, Feed intake (g f^{-1} d^{-1}) [4] WG, Weight gain (%); see Eq.2, [5] SGR, Specific Growth Rate (% d^{-1}); see Eq.3, [6] FCR, Feed Conversion Ratio; see Eq.5, [7] S, Survival (%); see Eq.1, [8] HSI, Hepatosomatic index; see Eq.6, [9] VSI, Viscerosomatic index; see Eq.7, [10] K, Condition factor; see Eq.4, [11] MR, Muscle ratio; see Eq.9, [12] IFR, Intraperitoneal fat ratio; see Eq.8, [13] GE, Gross energy, [14] NA, Not assessed, [15] T, Taurine, [16] M, Methionine, [17] T X M, Interaction of taurine and methionine

2.4.3 Growth

Significant interactions between dietary taurine and methionine content were observed ($P < .05$), when considering growth as response variable (**Table 2.3**). SGR was lowest in juvenile YTK fed the diet lowest in taurine and methionine content ($1.92 \pm 0.01\%$ d^{-1}).

The SGR then increased significantly with increasing taurine content and plateaued at a digestible taurine intake of 0.16 g $kgBW^{-1}$ d^{-1} and average digestible methionine intake of 0.25 g $kgBW^{-1}$ d^{-1} (**Figure 2.1**; **Table 2.3**). This equates to a dietary taurine content of 7.7 g kg^{-1} and an average methionine content of 10.9 g kg^{-1}. The SGR of YTK fed diets at an average methionine level of 17.2 g kg^{-1} were not significantly affected by the dietary taurine content. An increase in the dietary methionine content from 10.9 g kg^{-1} to 17.2g kg^{-1} resulted in an average SGR increase of 19% in juvenile YTK.

YTK fed the diet lowest in taurine and methionine had the poorest FBW (134.8 ± 3.0 g ind^{-1}) and WG ($151.8 \pm 1.2\%$). Both FBW and WG significantly increased with increasing dietary taurine content. Rising taurine contents had no further significant effect on FBW and WG at 8.5 g taurine kg^{-1} and above at an average methionine content of 10.9 g kg^{-1}. The FBW and WG of YTK fed diets containing on average 17.2 g methionine kg^{-1} were not significantly affected by the taurine content in the diets. YTK fed a relatively higher level of methionine showed an average increase of 22% in FBW and 32% in WG compared to YTK fed a lower methionine level.

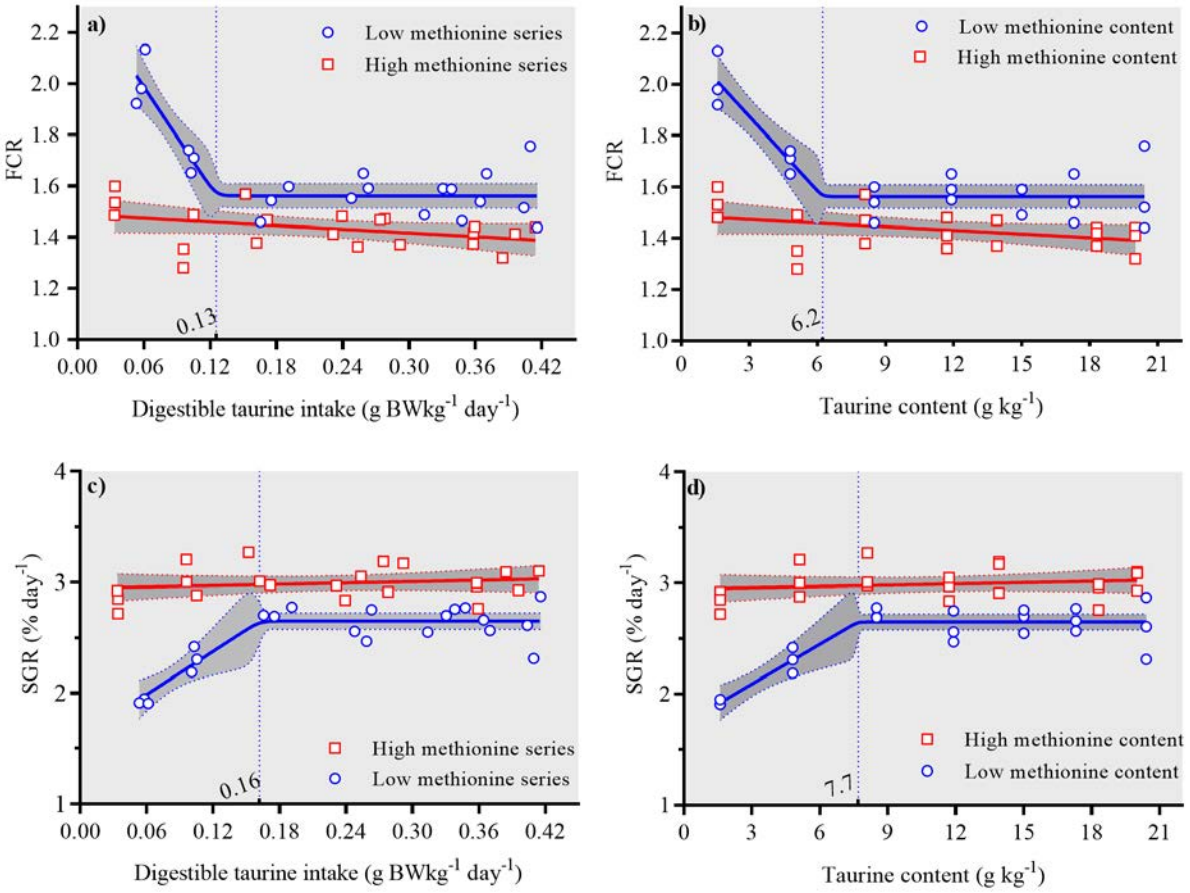

Figure 2.1 FCR response relative to (a) digestible taurine intake or; (b) dietary taurine content (b). SGR response relative to (c) digestible taurine intake or; (d) dietary taurine content. Dark grey areas indicate the 95% confidence interval. Dotted, vertical line indicates the minimum taurine requirement of YTK fed diets containing a low level of dietary methionine (10.9 g kg^{-1}; blue circles). No break point was identified at the high dietary methionine level (17.2 g kg^{-1}; red squares).

2.4.4 Feed conversion ratio

The effect of taurine on the FCR of juvenile YTK varied significantly depending on the dietary methionine content ($P < 0.05$; **Table 2.3**). The FCR performance in YTK fed the diet lowest in taurine and methionine was the poorest (2.01 ± 0.05), which then significantly improved with an increase of taurine content at an average methionine content of 10.9 g kg^{-1}. The FCR plateaued at a taurine content of 6.2 g kg^{-1} at an average methionine content of 10.9 g kg^{-1} (**Figure 2.1**). The FCR in YTK fed diets containing on average 17.2 g methionine kg^{-1} was 1.43 ± 0.03 and did not differ among dietary taurine treatments. Nevertheless, YTK fed diets containing more methionine showed on average 13% better FCRs than YTK fed diets with less methionine.

2.4.5 Morphometric indices

When considering the hepatosomatic index (HSI), a significant cross-over interaction between taurine and methionine levels were detected ($P < 0.05$; **Table 2.3**). There were no significant main effects among taurine and methionine treatments. No visual evidence of green liver syndrome was detected in YTK fed any of the dietary treatments.

The effect of methionine on viscerosomatic index (VSI) was dependent on the taurine level ($P < 0.05$). Additionally, significant differences between YTK fed different diets were found. The VSI decreased with an increase in dietary taurine and then showed no significant difference between 8.1 and 20 g taurine kg^{-1} with an average methionine content of 17.2 g kg^{-1}. The VSI ranged from 5.3% to 6.6%.

The effect of methionine on muscle ratio (MR) was dependent on the taurine level ($P < .05$). The MR ranged from 32.9 to 37.5%, with a tendency to improved performance with increasing taurine content, while the dietary methionine content did not seem to influence the MR of YTK.

No significant interaction between dietary taurine and methionine was found when considering the condition factor K. The effect of dietary methionine on K was significant ($P < 0.05$). YTK fed diets containing on average more methionine had a better K and in some instances these differences were significant (diet 11 compared to diet 1, 3, 4 and 7; $P < .05$; **Table 2.3**). The K values ranged from 1.3 to 1.5.

No significant interaction between dietary taurine and methionine was found when considering intraperitoneal fat ratio (IFR). However, IFR were significantly higher in YTK fed diets containing on average 17.2 g methionine kg^{-1} diet than YTK fed diets containing on average 10.9 g methionine kg^{-1} diet. The IFR ranged from 0.33 to 0.65.

2.4.6 Apparent digestibility

The ADC of crude protein, total lipid and gross energy were not significantly affected by the level of taurine nor methionine (**Table 2.3**). The average ADC for crude protein was 80.5 ± 2.0%. The average ADC for total lipid was 91.7 ± 1.2%. The average ADC for gross energy was 68.2 ± 3.1%. The ADC for taurine and methionine was significantly lower in YTK fed diets low in taurine and methionine (**Table 2.3**). The lowest ADC for taurine coincided with the lowest inclusion of taurine and was 51.9% and 40.8% in diets 1 and 8, respectively. In contrast, the

ADC of taurine of YTK fed higher levels of taurine was 90.2% and 89.9% for diets 7 and 14 respectively. YTK fed diets containing low levels of methionine had significantly lower ADC for methionine in comparison to YTK fed diets containing more methionine.

2.4.7 Whole carcass compositional analyses

2.4.7.1 Proximate composition

There were no significant interactions between dietary taurine and methionine level in the proximate whole carcass composition of YTK (**Table 2.4**). YTK average whole carcass proximate analysis (dry matter) was ash 127.3 g kg^{-1}, total lipid 141.9 g kg^{-1}, protein 740.0 g kg^{-1} and energy 22.5 MJ kg^{-1}.

2.4.7.2 Amino Acid composition

There were no significant interactions between dietary taurine and methionine level in the amino acid whole carcass composition of YTK. Moreover, there were no significant differences among YTK fed different dietary treatments, considering amino acid whole carcass composition except for taurine and arginine whole carcass content (**Table 2.4**). Amino acid whole carcass content was on average (dry matter): arginine 27.5 g kg^{-1}, histidine 20.1 g kg^{-1}, isoleucine 15.0 g kg^{-1}, leucine 24.3 g kg^{-1}, lysine 27.1 g kg^{-1}, methionine 10.0 g kg^{-1}, phenylalanine 14.8 g kg^{-1}, threonine 15.4 g kg^{-1}, valine 16.8 g kg^{-1}, and non-essential amino acids; alanine 21.3 g kg^{-1}, aspartic acid 32.7 g kg^{-1}, glutamine 46.2 g kg^{-1}, glycine 24.1 g kg^{-1}, serine 14.2 g kg^{-1}, and tyrosine 12.2 g kg^{-1}, and conditionally essential amino acids; cysteine 3.7 g kg^{-1}, proline 16.2 g kg^{-1}. YTK fed diets lowest in taurine had significantly lower whole carcass taurine level than YTK fed diets highest

in taurine ($P<.05$; **Table 2.4**). The inclusion level of dietary methionine did not significantly affect the taurine content in the whole carcass. The whole carcass taurine content ranged from 4.1 g kg^{-1} to 13.1 g kg^{-1}.

Table 2.4 Whole carcass proximate and amino acid composition of juvenile yellowtail kingfish (*Seriola lalandi;*), (g kg^{-1} on dry matter basis unless stated otherwise). Data are displayed mean ± SE (n=2) except for Diet 6 and 8 (n= 1). Taurine, methionine, and cysteine fecal content (g kg^{-1} on a dry matter basis). No significant interaction of taurine and methionine was detected. Different superscript letters indicate significant differences among the diet means for the respective main factor. The significant effects were determined by two-way ANOVA and levels of significance are with respect to P< .05 (*), P< .01 (**), and P< .001 (***).

	Low methionine diets (10.9 g kg^{-1})							High methionine diets (17.2 g kg^{-1})							P - value		
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12	Diet 13	Diet 14	Tau[6]	Met[7]	T xM[9]
Proximate values (g kg^{-1})																	
Ash	152.5± 4.3[a]	139.9± 5.0[a]	133.2±2 .9[a]	145.2± 4.3[a]	141.5± 6.2[a]	127.9	142.9± 1.0[a]	129.6	146.3± 5.3[b]	130.1± 2.4[b]	130.7± 0.2[b]	130.1± 2.8[b]	130.3± 2.4[b]	118.9± 4.4[b]	NS	*	NS
T. Lipid	131.5± 2.5[a]	144.4± 15.0[a]	137.8± 4.7[a]	128.9± 9.6[a]	127.6± 14.1[a]	130.3	123.0± 1.4[a]	171.8	137.2± 12.7[b]	137.0± 5.5[b]	148.6± 0.01[b]	151.1± 2.5[b]	138.9± 4.3[b]	179.7± 6.2[b]	NS	*	NS
CP	733.3± 2.0	736.9± 8.4	736.7± 0.1	750.3± 11.3	737.8± 7.5	772.7	750.6± 2.4	705.5	722.8± 18.5	750.7± 4.1	729.7± 9.0	734.6± 7.5	741.8± 4.3	716.6± 2.3	NS	NS	NS
GE[1]	21.8± 0.2[a]	22.4± 0.4[a]	22.2± 0.2[a]	22.1± 0.2[a]	22.3± 0.7[a]	22.0	21.9± 0.1[a]	23.6	22.5± 0.2[b]	22.2± 0.1[b]	22.8± 0.2[b]	22.7± 0.2[b]	22.4± 0.02[b]	23.7± 0.2[b]	NS	*	NS
EAA[2]																	
Arg	69.4±1.1[a]	76.1±0.4[ab]	73.2±1.5[ab]	78.9±1.3[b]	73.7±0.3[ab]	74.7	72.3±2.5[a]	69.7	70.5±2.2[ab]	77.2±1.0[ab]	77.4±0.6[b]	72.9±2.1[ab]	75.1±0.1[ab]	70.4±1.5[a]	*	NS	NS
His	36.8±0.2	42.7±0.1	43.1±1.1	39.7±0.6	38.8±1.6	43.0	39.3±0.9	37.2	40.2±2.2	38.3±1.7	39.7±0.9	39.2±2.2	41.3±0.4	36.0±0.0	NS	NS	NS
Ile	29.6±0.8	29.9±1.2	29.3±0.4	30.8±1.3	31.5±0.7	30.7	28.2±0.3	28.1	27.7±0.6	29.7±1.2	29.5±1.5	29.9±0.6	29.6±0.9	28.7±0.2	NS	NS	NS
Leu	47.5±1.4	49.5±1.6	47.9±0.1	51.1±2.3	50.1±1.0	47.8	46.1±0.2	44.5	43.6±1.0	47.9±1.5	47.7±1.8	48.6±0.9	46.9±1.0	48.2±0.4	NS	NS	NS
Lys	50.9±0.4	54.1±0.9	54.9±1.5	56.9±2.3	56.7±2.3	53.7	52.2±0.7	51.2	48.7±1.9	52.4±0.5	57.4±1.6	53.3±0.3	57.5±0.6	49.5±2.0	NS	NS	NS
Met	19.8±0.1	20.5±0.1	19.5±0.3	19.7±0.5	20.6±0.2	22.0	19.8±0.5	19.6	19.1±0.2	17.9±0.1	20.1±0.6	19.8±0.4	20.6±0.5	18.4±0.6	NS	NS	NS
Phe	28.9±0.7	29.6±0.5	28.8±0.1	30.4±1.1	30.8±0.3	29.4	28.7±0.1	28.1	27.3±0.6	29.1±0.9	29.2±1.1	29.3±0.4	28.7±0.6	28.5±0.2	NS	NS	NS
Thr	29.9±0.6	31.1±0.1	30.1±0.6	30.9±0.4	31.5±0.6	32.4	30.5±0.4	28.9	29.0±0.9	30.9±0.2	30.6±0.5	29.9±0.5	30.8±0.5	29.2±0.5	NS	NS	NS
Val	32.6±0.8	33.5±0.7	32.5±0.1	34.0±1.3	34.8±0.7	34.0	31.9±0.2	31.4	31.0±0.2	32.9±1.1	33.7±0.7	33.1±0.0	33.3±0.1	32.9±0.3	NS	NS	NS
NEAA[3]																	
Ala	42.5±0.3	42.9±1.9	42.5±1.0	42.2±0.2	43.8±1.0	43.7	41.8±0.1	41.1	40.9±1.1	42.5±0.3	42.3±0.0	41.3±0.6	41.5±0.3	39.5±0.1	NS	NS	NS
Asp	64.1±1.4	66.7±1.8	62.3±0.5	64.8±0.9	67.8±0.8	68.7	62.5±0.4	60.1	61.8±2.1	66.0±1.0	65.2±1.6	64.1±0.7	65.1±0.4	62.6±0.7	NS	NS	NS
Glu	92.3±2.4	92.7±2.7	90.3±1.8	91.8±1.0	94.0±0.1	93.8	90.2±0.1	86.4	87.9±0.8	93.1±0.3	90.9±1.0	89.1±1.6	93.7±0.7	87.6±0.7	NS	NS	NS

	Low methionine diets (10.9 g kg^{-1})							High methionine diets (17.2 g kg^{-1})							P - value		
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12	Diet 13	Diet 14	Tau[6]	Met[7]	T xM[9]
Gly	47.8±1.4	46.6±2.7	48.1±1.5	47.2±1.3	49.4±2.7	50.5	49.4±0.4	46.9	48.4±1.1	48.7±1.2	45.8±2.4	47.1±0.8	48.6±0.6	43.3±0.8	NS	NS	NS
Ser	28.8±0.5	29.0±0.3	28.4±0.0	28.5±0.5	28.9±0.2	29.0	27.6±0.5	27.1	27.7±1.5	28.7±0.9	27.7±0.3	26.8±0.3	27.9±0.4	26.9±0.4	NS	NS	NS
Tyr	24.0±1.2	24.8±0.3	22.8±0.3	25.0±0.8	24.8±0.5	26.0	23.3±0.4	22.9	22.4±0.3	24.1±0.5	24.5±0.3	23.5±0.5	24.6±0.2	24.0±0.1	NS	NS	NS
CEAA[4]																	
Cys	7.9±0.4	7.6±0.3	7.2±0.1	7.6±0.2	7.6±0.1	8.4	6.9±0.0	6.1	6.5±0.3	7.2±0.28	7.5±0.07	6.8±0.1	7.1±0.4	7.1±0.2	NS	NS	NS
Pro	32.3±0.6	32.8±1.7	33.0±1.0	33.0±0.3	34.6±1.6	34.3	33.6±0.4	32.4	32.7±0.7	33.3±0.1	32.0±0.4	32.8±0.6	33.5±0.5	30.3±0.3	NS	NS	NS
Tau	4.6±0.4^{a}	9.1±1.7^{ab}	10.6±0.2^{b}	9.5±1.1^{ab}	11.7±1.6^{b}	13.1	12.9±0.2^{b}	4.1	9.9±0.6^{a}	11.1±0.7^{ab}	9.8±1.4^{b}	11.9±0.4^{b}	12.2±0.6^{b}	8.7±0.1^{b}	*	NS	NS
AA[5] fecal content (g kg^{-1})																	
Tau[6]-feces	2.0±0.2^{a}	NA	NA	3.5±0.1^{b}	NA	NA	4.2±0.9^{b}	2.5±0.3^{a}	NA	NA	3.5±0.1^{b}	NA	NA	4.7±0.2^{b}	***	NS	NS
Met[7]-feces	6.5±0.8	NA	NA	5.5±1.0	NA	NA	4.7±0.6	4.4±1.0	NA	NA	4.0±0.1	NA	NA	4.4±0.3	NS	NS	NS
Cys[8]-feces	6.5±0.01	NA	NA	6.2±0.03	NA	NA	7.5±0.1	6.2±0.01	NA	NA	7.7±0.02	NA	NA	7.02±0.04	NS	NS	NS

[1] GE, Gross energy (MJ kg^{-1}), [2] EAA, essential amino acids, [3] NEAA, non-essential amino acids, [4] CEAA, conditionally essential amino acids, [5] AA, amino acids, [6] Tau, Taurine, [7] Met, Methionine, [8] Cys, Cysteine, [9] T X M, Interaction of taurine and methionine

2.4.8 Regression analyses

The Koops and Grossman (1993) regression model was applied to describe the relationships of SGR or FCR to incremental increases in taurine (content and intake) for each methionine series (**Figure 2.1**). The regression model applied to the lower methionine content series in response to SGR had an AIC value of 77.85, total sums of squares of 0.31, and an r^2 of 0.82. The regression model applied to the lower methionine content series in response to FCR had an AIC value of 95.03, total sum of square of 0.14 and an r^2 of 0.79. The applied models indicate that a daily intake between 0.13 and 0.16 g digestible taurine kg BW^{-1} d^{-1} when dietary methionine levels are on average 10.9 g kg^{-1} diet will optimize SGR and FCR in juvenile YTK respectively.

2.5 Discussion

This study determined the taurine requirement of juvenile YTK and revealed two key findings: that dietary methionine at adequate levels can spare taurine; and that the industry standard dietary methionine inclusion of 11.1 g kg^{-1} diet may not be sufficient to optimize growth in YTK. The results demonstrate that both taurine and methionine have critical functions in the metabolism and health of YTK. Growth, feed and morphometric responses of YTK from this study indicate that taurine and methionine are highly interactive; methionine (taurine's metabolic precursor) potentially spares taurine. The results of this study indicate that there is no benefit in supplementing taurine into low taurine diets for YTK when the methionine intake is adequate. However, if the digestible methionine intake approaches or is below 0.25 g $kgBW^{-1}$ d^{-1}, then the digestible taurine intake should be at least 0.16 g $kgBW^{-1}$. This equates to a dietary methionine content of 10.9 g kg^{-1} diet and a dietary taurine content of 7.7 g kg^{-1}.

Taurine is derived from the bioconversion of methionine and cysteine, which is enzyme facilitated. If enzymes are present and sufficiently active to synthesize taurine *de novo*, then dietary methionine can spare taurine as a precursor (Kim et al., 2008b). The sparing effects of sulfur amino acids on taurine requirements have been demonstrated in other finfish species. Ferreira et al. (2015) demonstrated that supplementary taurine does not need to be supplied in diets for rock bream when the TSAA levels are at 27 g kg^{-1} diet and suggested that rock bream may have some capacity to biosynthesize taurine from methionine and cysteine, but not at a rate that can meet requirements. The rate of endogenously synthesized taurine in fish differs among species and depends on two controlling enzymes: cysteine dioxygenase (CDO) and cysteine sulfinate decarboxylase (CSD) (Gaylord et al., 2007; Yokoyama et al., 2001). Because of recent developments in the understanding of SAA metabolism, taurine's conventional definition as a non-essential amino acid has been challenged and re-evaluated to be considered conditionally essential for many fish species (Li et al., 2009b; Salze et al., 2011; Takagi et al., 2008). Nevertheless, our results indicate that taurine supplementation is conditionally essential in YTK. Adequate amounts of dietary methionine spare taurine and make taurine supplementation in low taurine diets unnecessary. Indeed, the greater amberjack (*Seriola dumerili*) and YTK have been shown to carry the protein sequence for the CSD enzyme (UniProt: A0A3B4UMV9, A0A3B4WPE6, A0A0N9E6M5) and CDO enzyme (UniProt: A0A3B4VM04, A0A3B4X7Z8; The UniProt Consortium, 2019). The Japanese yellowtail also carries the CSD enzyme (The UniProt Consortium, 2019); however, the CDO enzyme has not yet been identified for this species.

The taurine requirement in juvenile YTK was estimated by Salze et al. (2018) to be between 2.6 to 10.2 g kg^{-1} diet, depending on the response variable or regression model that was applied, with the diets containing 10.5 g methionine kg^{-1} and 6.3 g cysteine kg^{-1}. This generally supports the findings of the present study, i.e. taurine supplementation is required when dietary specification of methionine is approximately 10 g kg^{-1} in diets for YTK. Salze et al. (2018) used poultry and soybean meal as the two main dietary protein sources to determine taurine requirement. Similarly, Jirsa et al. (2014) and Takagi et al. (2008) observed inferior physiological development of Japanese and YTK fed taurine deficient all-plant protein diets. However, Nguyen et al. (2015) showed that despite 15 g kg^{-1} taurine supplementation into soybean meal-based diets, Japanese yellowtail still experienced inferior growth due to antinutritional factors reducing the bile acid reabsorption and secretion and pancreatic digestive enzyme production. Kaushik et al. (2004) successfully reduced fishmeal to 5% using plant proteins in diets for European sea bass, also a marine carnivore, without supplementary taurine. The dietary amino acid profile was not presented in that study; therefore, it is not possible to conclude if taurine sparing was occurring. However, European sea bass seem to have some capacity to synthesize taurine to meet requirements. Martins et al. (2018) determined a taurine requirement in European sea bass of 4.7 to 5.0g kg^{-1} diet at a methionine level of 10 - 12 g kg^{-1} dry diet. Understanding the TSAA content and interactions is important for assessing the absolute requirement of taurine.

Methionine can be deficient in some dietary protein sources and may not meet requirements for certain species. Current industry practice in Australia is to formulate to a minimum of about 11.0 g methionine kg^{-1} diet for YTK, which is loosely based on the study by Ruchimat et al. (1997) who reported the methionine requirement of Japanese yellowtail to be 11.1 g methionine

kg^{-1}. However, our results indicated that YTK growth was relatively greater at a higher methionine inclusion, which may be a species-specific difference. Interestingly, if non-linear regression analyses (quadratic) are applied to the data of Ruchimat et al. (1997) a requirement value of 12.9 g kg^{-1} diet is obtained for Japanese yellowtail. Further, it is important to note that Ruchimat et al. (1997) qualifies the methionine requirement value in the presence of 3.1 g cysteine kg^{-1} diet i.e. a minimum TSAA specification of 14.2 g kg^{-1} diet. The TSAA contents of the high methionine series of diets in the current study were on average 22.7 g kg^{-1} diet and indicate improved growth and feed performance in comparison to the diet containing methionine and cysteine at current industry practice (16.8 g TSAA kg^{-1} diet). Follow-up studies to quantify the methionine requirement and the sparing capacity of cysteine in YTK would be pertinent.

The partitioning or metabolic allocation of ingested taurine, methionine and cysteine is not well understood in YTK. The whole carcass taurine content increased and then plateaued to an incremental increase of digestible taurine intake, indicating a maximum retention of the free taurine pool in whole carcass of YTK. Beyond the saturation point excess taurine may have been excreted. In rats this occurs via kidneys (Sved et al., 2007). In YTK, fecal taurine increased with increasing dietary taurine intake (**Table 2.4**); however, generally, the excretory mechanism of excess dietary taurine in fish is not well understood. Increased levels of dietary methionine did not significantly increase the whole carcass taurine content, indicating no accumulation in whole carcass total amino acid pool of methionine derived taurine. In turbot, graded levels of dietary taurine led to a respective change in taurine content of whole carcass (Qi et al., 2012). Yuzhe et al. (2013) found that an increase of dietary methionine induced an increase of taurine when testing for the free amino acid pool in muscle tissue of Japanese flounder, whereas dietary

methionine did not affect the taurine content when testing the total amino acid composition. The underlying mechanisms of methionine derived taurine in fish and YTK are not well understood and require further research. Further, fecal methionine and cysteine content remained consistent regardless of the methionine intake while fecal taurine content increased with increasing digestible taurine intake, regardless of the digestible methionine intake.

Studies on methionine and cysteine derived taurine in fish are rare. However, the liver appears to be a major organ in transforming sulfur amino acids to taurine and more than 50 other transmethylation and sulfuration reactions in fish (Liu et al., 2017), piglets (Robinson et al., 2016), rats (Stipanuk et al., 2013), and broiler chicks (Robinson et al., 2016; Saunderson, 1985). In piglets, of the total 100% ingested dietary methionine, ~80% reaches the liver and is further metabolized to ~30% phosphatidylcholine and creatine, ~50% proteins, and ~20% tissue proteins (Robinson et al., 2016). However, ~20% of the non-redirected methionine to the liver, ~6% were metabolized in gastrointestinal tissue to homocysteine (Riedijk et al., 2007). In total, ~41.2% of ingested methionine by piglets can potentially become taurine. Robinson et al. (2016) found that restricted availability of dietary methyl donors, such as methionine, increased by threefold the methionine partitioning to homocysteine, an intermediate metabolite for the cysteine production. Further cysteine oxidation produces sulfate and taurine (~2:1 ratio; Stipanuk and Ueki, 2011). In fish, CDO and CSD activity is species-specific and can restrict taurine production to the extent that fish are not capable of meeting taurine requirements (Salze and Davis, 2015). Therefore, taurine is considered either essential (Magalhães et al., 2019), non-essential (Kim et al., 2008b) or conditionally essential (Poppi et al., 2018). To date, studies of enzyme derived pathways of the taurine synthesis have focused on the enzymes CDO and CSD. However, Gonzales-Plasus et

al. (2018) hypothesized that carp may synthesize taurine through a non-cysteinesulfinic acid related pathway, emphasizing the presence of cysteamine decarboxylase in carp. This hypothesis is based on the concept that carp have no demonstrable taurine requirement (Kim et al., 2008b), retain relatively high amounts of taurine in body tissue when fed low levels of dietary taurine (Hujita, 1988; Kim et al., 2008b) and have limited CSD activity (Yokoyama et al., 2001). The involvement of specific enzymes and *de novo* synthesis of sulfur related compounds from dietary supplements is not well understood in aquaculture species. Further research is needed to understand the partitioning of methionine and cysteine toward metabolites and the *de novo* synthesis of taurine in YTK and fish species in general.

Dietary taurine and methionine did not significantly affect the digestibility of lipid, protein and energy in YTK. However, there was a significant correlation in taurine and methionine digestibility with increasing digestible taurine and methionine intake. The ADC of taurine and methionine in YTK fed diets containing no and low amounts of crystalline taurine and methionine was significantly lower than the ADC of YTK fed diets containing higher amounts of crystalline amino acids, likely because amino acids in this form are absorbed faster than protein-bound amino acids (Nunes et al., 2014). Ambardekar et al. (2009) demonstrated in channel catfish increased ADC of crystalline methionine (~100%) compared to the ADC of protein-bound methionine in blood meal (77.2 ± 8.2%) and fishmeal (92.8 ± 5.6), indicating an ingredient-specific ADC of methionine; however, methionine from intact proteins in form of corn grain, soybean meal and wheat middlings demonstrated equally good ADC to crystalline methionine (~100%). Overall, research on the digestibility of different taurine and methionine sources in fish

is scant and further research is required to understand the complex interactions between these compounds.

2.6 Conclusion

To conclude, our results indicate that juvenile YTK require at least 0.16 g digestible taurine $kgBW^{-1}$ d^{-1} at an average digestible methionine intake of 0.25 g $kgBW^{-1}$ d^{-1}. This equates to a minimum taurine content of 7.7 g kg^{-1} diet at an average methionine level of 10.9 g kg^{-1} diet to optimize growth rate. To optimize FCR we recommend a minimum daily intake of 0.13 g digestible taurine $kgBW^{-1}$ d^{-1} at an average digestible methionine daily intake of 0.25 g $kgBW^{-1}$ d^{-1}. The results indicate that supplementation of taurine in a low taurine diet is not necessary if an average digestible methionine intake of 0.34 g $kgBW^{-1}$ d^{-1} is provided, which equates to an average methionine content of 17.2 g kg^{-1} diet. YTK fed diets containing 17.2 g methionine kg^{-1} diet performed better than those fed a standard inclusion of 10.9 g kg^{-1} diet, indicating that current industry practice is likely inadequate; however, further research is required to precisely quantify the dietary methionine requirement of YTK. These recommendations are relevant for the size and culture conditions undertaken in this study. We further recommend investigation of the impacts of ontogenetic and abiotic factors on the TSAA requirements in YTK.

Chapter 3. The sulfur amino acid requirements of juvenile yellowtail kingfish (*Seriola lalandi*)[2]

3.1 Abstract

The dietary methionine and cysteine requirements of yellowtail kingfish (YTK) are unknown. Methionine, an essential sulfur-containing amino acid, acts as a sulfur and methyl donor for key metabolites, such as cysteine and taurine. Cysteine, a conditionally essential sulfur-containing amino acid, can spare significant amounts of methionine in the total sulfur amino acid (TSAA) requirement. Both methionine and cysteine are an integral part of YTK aquafeeds, in which methionine supplementation levels are based on the requirements of the closely related Japanese Yellowtail (*Seriola quinqueradiata*) at approximately 11.1 g kg^{-1} diet. However, recent research has demonstrated better growth and feed efficiency in YTK fed diets containing more than 11 g methionine kg^{-1}, although the precise methionine requirement and potential interactions with cysteine remain unknown. Therefore, the present study was designed to (1) elucidate the methionine requirement of YTK at low and high dietary cysteine contents and to (2) quantify cysteine's sparing capacity for methionine in the TSAA requirement. These requirements and relationships were established by feeding ten isonitrogenous and isoenergetic diets, made from common feed ingredients, to triplicate groups of 12 fish (initial 52.6 ± 1.0 g $fish^{-1}$) over 54 days.

[2] Data from this chapter were published as: Candebat, C. L., Booth, M., & Pirozzi, I. (2021). The sulfur amino acid requirements of juvenile yellowtail kingfish (*Seriola lalandi*). Aquaculture, 534, 736234.

The orthogonal design consisted of two levels of dietary cysteine (5.6 & 13.9 g kg^{1}), crossed with five levels of dietary methionine, increasing from 7.9 to 25.2 g kg^{-1}. Non-linear regression analysis indicated an average digestible TSAA requirement of 0.70 g $kgBW^{-1}$ d^{-1} (0.56 g Met $kgBW^{-1}$ d^{-1} & 0.14 g Cys $kgBW^{-1}$ d^{-1}) based on feed conversion ratio, specific growth rate, and protein retention efficiency. This approximates to an average dietary sulfur amino acid specification of 24.5 g kg^{-1} (18.9 g Met kg^{-1} & 5.6 g Cys kg^{-1}). Cysteine spared 40.4 - 49.2% of methionine in the TSAA requirement on an equimolar sulfur basis. Sub- and supraoptimal levels of dietary methionine and cysteine induced inferior growth and feed efficiency. Additionally, fish fed the diet lowest in dietary methionine and cysteine indicated early stages of cataract. This study provides quantitative data on the sulfur amino acid requirements of juvenile YTK and will facilitate the formulation of better diets for this species.

3.2 Introduction

Methionine and cysteine are sulfur amino acids that enable normal growth and performance of fish. Methionine is incorporated into proteins and is the initiating amino acid in the synthesis of practically all eukaryotic proteins (Brosnan and Brosnan, 2006). Methionine is considered an essential amino acid as fish are unable to produce sufficient methionine via *de novo* synthesis to meet their metabolic requirements. Therefore, methionine must be obtained through the diet. Methionine is involved in the transmethylation and transulfuration of two key intermediates; S-adenosylmethionine and cysteine (Finkelstein et al., 1988). S-adenosylmethionine is a versatile methyl and methylene donor for e.g. proteins, lipids, creatine, phosphatidylcholine, carnitine, DNA, and RNA intermediates (Lu, 2000). Assuming that methionine can convert 100% into cysteine means that it can cover 100% of the TSAA requirement (Ball et al., 2006).

The TSAA (Met) requirement is the absolute requirement for SAA that is completely met by methionine in the absence of cysteine (Ball et al., 2006). The TSAA (Met) requirement can also be met by a combination of methionine and cysteine which is the TSAA (Met + Cys) requirement (National Research Council, 2011a); however, the TSAA (Met + Cys) requirement must contain sufficient methionine to meet the minimum obligatory requirement (MOM) for methionine which cannot be provided by cysteine. Cysteine, like methionine, serves as a building block in the protein biosynthesis (Yin et al., 2016). However, unlike methionine, cysteine is a conditionally EAA, which means its requirement can be completely spared by supplying dietary methionine, its metabolic precursor. Cysteine can only spare the proportion of methionine in the TSAA requirement that would be used to form cysteine itself (Courtney-Martin et al., 2012),

due to the irreversible intermediate synthesis of L-homocysteine to L-cystathionine. Metabolically, cysteine is important as a precursor for the synthesis of taurine, sulfate, and glutathione (Bertolo and McBreairty, 2013) and can spare its derivatives, if not enzymatically restricted. The metabolic relatedness, interactions and sparing effects of SAA imply that quantifying the requirement for one essential SAA must be done with reference to the other SAA and derivatives present in the diet (Ball et al., 2006).

Yellowtail kingfish, *Seriola lalandi* (hereafter referred to as YTK), is a circum-globally distributed, high value aquaculture species, which global commercialization in recirculating aquaculture systems and sea cages are becoming increasingly popular (Symonds et al., 2014). Little is known about the EAA requirements of YTK and aquafeed formulations are mainly based on the known requirements of other carnivorous fish species. For example, current recommended levels of dietary methionine for YTK are based on the study of Ruchimat et al. (1997), who found the methionine requirement of the closely related Japanese yellowtail was about 11.1 g kg^{-1} diet. However, their study did not account for dietary interactions among SAA compounds. Reliance of the established requirements of other species, even closely related ones, requires caution. For instance, Candebat et al. (2020) recently demonstrated that YTK may have a higher dietary methionine requirement than Japanese Yellowtail, as YTK fed diets containing 17.2 g methionine kg^{-1} exhibited better growth and feed efficiency, than YTK fed diets containing 11 g methionine kg^{-1} diet. This indicates that the current methionine content of YTK aquafeeds might be insufficient to provide the methionine or indeed the TSAA requirement necessary to optimize YTK growth and feed efficiency.

Fish feed producers and formulators are making a concerted effort to develop more cost-effective and sustainable aquafeeds, moving away from fishmeal as a primary protein source, especially in carnivorous species (Naylor et al., 2009). Hence, alternative feedstuffs, such as plant protein meals and concentrates and rendered animal proteins are routinely being utilized in aquafeed manufacture. However, alternative protein sources can have unbalanced EAA profiles and are often deficient in methionine (Ayadi et al., 2012). To correctly formulate nutritionally balanced diets the nutrient requirements of the animal must be known. Currently, a comprehensive understanding of the quantitative requirements for SAA are missing for YTK. Therefore, the present study was designed to elucidate the methionine requirement of juvenile YTK in the presence of low or higher dietary cysteine content and to determine the sparing effect of cysteine for dietary methionine in the TSAA requirement.

3.3 Methods

3.3.1 Ethics

This experiment was performed under the NSW Department of Primary Industries (DPI) Fisheries Animal Care & Ethics (ACEC) Research Authority known as 'Aquaculture Nutrition ACEC 93/5–Port Stephens' (ACEC, 2009).

3.3.2 Experiment design

A factorial dose-response design was applied to quantify the dietary methionine requirement of juvenile YTK relative to dietary cysteine content and cysteine's sparing effect for methionine in the TSAA requirement.

All ten diets were made, using practical feed ingredients and formulated to be isonitrogenous (624.1 ± 8.2 g crude protein kg^{-1}) and isoenergetic (22.2 ± 0.1 MJ GE kg^{-1}; **Table 3.1**) to meet the protein and energy requirements of juvenile YTK (Booth et al., 2010; Pirozzi et al., 2019). Desired dietary SAA specifications for each diet were achieved by using raw ingredients, crystalline DL-methionine, and L-cysteine (**Table 3.2**). The design established five incremental levels of methionine, ranging from 7.9 to 25.2 kg^{-1} diet, crossed with two levels of cysteine (5.5 or 13.9 g kg^{-1} diet; **Table 3.1**). Additionally, taurine was added to each treatment, at 7g kg^{-1} diet, to meet the known taurine requirement of YTK (Candebat et al., 2020) and 1 g yttrium oxide kg^{-1} diet was included in each diet as an inert digestibility marker (**Table 3.2**).

Table 3.1 Compositional analysis of experimental diets (g kg^{-1} dry matter).

	Low cysteine series					High cysteine series				
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10
Proximate composition										
Dry matter	928.0	937.7	906.3	928.3	909.9	928.2	927.8	928.4	896.0	909.1
Crude protein	638.8	607.2	582.6	636.6	619.5	637.9	591.5	601.7	674.4	651.1
Total nitrogen	102.2	97.1	93.2	101.9	99.1	102.1	94.6	96.3	107.9	104.2
Total lipid	135.8	119.2	134.0	135.6	135.2	130.2	128.9	130.7	128.6	138.1
Ash	107.3	106.2	106.8	106.9	108.2	100.0	99.8	103.0	104.5	104.3
Gross energy (MJ kg^{-1})	21.8	22.1	22.1	22.2	22.2	22.4	22.1	22.4	22.2	22.4
Amino acid composition										
Alanine	30.6	29.7	29.2	29.7	28.7	29.0	30.0	29.3	29.6	28.0
Arginine	39.0	38.5	37.3	38.7	37.4	37.5	38.9	38.3	38.2	36.6
Aspartic acid	44.7	44.0	41.7	44.0	42.2	42.1	43.2	43.5	43.1	41.4
Cysteine	5.8	5.6	5.0	5.4	5.9	13.7	14.1	14.4	13.5	13.9
Glutamic acid	84.4	83.9	79.4	83.9	80.6	80.3	82.4	82.7	82.1	79.0
Glycine	53.1	50.2	51.5	50.0	48.2	50.2	52.2	48.9	51.1	47.2
Histidine	13.1	13.3	12.5	13.4	13.1	12.6	13.2	13.3	12.9	12.6
Hydroxyproline	18.5	16.9	18.3	16.8	16.0	17.3	18.1	16.4	17.8	15.7
Isoleucine	21.8	22.1	20.7	22.3	21.8	21.0	21.8	21.8	21.4	21.1
Leucine	39.8	40.4	37.8	40.5	39.5	38.4	39.8	40.0	39.2	38.3
Lysine	33.9	33.8	32.1	34.0	32.9	32.6	33.4	33.9	33.4	32.2
Methionine	8.8	12.7	16.5	22.4	24.7	7.9	12.7	17.6	21.2	25.2
Phenylalanine	23.0	23.2	21.8	23.4	22.8	22.2	23.0	23.1	22.6	22.1
Proline	45.8	44.7	44.0	44.6	43.1	43.8	45.4	43.8	44.6	42.1
Serine	26.9	27.1	25.5	27.0	26.0	26.2	26.8	26.9	26.6	25.6
Taurine	7.0	7.0	6.9	7.1	6.9	7.0	7.3	7.3	7.2	6.9
Threonine	20.2	20.4	19.2	20.5	19.8	19.6	20.2	20.3	19.9	19.3
Tryptophan	4.9	4.9	4.5	4.9	4.7	5.1	4.8	5.1	4.8	4.4
Tyrosine	15.2	15.3	14.3	15.5	15.0	14.5	15.2	15.5	15.2	14.7
Valine	26.4	26.8	25.1	26.9	26.3	25.4	26.5	26.4	25.9	25.5

Table 3.2 Experiment diet formulations (g kg^{-1} dry matter).

Ingredient	Low cysteine series					High cysteine series				
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10
Dehulled Lupins[1]	199.1	199.1	199.1	199.1	199.1	199.1	199.1	199.1	199.1	199.1
Soy protein concentrate[1]	152.0	147.1	152.0	152.0	152.0	151.4	152.0	152.0	152.0	152.0
Fishmeal[1]	126.0	129.7	129.7	129.7	129.7	129.7	129.7	129.7	129.7	129.7
Gelatin[2]	143.3	134.9	127.3	127.3	127.3	134.5	123.5	123.5	123.5	118.6
Sodium Caseinate[3]	97.8	101.0	101.0	101.0	101.0	97.8	101.0	101.0	101.0	101.0
Fish Oil[1]	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Blood Meal[1]	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Diatom. Earth	51.7	51.4	51.2	51.2	51.3	44.0	44.9	46.9	49.0	45.4
Feather Meal	28.7	29.4	28.5	28.5	28.5	30.0	30.0	30.0	30.0	32.0
Maize starch	25.0	25.7	24.2	18.9	13.3	26.0	27.0	19.7	12.3	13.3
NaH2PO4	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Vit-min premix	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Choline Chloride (70%)	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Rovimix Stay-C	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Lysine	2.9	2.9	2.9	2.9	2.9	2.9	2.9	2.9	2.9	2.9
Yttrium oxide (Y_2O_3)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Methionine[4]	0.0	5.3	10.6	15.9	21.4	0.0	5.3	10.6	15.9	21.5
Cysteine[5]	0.0	0.0	0.0	0.0	0.0	11.1	11.1	11.1	11.1	11.0
Taurine[6]	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5

[1]Ridley Agriproducts Pty Ltd, Australia, [2]J.L. Stewart & Son Pty Ltd, Glendenning, NSW, Australia, [3]Total FoodTec Pty Ltd, Darra, QLD, Australia, [4]99.8 % purity, Ridley Agriproducts Pty Ltd, Australia, [5]98.5 % purity, Spectrum Chemical, USA, [6]96.5 % purity, Bulkpowders, Australia

Triplicate groups of 12 YTK (52.6 ± 1.0 g $fish^{-1}$) were stocked into 200 L experiment tanks integrated within a research scale recirculating aquaculture system (RAS). A random sample of ten fish were also taken to determine the initial chemical composition of juvenile YTK. Experimental diets were randomly allocated and YTK were hand fed to apparent satiation twice daily (9 AM and 3 PM) during weekdays and once daily (9 AM) on weekends for 54 days. After feeding events, any uneaten pellets that settled to the tank floor were automatically flushed into a pellet trap and collected, ensuring the integrity of uneaten pellets. The collected pellets were pooled within tank over the duration of the trial and stored at -18°C. At the conclusion of the trial the total dry weight of the uneaten pellets was determined by oven drying for approximately 48 h at 80°C until weights remained stable. The feed intake of each tank was then calculated by deducting the total dry matter value of uneaten pellets from the dry matter amount of feed delivered to each tank.

3.3.3 Diet manufacture

All experiment diets were made using laboratory scale equipment. Prior to making the experiment diets, each raw material was finely milled using a high-speed hammer mill, fitted with a 1.6 mm screen (Raymond Laboratory Mill, Transfield Technologies, Rydalmere, NSW, Australia). Wheat flour was autoclaved at 121°C for two min. Supplements and processed raw materials were then intensively dry mixed in a Hobart mixer (Hobart Mixer; Troy Pty Ltd, Ohio, USA) before the addition of oil and fresh water, forming a moist dough. The dough was then cold extruded into 6mm sinking pellets (Dolly, La Monferrina, Castell'Alfero, Italy) and oven dried at ~60°C reducing the moisture content <10%.

3.3.4 Fish handling and experimental system

All YTK used in the experiment were progeny of wild-caught broodstock held at the NSW DPI PSFI NSW, Australia. Prior to stocking, juvenile YTK were held in 10 kL tanks at 15°C – 19°C and fed daily with commercial YTK 4mm floating pellets (specified crude protein 50%, crude fat 14%, and crude fiber 4%). Prior to handling YTK were sedated using the recommended dose of Aqui-S® (540 g L^{-1} isoeugenol; Aqui-S New Zealand Ltd.). Fish were fasted for 24h prior to weighing and termination.

The experiment was conducted under controlled conditions within a RAS. All 30 x 200 L tanks were partially covered with black plastic to reduce disturbance of fish and each tank was covered with netting, preventing YTK from escaping. Effluent water was continuously exchanged with filtered and disinfected estuarine water at a rate of approximately 10% d^{-1}. YTK received a prophylactic hydrogen peroxide treatment (150 ppm for 30 min) against skin and gill fluke at week three and week six. The laboratory light regime was based on the natural light regime of the season (11L: 13D) using dimmed, overhead LED lamps. Dissolved oxygen was supplied using industrial oxygen (BOC) diffused directly into each experiment tank. Water quality in the RAS was monitored daily in the morning for water temperature (21.2 ± 0.6°C), salinity (32.9 ± 3.2), dissolved oxygen (12.1 ± 3.0 mg L^{-1}), pH (7.4 ± 0.4), and TAN (≤ 0.25 mg L^{-1}).

3.3.5 Performance variables and calculations

The following variables were used to assess the effects of methionine and cysteine concentration on the performance of juvenile YTK. Calculations are based on the comparative slaughter assay technique (**Table 3.3**). In addition, the eyes of all YTK were visually inspected at harvest and scored for the presence or absence of cataracts, a clouding of the lens.

$$Survival\ (\%) = \left(\left(\frac{100}{\#\ stocking\ fish}\right) * \#fish\ at\ harvest\right) \quad \text{(Eq. 1)}$$

$$Weight\ gain\ (WG)(\%) = \left(\frac{FBW - IBW}{IBW}\right) * 100 \quad \text{(Eq. 2)}$$

where FBW is final body weight (g) and IBW is initial body weight (g)

$$Specific\ growth\ rate\ (SGR)(\%\ day - 1) = \left(\frac{Ln\ FBW - Ln\ IBW}{days}\right) * 100 \quad \text{(Eq. 3)}$$

$$Thermal - unit\ growth\ coefficient\ (TGC) = \left(\frac{FBW^{\frac{1}{3}} - IBW^{\frac{1}{3}}}{T * t}\right) * 100 \quad \text{(Eq. 4)}$$

where T is temperature in °C and t is time in days

$$Feed\ conversion\ ratio\ (FCR) = \frac{feed\ intake\ (g\ fish^{-1})}{weight\ gain\ (g\ fish^{-1})} \quad \text{(Eq. 5)}$$

$$Condition\ factor\ (K) = \left(\frac{FBW}{fork\ length\ (cm)^3}\right) * 100 \quad \text{(Eq. 6)}$$

$$Hepatosomatic\ index\ (HSI) = \left(\frac{liver\ weight\ (g)}{FBW}\right) * 100 \quad \text{(Eq. 7)}$$

$$Viscerosomatic\ index\ (VSI) = \left(\frac{viscera\ weight\ (g)}{FBW}\right) * 100 \quad \text{(Eq. 8)}$$

where viscera include liver, organs, and intraperitoneal fat

$$Protein\ retention\ efficiency\ (PRE) = \frac{CP\ gain\ (g\ fish^{-1})}{CP\ intake\ (g\ fish^{-1})} \quad \text{(Eq. 9)}$$

$$Methionine\ retention\ efficiency\ (MRE) = \frac{Met\ gain\ (g\ fish^{-1})}{Met\ intake\ (g\ fish^{-1})} \quad \text{(Eq. 10)}$$

The sparing potential of cysteine on methionine in the TSAA requirement of juvenile YTK was based on Ball et al. (2006) and Humayun et al. (2007) and was calculated as follows:

$$Cys.sparing\ =\ Met\ req.at\ low\ cys\ (Met + Cys) -\ Met\ req.at\ high\ cys\ (Met) \quad \text{(Eq. 11)}$$

Ball et al. (2006) defines the measured methionine requirement at low/absent cysteine content as the TSAA requirement, assuming the low cysteine proportion of the TSAA requirement is

converted to the molar mass of methionine and added. Furthermore, Ball et al. (2006) suggests that the methionine requirement at excess cysteine content defines the MOM requirement. Using the terminology suggested by Ball et al. (2006), the following equation was used to calculate cysteine sparing:

$$Cys\ sparing\ =\ TSAA\ requirement\ (Met) -\ MOM\ requirement\ (Met) \quad \text{(Eq. 12)}$$

3.3.6 Apparent digestibility and digestible intake of dietary treatments

Following the conclusion of the growth experiment the apparent digestibility coefficient of diets representing low (Diet 1 and Diet 6), medium (Diet 3 and Diet 8) and high (Diet 5 and Diet 10) levels of dietary methionine within each cysteine series were determined by using stripping techniques (Booth and Pirozzi, 2018). Prior to stripping, YTK were anesthetized in their respective tanks using 5- 25mg L^{-1} Aqui-S®. Feces was collected from the posterior intestine of individuals by applying gentle abdominal pressure. Urine contamination was minimized by not collecting from the initial stripping action which often expels urine first. Mucosal contamination was minimized by avoiding scraping of the skin when collecting feces. Fecal samples were stored at -20 °C. This procedure was repeated twice a week until ~2 g dry fecal matter was obtained. The fecal material collected from individual YTK was pooled within tank.

ADCs were calculated according to the equation-based on Cho et al. (1982), with the exception that yttrium was used as the inert marker.

$$Apparent\ digestibility\ coefficient\ (ADC\ \%) = \ [1 - ((F/D) * (Dmarker/Fmarker))] * 100$$

(Eq. 13)

where F is % nutrient in feces; D is % nutrient in diet; Dmarker is % marker in diet; Fmarker is % marker in feces.

Furthermore, ADC (%) of methionine and cysteine were used to calculate methionine's and cysteine's daily digestible intake (g $kgBW^{-1}$ d^{-1}) and were calculated as follows:

Daily digestible feed intake (g $kgBW^{-1}$ d^{-1})

= ((dietary content*intake per fish/100) * ADC[$\bar{x}$])/d^{-1}/ weight[kg^{-1}] (Eq. 14)

where methionine's ADC [$\bar{x}$] was 82.61% and cysteine's ADC [$\bar{x}$] was 74.78%.

3.3.7 Compositional analyses of whole carcass, diets, and fecal material

Five juvenile YTK were randomly selected from each replicate tank at the end of the growth phase for compositional analyses. Whole carcass samples were autoclaved for five minutes at 120 °C, dried and finely milled. Diets, whole carcass and fecal samples were analyzed for dry matter, nitrogen (crude protein), gross energy, total lipid, ash and amino acid contents according to routine methods (AOAC International, 2016, 2005). Diets and fecal material were also analyzed for yttrium to allow calculation of apparent digestibility coefficients, via inductively coupled plasma mass spectrometer (LCMS-8030, Shimadzu). The compositional analysis of whole carcass and diet samples were conducted by CSIRO (Agriculture and Food, Carmody Road, St Lucia,

QLD 4067, Australia). Protein content was determined by multiplying the nitrogen content of each sample by 6.25. Total lipid content was gravimetrically determined after chloroform: methanol (2:1) sample extraction using the Folch method (Folch et al., 1953). Ash was gravimetrically determined after incineration at 550 °C in a muffle furnace. The amino acid profile of experimental diets was done by the Australian Proteome Analysis Facility (Macquarie University, Sydney, NSW, Australia) after hydrolyzing of samples, labelling amino acids using Waters AccQTag Ultra chemistry, and detecting amino acids via UPLC.

3.3.8 Data analysis

Raw data were processed via Microsoft Excel for Office 365 and further modelled via GraphPad Prism Version 8.3.0 (La Jolla, CA, USA). Food conversion ratio (FCR), specific growth rate (SGR), protein retention efficiency (PRE), and methionine retention efficiency (MRE) were used as dependent variables to estimate the methionine and cysteine content and digestible intake that optimized these performance criteria in juvenile YTK. Various non-linear regression models were applied to the data and then visually and statistically cross-validated to assess the quality of fit (i.e. Akaike's Information Criterion (AICc), sum of squares (SS) and the standard error of estimate (SEE)). The best-fitting regression models (second order polynomial, power series, lognormal) were then used to calculate the inflection points of the respective response variables.

Second order polynomial:

$$Y = B0 + B1 * X + B2 * X^2 \quad \text{(Eq. 15)}$$

where the inflection point is calculated as:

$$X = -(\mathrm{B1})/(2 * B2) \quad \text{(Eq. 16)}$$

Power series:

$$Y = A * X^B + C * X^D \quad \text{(Eq. 17)}$$

where the inflection point is calculated as:

$$X = \left(-(D * C)/(B * A)^{1/B - D}\right) \quad \text{(Eq. 18)}$$

Lognormal:

$$Y = (A / X) * \exp(-0.5 * (\ln(X / GeoMean) / (\ln(GeoSD))\text{^}2) \quad \text{(Eq. 19)}$$

where the inflection point is calculated as:

$$X = \exp(\ln(GeoMean) - (\ln(GeoSD)\text{^}2) \quad \text{(Eq. 20)}$$

3.3.9 Statistical analysis

Statistical analyses were performed using the R language and the R software environment for statistical computing (2.13.) with the packages car, ggplot2, ggpubr, multcompView, plyr, and PMCMRplus. All response variables were validated for assumptions of normality and constant variance via Shapiro-Wilk normality test and Levene's test for homogeneity of variance, respectively. If assumptions were not met, data were sine, square root or inverse transformed. All response variables were then subject to a two-way analysis of variance (ANOVA), elucidating the effect of five methionine levels, two cysteine levels and their interactions. In the event of a significant interaction, all ten dietary treatments means were compared via Tukey HSD posthoc test. However, if no significant interaction was found but only a main factor had a significant effect, the respective factor level means were examined via Tukey HSD posthoc test (Wei et al.,

2011). Effects were considered significant at $P < 0.05$. Data were expressed as mean ± standard error of three replicates

3.4 Results

3.4.1 Survival

The survival of YTK was unaffected by methionine and cysteine; however, a significant interaction between methionine and cysteine was detected (**Table 3.3**). The interaction was primarily driven by the low survival of YTK fed diet 1 (80.6%) and the complete survival of YTK fed diet 6 (100%); diet 1 having the lowest level of methionine in the low cysteine series and diet 2 having the lowest level of methionine in the high cysteine series. The survival of all treatments ranged between 80.6 to 100.0% (**Table 3.3**).

3.4.2 Feed efficiency

The FI of YTK depended on the level of dietary methionine ($P < 0.05$) but was unaffected by the level of dietary cysteine or interactions (**Table 3.3**). FI was significantly lower ($P< .05$) in YTK fed diet 1 (197.4 ± 10.3 g $fish^{-1}$) and diet 6 (231.4±7.3 g $fish^{-1}$) than in YTK fed diet 3 (271.7 ± 10.4 g $fish^{-1}$) and diet 8 (245.2 ± 11.9 g $fish^{-1}$; Table 3.3). The FCR of YTK strongly depended on the dietary methionine content ($P< .05$) but also indicated significant interactions between the main factors (**Table 3.3**). FCR was significantly higher (worse) in YTK fed diet 1 (1.01 ± 0.01) and diet 6 (1.07 ± 0.01) than it was in most other diets, where FCR was lower (better) and ranged narrowly between 0.93 and 0.99 (**Table 3.3**).

Table 3.3 Biometric performance of juvenile yellowtail kingfish.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Probability (P value)[2]		
											M	C	Mx C
Met (g kg^{-1} DM)	8.8	12.7	16.5	22.4	24.7	7.9	12.7	17.6	21.2	25.2			
Cys (g kg^{-1} DM)	5.8	5.6	5.0	5.4	5.9	13.7	14.1	14.4	13.5	13.9			
Growth and feed performance													
IBW (g $fish^{-1}$)	52.6±1.0	52.9±1.1	52.2±1.0	52.7±1.1	52.6±1.0	52.1±1.1	52.8±1.0	52.7±1.1	52.8±1.0	52.7±1.0	NS	NS	NS
FBW(g $fish^{-1}$)	250.4±10.4^{a}	303.0±11.5^{b}	341.4±9.6^{b}	310.1±12.2^{b}	303.0±12.4^{b}	268.8±8.2^{a}	313.6±9.5^{b}	309.4±9.0^{b}	301.6±9.9^{b}	289.3±13.2^{b}	***	NS	NS
Feed intake (g $fish^{-1}$)	197.4±10.3^{a}	245.8±8.0^{ab}	271.7±10.4^{b}	239.8±6.8^{ab}	248.2±8.8^{ab}	231.4±7.3^{a}	249.3±20.0^{ab}	245.2±11.9^{b}	239.3±15.3^{ab}	227.2±19.5^{ab}	*	NS	NS
WG[3] (%)	373.6±22.8^{a}	473.7±14.9^{b}	554.2±25.2^{b}	488.6±0.6^{b}	476.2±14.0^{ab}	416.4±13.5^{a}	491.4±35.0^{b}	486.1±22.7^{b}	469.5±28.8^{b}	449.0±40.2^{ab}	**	NS	NS
SGR[4] (% d^{-1})	2.88±0.09^{a}	3.23±0.05^{b}	3.48±0.07^{b}	3.28±0.00^{b}	3.24±0.05^{ab}	3.04±0.05^{a}	3.28±0.11^{b}	3.27±0.07^{b}	3.22±0.10^{b}	3.14±0.14^{ab}	***	NS	NS
FCR[5]	1.01±0.01^{ab}	0.98±0.02^{bc}	0.94±0.00^{bc}	0.93±0.02^{c}	0.99±0.03^{bc}	1.07±0.01^{a}	0.96±0.01^{bc}	0.96±0.00^{bc}	0.97±0.00^{bc}	0.96±0.00^{bc}	***	NS	*
TGC[6]	0.22±0.01^{a}	0.26±0.00^{b}	0.28±0.01^{b}	0.26±0.00^{b}	0.26±0.00^{ab}	0.24±0.00^{a}	0.26±0.01^{b}	0.26±0.01^{b}	0.26±0.01^{b}	0.25±0.01^{ab}	***	NS	NS
PRE[7]	0.31±0.01^{ab}	0.33±0.01^{abcd}	0.38±0.01^{d}	0.35±0.01^{bcd}	0.33±0.01^{abc}	0.28±0.00^{a}	0.36±0.01^{cd}	0.37±0.01^{cd}	0.31±0.01^{ab}	0.33±0.00^{abcd}	***	NS	*
MRE[8]	0.65±0.01^{a}	0.45±0.02^{b}	0.39±0.01^{c}	0.29±0.01^{d}	0.24±0.01^{d}	0.67±0.02^{a}	0.49±0.02^{b}	0.36±0.02^{c}	0.26±0.05^{d}	0.25±0.01^{d}	***	NS	NS
Morphometric and other indices													
K[9]	2.01±0.37	2.64±0.97	0.90±0.10	1.38±0.28	1.26±0.19	1.58±0.17	1.11±0.25	1.06±0.11	1.40±0.48	1.65±0.33	NS	NS	NS
HSI[10](%)	0.81±0.02	0.74±0.03	0.72±0.02	0.77±0.03	0.79±0.02	0.79±0.04	0.78±0.02	0.77±0.02	0.81±0.03	0.85±0.05	NS	NS	NS
VSI[11] (%)	5.50±0.24	5.29±0.73	6.48±0.28	5.70±0.46	6.08±0.41	5.56±0.23	6.38±0.40	7.44±1.19	6.35±0.51	6.22±0.38	NS	NS	NS
Survival (%)	80.6±2.8^{a}	97.2±2.8^{ab}	94.4±2.8^{ab}	94.4±5.6^{ab}	97.2±2.8^{ab}	100±0.0^{b}	94.4±5.6^{ab}	91.7 ±4.8^{ab}	97.2±2.8^{ab}	88.9±2.8^{ab}	NS	NS	*
Bilateral cataract (%)	82.2±9.7^{a}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	***	***	***

[1]Data were expressed as mean ± SE from triplicate groups. Treatments within rows sharing superscript letters are not significantly different, [2]Significant effects determined via two-way ANOVA. M x C, interaction; NS, not significant. Levels of significance are with respect to P< .05 (*), P< .01 (**), and P< .001 (***), [3]WG, Weight gain , [4]SGR, specific growth rate Eq. 3, [5]FCR, feed conversion ratio Eq. 5, [6]TGC, thermal growth coefficient Eq. 4, [7]PRE, Protein retention efficiency Eq. 9, [8]MRE, Methionine retention efficiency Eq. 10, [9]K, Condition factor Eq. 6, [10]HSI, hepatosomatic index Eq. 7, [11]VSI, viscerosomatic index Eq. 8

3.4.3 Growth responses

The growth of juvenile YTK (i.e. FBW, WG, SGR, and TGC) depended on the dietary methionine content of the diets ($P < 0.05$) but was not affected by cysteine content or the interaction of the main factors (**Table 3.3**). Overall, growth of YTK was poorest in those groups fed the diet with the lowest amount of methionine and cysteine (i.e. diet 1), with a FBW of 250.4 ± 10.4 g $fish^{-1}$, WG of 373.3 ± 22.8%, SGR of 2.88 ± 0.09% d^{-1} and TGC of 0.22 ± 0.01 (**Table 3.3**). Nonetheless, YTK fed on diet 1 increased their initial body weight by approximately 374% over the duration of the experiment. Growth parameters were numerically highest in YTK fed diet 3, but they were not significantly different from groups of YTK fed diets 2, 4, 5, 7, 8, 9 or 10 (**Table 3.3**). The growth of YTK fed diet 6, which was also lowest in dietary methionine, was not significantly different to diet 1 (**Table 3.3**).

3.4.4 Retention efficiency

The retention efficiency of protein and methionine of juvenile YTK depended on the dietary methionine content of the diets ($P < 0.05$); however, only the protein retention efficiency was significantly affected by a methionine - cysteine interaction (**Table 3.3**). The protein retention efficiency of YTK was poor in those groups fed the diets lowest in methionine and cysteine (diet 1), lowest in methionine but high in cysteine (diet 6), and relatively high in methionine and cysteine (diet 9) with a PRE of 0.31 ± 0.01, 0.28 ± 0.00, and 0.31 ± 0.01 respectively (**Table 3.3**). The protein retention efficiency was significantly higher in YTK fed diets 3, 7 and 8 (**Table 3.3**). The PRE range across all of the diet treatments was relatively small at 0.28 - 0.38 representing diets 6 and 3 respectively (**Table 3.3**). The methionine retention efficiency

significantly decreased with an increase in methionine intake ($P < 0.05$, **Table 3.3**) but did not differ among cysteine levels ($P > .05$).

3.4.5 Morphometric indices and cataracts

K, HSI, and VSI were not significantly affected by the level of dietary methionine, level of dietary cysteine or the interaction of the main effects ($P > .05$; **Table 3.3**). The K of YTK was lowest in fish fed diet 3 ($0.90 \pm 0.10\%$) and diet 8 ($1.06 \pm 0.11\%$), whereas the K ranged between 1.11 to 2.64% in fish fed the other dietary treatments. HSI was numerically lowest in YTK fed diet 3, but overall, there was little variation among the HSI of different treatments, which ranged between 0.72 to 0.85% (**Table 3.3**). The VSI of YTK was also very similar among dietary treatments, ranging from 5.29 to 7.44% (**Table 3.3**). 82.2 % of YTK fed diet 1 for 54 days developed bilateral cataracts. This symptom was not recorded in fish fed any of the other dietary treatments (**Table 3.3**; **Figure 3.1**).

3.4.6 Apparent digestibility coefficients and digestible SAA intake

The apparent digestibility coefficients for crude protein, lipid, and gross energy were unaffected by dietary methionine or cysteine contents and no interaction of the main factors was detected ($P > .05$; **Table 3.4**). However, the ADC of methionine was dependent on the dietary methionine content ($P < 0.05$), increasing significantly at higher dietary inclusion levels (i.e. diet 3, diet 5, diet 8 and diet 10; **Table 3.4**). The ADC of methionine was unaffected by dietary cysteine contents and no interaction of the main factors was detected. The ADC of cysteine was affected by a methionine-cysteine interaction ($P < 0.05$), whereby the ADC for cysteine

increased when fed increasing contents of methionine when the dietary content of cysteine was low. The digestibility of cysteine was higher in the series of diets containing the higher level of cysteine (**Table 3.4**).

Figure 3.1 Cataract (indicated by arrow) in a juvenile yellowtail kingfish (*Seriola lalandi*) exposed to low levels of dietary methionine and cysteine (Diet 1) after eight weeks.

Table 3.4 Apparent digestibility coefficients (%) and digestible intake (g $kgBW^{-1}$ d^{-1}) of key nutrients in juvenile yellowtail kingfish (*Seriola lalandi*)

	Low cysteine series					High cysteine series					P value[2]		
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	M	C	Mx C
Apparent digestibility coefficient													
CP	73.1±1.0	NA[3]	75.0±0.7	NA	76.2±1.8	74.5±0.5	NA	78.0±1.0	NA	75.1±3.9	NS	NS	NS
Lipid	76.5±0.9	NA	83.3±0.7	NA	81.6±2.1	80.2±0.9	NA	84.0±0.7	NA	80.8±1.6	NS	NS	NS
GE	60.6±0.6	NA	68.6±0.2	NA	67.1±1.8	63.5±0.9	NA	70.2±1.6	NA	65.5±2.8	NS	NS	NS
Meth	76.8±1.2^{a}	NA	84.6±1.1^{b}	NA	86.2±0.4^{b}	77.7±0.7^{a}	NA	87.7±0.9^{b}	NA	82.4±5.3^{b}	*	NS	NS
Cys	33.6±3.5^{a}	NA	46.0±1.3^{ab}	NA	52.5±3.1^{b}	74.0±0.7^{c}	NA	77.5±0.3^{c}	NA	72.8±4.3^{c}	**	***	**
Tau	51.4±5.7	NA	46.2±1.7	NA	37.2±0.4	51.6±4.7	NA	42.6±4.2	NA	49.6±5.6	NS	NS	NS
Digestible Intake[4]													
Met	0.23±0.01^{a}	0.38±0.01^{b}	0.51±0.01^{c}	0.64±0.02^{d}	0.74±0.02^{e}	0.24±0.00^{a}	0.38±0.02^{b}	0.52±0.02^{c}	0.62±0.02^{d}	0.71±0.03^{e}	***	NS	NS
Cys	0.14±0.00^{a}	0.15±0.00^{a}	0.14±0.00^{a}	0.14±0.00^{a}	0.16±0.00^{a}	0.37±0.01^{b}	0.38±0.02^{b}	0.38±0.01^{b}	0.35±0.01^{b}	0.35±0.02^{b}	NS	***	NS
Tau	0.10±0.00^{a}	0.12±0.00^{ab}	0.12±0.00^{b}	0.11±0.00^{ab}	0.12±0.00^{ab}	0.12±0.00^{ab}	0.12±0.01^{ab}	0.12±0.00^{b}	0.12±0.00^{ab}	0.11±0.01^{ab}	NS	NS	*

[1] Data were expressed as mean± SE from triplicate groups. Treatments within rows sharing superscript letters are not significantly different, [2] Significant effects determined via two-way ANOVA. Met x Cys, interaction; NS, not significant. Levels of significance are with respect to P< .05 (*), P< .01 (**), and P< .001 (***), [3] NA, not assessed. Please see section 2.5. for more information. [4] Digestible intakes were calculated by using the respective SAA content, SAA intake, average SAA ADC, experimental length and geomean body weight. Please see section 2.5. for more information.

The digestibility of taurine was not affected by dietary treatment nor the interaction of the main factors (**Table 3.4**).

The relative digestible intake of methionine and cysteine by YTK (i.e. g digestible amino acid $kgBW^{-1}$ d^{-1}) depended on the dietary content of the respective SAA being fed ($P < 0.05$; **Table 3.4**), where an increase in content was directly associated with an increase in digestible intake of that amino acid. The relative intake of digestible taurine was dependent on the methionine-cysteine interaction ($P < 0.05$), where the effect of methionine on the digestible intake of taurine depends on cysteine and vice versa.

3.4.7 Composition of whole carcass

3.4.7.1 Proximate composition

The moisture, crude protein, lipid, ash and gross energy content of YTK whole carcass were not statistically affected by the dietary content of methionine or cysteine or the interaction of the main factors ($P > .05$; **Table 3.5**). Results of the proximate analysis showed the average moisture, crude protein, lipid, ash and gross energy content of whole YTK was 695 g kg^{-1}, 202 g kg^{-1}, 65.3 g kg^{-1}, 35.6 kg^{-1} and 7.3 MJ kg^{-1}, respectively.

3.4.7.2 Whole carcass amino acid composition

Apart from valine, there were no significant differences in the amino acid composition of YTK with respect to the methionine and cysteine content of the diets or the interaction of the main

effects ($P > .05$; **Table 3.5**). The valine composition of YTK was significantly lower in fish fed diet 1 and diet 6 (8.4 - 8.5 g kg^{-1}) compared to the other diets.

Table 3.5 Whole carcass proximate and amino acid composition of initial yellowtail kingfish (*Seriola lalandi*) fed a commercial diet and juvenile yellowtail kingfish fed diets containing varying levels of methionine and cysteine (g kg^{-1} as is basis).

	Com	Low cysteine series					High cysteine series					P-value[2]		
		Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	M	C	M x C
Proximate values														
Moisture content	770.7	699.2±3.5	699.3±1.5	691.7±2.3	693.9±4.9	699.2±0.3	700.2±4.2	692.0±2.8	686.7±8.1	694.8±7.1	693.6±6.5	NS	NS	NS
Crude protein	166.3	199.6±2.7	195.3±2.2	205.8±6.0	206.3±8.5	199.4±2.7	193.1±2.1	206.5±4.2	210.8±4.8	201.0±9.9	204.2±1.9	NS	NS	NS
Total nitrogen	26.6	31.9±0.4	31.3±0.3	32.9±1.0	33.0±1.4	31.9±0.4	30.9±0.3	33.0±0.7	33.7±0.8	32.2±1.6	32.7±0.3	NS	NS	NS
Total lipid	23.4	62.1±2.3	67.5±6.1	66.4±5.5	66.4±5.5	57.7±3.1	71.5±1.6	67.4±3.3	66.0±3.3	65.0±5.0	65.4±7.4	NS	NS	NS
Ash	37.6	35.6±1.1	36.7±2.1	34.6±0.5	35.6±0.9	37.4±2.8	32.0±1.9	35.4±1.2	34.7±1.0	38.2±1.4	35.7±1.0	NS	NS	NS
Gross energy	4.96	7.2±0.2	7.1±0.1	7.4±0.1	7.2±0.2	7.0±0.2	7.4±0.1	7.4±0.1	7.5±0.2	7.2±0.1	7.4±0.3	NS	NS	NS
Amino acids														
Alanine	10.3	13.6±0.3	13.2±0.3	14.0±0.3	13.8±0.8	13.4±0.2	13.4±0.5	13.7±0.4	13.9±0.2	13.0±1.2	13.8±0.1	NS	NS	NS
Arginine	10.6	10.4±0.3	10.9±0.5	11.1±0.4	11.0±0.6	10.7±0.3	9.9±0.3	11.4±0.3	10.6±0.3	10.3±2.0	10.9±0.5	NS	NS	NS
Aspartic acid	16.2	18.1±0.4	18.3±0.2	19.0±0.4	18.6±0.5	18.5±0.4	17.7±0.6	19.1±0.1	18.9±0.2	18.2±1.5	18.6±0.1	NS	NS	NS
Cysteine	1.7	1.8±0.0	1.6±0.0	1.7±0.1	1.7±0.1	1.7±0.0	1.8±0.1	1.8±0.0	1.7±0.1	1.5±0.2	1.8±0.1	NS	NS	NS
Glutamic acid	25.1	27.2±0.6	27.7±0.3	28.7±0.5	28.3±1.0	27.9±0.6	26.4±0.9	28.2±0.4	29.0±0.5	27.5±2.2	28.5±0.2	NS	NS	NS
Glycine	12.9	16.5±0.3	16.4±0.2	16.4±0.3	16.1±0.7	15.9±0.1	16.0±0.5	16.4±0.4	16.2±0.3	15.5±1.7	15.8±0.3	NS	NS	NS
Histidine	95.0	5.5±0.2	5.9±0.2	5.9±0.1	5.7±0.1	5.5±0.2	5.2±0.1	5.8±0.1	6.0±0.1	5.6±0.5	5.4±0.2	NS	NS	NS
Isoleucine	7.2	7.5±0.1	7.8±0.1	8.3±0.2	8.0±0.0	7.9±0.3	7.4±0.3	8.1±0.0	8.1±0.2	7.9±0.5	8.1±0.1	NS	NS	NS
Leucine	12.2	12.6±0.2	12.9±0.1	13.4±0.4	13.3±0.2	13.2±0.4	12.5±0.4	13.4±0.0	13.3±0.2	12.8±1.0	13.3±0.1	NS	NS	NS
Lysine	12.6	15.2±0.9	15.8±0.3	16.7±0.2	16.3±0.2	15.5±0.8	15.1±0.7	16.1±0.3	16.0±0.5	16.4±1.1	15.8±0.5	NS	NS	NS
Methionine	5.0	5.6±0.1	5.5±0.1	5.9±0.1	5.8±0.3	5.6±0.2	5.5±0.2	5.8±0.1	5.8±0.3	5.3±0.8	5.9±0.1	NS	NS	NS
Phenylalanine	6.7	7.4±0.1	7.2±0.2	7.7±0.2	7.5±0.3	7.5±0.1	7.4±0.2	7.7±0.1	7.6±0.1	7.3±0.7	7.7±0.1	NS	NS	NS
Proline	8.1	9.8±0.1	9.8±0.1	9.8±0.3	9.8±0.3	9.7±0.2	9.4±0.2	9.9±0.2	9.8±0.2	9.2±1.1	9.6±0.0	NS	NS	NS
Serine	7.0	7.6±0.2	7.6±0.1	8.0±0.4	8.1±0.3	7.8±0.2	7.5±0.4	8.1±0.1	7.9±0.2	7.4±1.2	7.9±0.1	NS	NS	NS
Taurine	2.9	2.8±0.1	2.4±0.1	2.5±0.1	2.6±0.2	2.4±0.1	2.7±0.2	2.5±0.1	2.6±0.1	2.5±0.3	2.5±0.1	NS	NS	NS
Threonine	7.7	8.6±0.2	8.8±0.1	9.1±0.2	9.0±0.2	8.9±0.3	8.6±0.3	9.1±0.1	9.0±0.2	8.3±1.1	9.0±0.1	NS	NS	NS
Tyrosine	5.9	6.0±0.2	5.8±0.5	6.5±0.1	6.1±0.4	6.2±0.2	6.2±0.3	6.5±0.4	6.4±0.2	5.7±0.9	6.6±0.1	NS	NS	NS
Valine	8.2	8.5±0.1^{a}	9.0±0.0^{b}	9.3±0.2^{b}	9.1±0.1^{ab}	8.9±0.3^{ab}	8.4±0.3^{a}	9.2±0.0^{b}	9.3±0.2^{b}	8.9±0.5^{ab}	9.0±0.1^{ab}	*	NS	NS

[1] Data were expressed as mean ± SE from triplicate groups. Treatments within rows sharing superscript letters are not significantly different, [2] Significant effects determined two-way ANOVA (excluding initial YTK). Met x Cys, interaction; NS, not significant; Gross energy (MJ kg^{-1}). Levels of sig. are with respect to P< .05 (*), P< .01 (**), and P< .001 (***)

3.4.8 Non- linear regression analysis

3.4.8.1 Methionine requirement at low cysteine content

The regression analyses applied to FCR, SGR, PRE, and MRE in response to the lower cysteine series were all compared for the best-fitting model. The applied models indicated optimized FCR, SGR, and PRE at a dietary methionine content of 20.5 g kg^{-1}, 18.4 g kg^{-1}, and 17.8 g kg^{-1} respectively (average = 18.9 g kg^{-1}) when the average dietary cysteine content was 5.6 g kg^{-1} (**Figure 3.2**). Furthermore, the applied models indicated optimized FCR, SGR, and PRE at a daily digestible methionine intake of 0.60 g $kgBW^{-1}$ d^{-1}, 0.55 g $kgBW^{-1}$ d^{-1}, and 0.52 g $kgBW^{-1}$ d^{-1} respectively (average= 0.56 g $kgBW^{-1}$ d^{-1}) when the average daily digestible cysteine intake was 0.14 g $kgBW^{-1}$ d^{-1} (**Figure 3.3**).

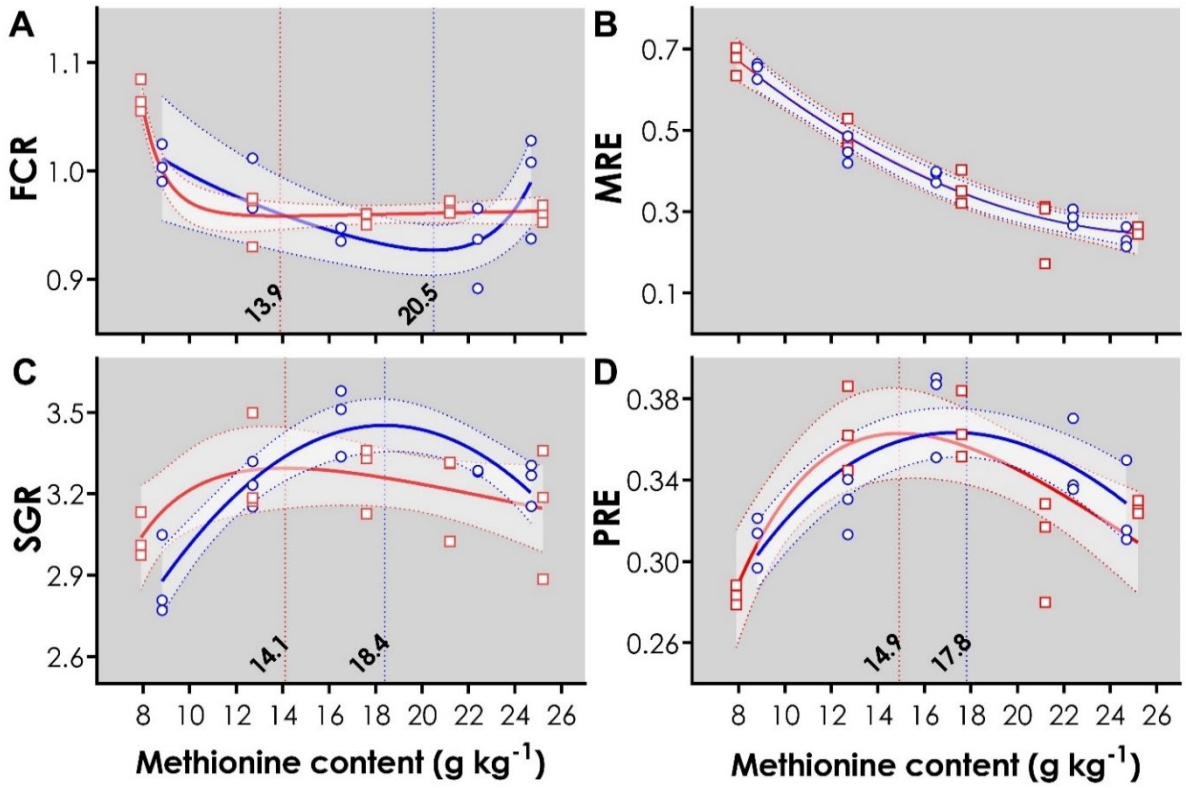

Figure 3.2 Dose-response curves fitted to five graded methionine and two graded cysteine levels. Curves and vertical lines (annotated breakpoint) in red identify the dietary methionine requirement at a dietary cysteine content of 13.9 g kg^{-1}, representative for the MOM requirement. Curves and vertical lines (annotated breakpoint) in blue identify the methionine requirement at a cysteine content of 5.6 g kg^{-1}, representative for the TSAA requirement. (A) Feed conversion ratio, (B) methionine retention efficiency, (C) specific growth rate, (D) protein retention efficiency responses relative to the dietary methionine content (g kg^{-1}). White semi-transparent areas indicate the 95% confidence interval. Regression models were selected according to goodness of fit.

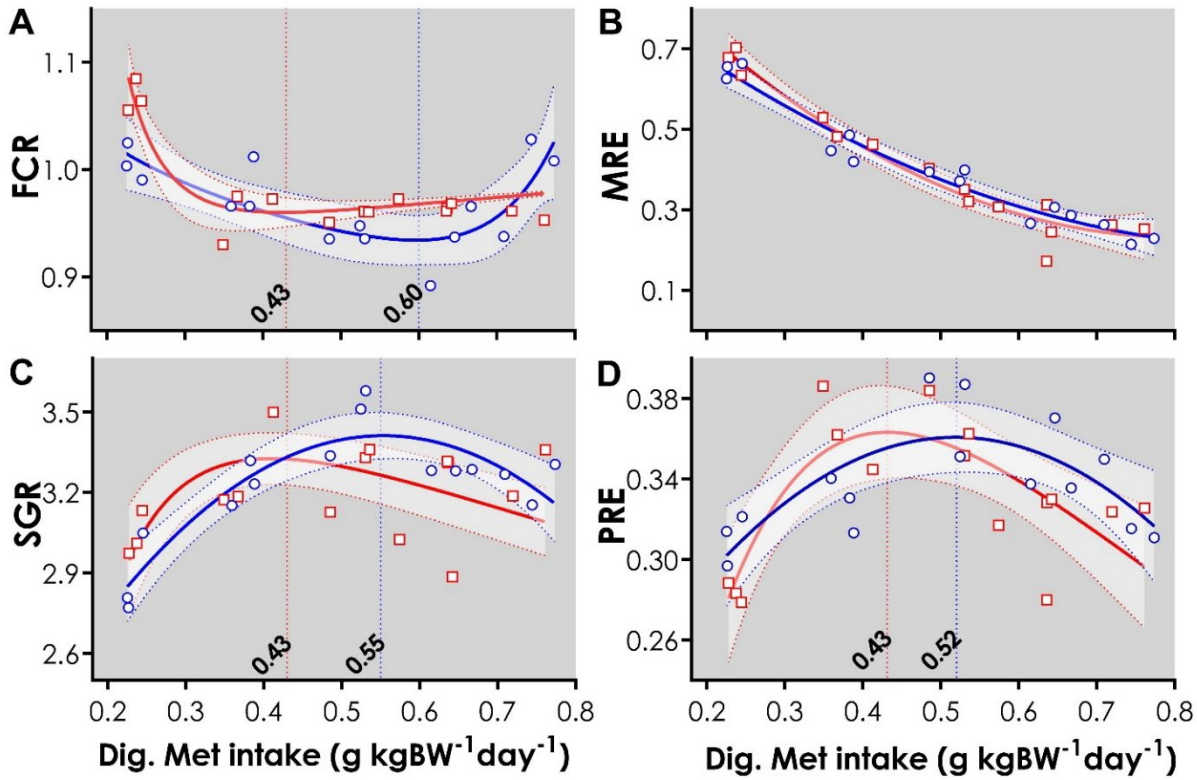

Figure 3.3 Dose-response curves fitted to five graded methionine and two graded cysteine levels. Curves and vertical lines (annotated breakpoint) in red identify the methionine requirement at a digestible cysteine intake of 0.37 g kgBW^{-1} d^{-1}, representative for the MOM requirement. Curves and vertical lines (annotated breakpoint) in blue identify the methionine requirement at a digestible cysteine intake of 0.14 g kgBW^{-1} d^{-1}, representative for the TSAA requirement. (A) Feed conversion ratio, (B) methionine retention efficiency, (C) specific growth rate, (D) protein retention efficiency responses relative to the digestible methionine intake (g kgBW^{-1} d^{-1}). White semi-transparent areas indicate the 95% confidence interval. Regression models were selected according to goodness of fit.

3.4.8.2 Methionine requirement at high cysteine content

The regression analyses applied to SGR, FCR, PRE, and MRE in response to the higher cysteine series were compared for the best-fitting regression model. The applied models indicated optimized FCR, SGR, and PRE at dietary methionine contents of 13.9 g kg^{-1}, 14.1 g kg^{-1}, and 14.9 g kg^{-1} (average = 14.3 g kg^{-1}) when the average dietary cysteine content was 13.9 g kg^{-1} (**Figure 3.2**).

Furthermore, the applied models indicated optimized FCR, SGR, and PRE at an a daily digestible methionine intake of 0.41 g $kgBW^{-1}$ d^{-1}, 0.43 g $kgBW^{-1}$ d^{-1}, and 0.43 g $kgBW^{-1}$ d^{-1}, respectively (average = 0.42 g $kgBW^{-1}$ d^{-1}) when the average daily digestible cysteine intake was 0.37 g $kgBW^{-1}$ d^{-1} (**Figure 3.3**).

3.4.9 TSAA requirement, MOM requirement and relative sparing by cysteine

Estimates on the measured methionine requirements at an average low cysteine content (5.6 g kg^{-1}) amounts to an TSAA (Met+Cys) requirement of 26.1 g kg^{-1}, 23.9 g kg^{-1}, and 23.4 g kg^{-1} based on FCR, SGR, and PRE respectively (average = 24.5 g kg^{-1}; **Table 3.6**). Furthermore, estimates on the methionine requirement at an average high cysteine content (13.9 g kg^{-1}) were 13.9 g kg^{-1}, 14.1 g kg^{-1}, and 14.9 g kg^{-1} based on FCR, SGR, and PRE respectively (average = 14.3 g kg^{-1}) represents the MOM requirement (**Table 3.6**). Calculations and estimates on TSAA and MOM requirements on a digestible intake basis are presented in **Table 3.6**. The sparing effect of cysteine for methionine in the TSAA requirement was estimated to be between 40 - 49 % on an equimolar sulfur basis (**Table 3.6**).

Table 3.6 Determination of methionine requirements at low and high levels of cysteine for yellowtail kingfish (*Seriola lalandi*). (1) TSAA [Met+ Cys], (2) TSAA [Met], and (3) MOM [Met] requirement and (4) cysteine's sparing potential on the TSAA requirement [Met].

			Dietary content (g kg^{-1})			Digestible intake (g kgBW^{-1} d^{-1})		
			FCR[6]	**SGR**[7]	**PRE**[8]	**FCR**	**SGR**	**PRE**
1	**TSAA (Met+Cys) requirement**	Estimated Met[1] requirement at:	20.50	18.38	17.81	0.60	0.55	0.52
		+ low Cys[2] level	+ 5.56	+ 5.56	+ 5.56	+ 0.14	+ 0.14	+ 0.14
		= TSAA (Met + Cys) requirement[3]	**26.06**	**23.94**	**23.37**	**0.74**	**0.69**	**0.66**
2	**TSAA (Met) requirement**	Estimated Met requirement at:	20.50	18.38	17.81	0.60	0.55	0.52
		+ low Cys level* molecular weight adjustment[4]	+ 5.56 * 1.23	+ 5.56 * 1.23	+ 5.56 * 1.23	+ 0.14 * 1.23	+ 0.14 * 1.23	+ 0.14 * 1.23
		= TSAA (Met) requirement[5]	**27.35**	**25.23**	**25.96**	**0.77**	**0.72**	**0.72**
3	**MOM (Met) requirement**	Estimated Met requirement at:	13.89	14.10	14.92	0.43	0.41	0.43
		(high Cys level, redundant for calculation)	(13.91)	(13.91)	(13.91)	(0.37)	(0.37)	(0.37)
		= MOM (Met) requirement[6]	**13.89**	**14.10**	**14.92**	**0.43**	**0.41**	**0.43**
4	**Cys sparing potential**	TSAA (Met) requirement	27.35	25.23	25.97	0.77	0.72	0.72
		- MOM (Met) requirement	- 13.89	- 14.10	- 14.92	- 0.43	- 0.41	- 0.43
		= Cys sparing of the TSAA (Met) requirement	**13.46**	**11.13**	**11.05**	**0.34**	**0.31**	**0.29**
		= Cys sparing (%) On an equimolar sulfur basis	**49.21**	**44.11**	**42.54**	**44.37**	**43.29**	**40.44**

[1]Met, Methionine, [2]Cys, Cysteine, [3]TSAA (Met + Cys) requirement, the absolute requirement for sulfur amino acids that is met with a cysteine-methionine combination, [4]Adjustment of cysteine's molecular weight of 121.16 g/mol to methionine's molecular weight of 149.21 g mol^{-1} to receive a uniform TSAA requirement, [5]TSAA (Met) requirement, the absolute requirement for sulfur amino acid that is completely met by methionine, [6]MOM (Met) requirement, the minimum obligatory requirement for methionine, that can only be met by methionine, [7]FCR, Feed conversion ratio Eq. 5, [8]SGR, specific growth rate Eq. 3, [9]PRE, Protein retention efficiency Eq. 9

3.5 Discussion

This study determined the methionine requirement of juvenile YTK at two levels of dietary cysteine (i.e., 5.6 g kg^{-1} and 14.1 g kg^{-1}). Our study revealed four key findings of importance to the Australian YTK industry: (1) the standard inclusion level of approximately 11.0 g dietary methionine kg^{-1} commercial diet is below the MOM requirement for YTK and consequently, dietary cysteine cannot substitute for this missing proportion of the TSAA requirement, even when provided in excess; (2) dietary cysteine can spare 40 - 49% of the TSAA requirement of YTK on a equimolar sulfur basis; (3) dietary methionine and cysteine at supraoptimal levels resulted in inferior feed efficiency and growth rate, indicating an upper threshold of dietary SAA for YTK; (4) a diet low in both methionine and cysteine content (i.e. diet 1), was associated with the occurrence of bilateral cataracts in YTK, which is the clouding of the lens.

The dietary methionine requirement at low levels of dietary cysteine (5.6 g kg^{-1}) in juvenile YTK for FCR, SGR, and PRE was met at 20.5, 18.4, and 17.8 g methionine kg^{-1} diet respectively (**Figure 3.2**). The daily digestible methionine requirement at low levels of digestible cysteine (0.14 g $kgBW^{-1}$ d^{-1}) was found to be optimal for FCR, SGR, and PRE at 0.60, 0.55, and 0.52 g $kgBW^{-1}$ d^{-1} respectively. Hence, the average dietary or daily digestible TSAA requirements (Met + Cys) are 24.5 g kg^{-1} or 0.70 g $kgBW^{-1}$ d^{-1}, respectively (**Table 3.6**). Our results confirm suggestions of Candebat et al. (2020) that the current methionine inclusion level for YTK aquafeed of 11 g kg^{-1} is not sufficient. In that study a methionine deficiency provoked a taurine requirement, confirming that taurine is conditionally essential for YTK (Candebat et al., 2020) as has been demonstrated in other species (e.g. Gaylord et al., 2006; Li et al., 2009b; Lunger et

al., 2007). Additionally, our current results indicate that a dietary methionine deficiency induces a requirement for cysteine, confirming cysteine's well-known role as conditionally essential amino acid (Brosnan and Brosnan, 2006). Requirement estimates for methionine (11.1 g kg^{-1}) and TSAA (Met + Cys; 14.2 g kg^{-1}), determined by Ruchimat et al. (1997) for Japanese Yellowtail, are too low to be used for YTK aquafeed formulations to maximize growth. Previous studies have shown that SAA requirements are size-specific (Michelato et al., 2013) and species-specific (Nguyen and Davis, 2009a; Poppi, 2017), which potentially explains the discrepancy between the SAA requirements quantified by Ruchimat et al. (1997) for Japanese Yellowtail (initial 23.3 g & end 34.3 - 108.9 g) and those quantified by this study for YTK (initial 52.6 g & end 250.4 - 341.4 g). The TSAA (Met + Cys) requirement of yellowtail kingfish from this study is in the upper requirement range in comparison to other commercially important fish species that have relatively lower TSAA (Met + Cys) requirements, e.g. channel catfish (*Ictalurus punctatus*; 14.9 g kg^{-1}; Ahmed, 2014), Tilapia (*Oreochromis mossambicus*; 16.0 g kg^{-1}; Jackson and Capper, 1982), Nile Tilapia (*Oreochromis niloticus*; 9.4 g kg^{-1}; Nguyen and Davis, 2009a), and rainbow trout; *Oncorhynchus mykiss*; 8 g kg^{-1}; Kim et al., 1992). However, there are other commercially important fish species that have relatively higher TSAA (Met + Cys) requirements, e.g. Asian sea bass (*Lates calcarifer*; 20.2 g kg^{-1}; Poppi et al., 2017), Indian major carp (*Cirrhinus mrigala*; 25.4 g kg^{-1}; Khan and Abidi, 2013), black sea bream (*Sparus macrocephalus*; 20.2 g kg^{-1}; Zhou et al., 2011), and European sea bass (*Dicentrarchus labrax*; 22.0 g kg^{-1}; Hidalgo et al., 1987). The above mentioned TSAA requirements of other species measured the TSAA requirement at dietary cysteine contents as low as 3.0 g kg^{-1} diet and as high as 12.9 g kg^{-1} diet. In human (Courtney-Martin et al., 2010; di Buono et al., 2001a; Humayun et al., 2006) and livestock (Graber et al., 1971) nutrition research, it is common practice to define the TSAA requirement

as the dietary methionine intake in the absence of cysteine at an optimum response variable, as methionine can meet 100% of the metabolic cysteine requirement (Ball et al., 2006). However, in fish nutrition research, it is common practice to describe the TSAA requirement as the sum of dietary methionine and cysteine intake inducing an optimum response of a selected performance variable (National Research Council, 2011b), where more dietary cysteine will reduce the methionine requirement. It is important to note that while a methionine and cysteine molecule each contain a single sulfur atom, that they differ in molecular weight (cysteine 121.16 g mol^{-1} & methionine 149.21 g mol^{-1}) by virtue of the different number of carbon and hydrogen atoms. This means that 10 g of methionine contains less sulfur atoms than 10 g of cysteine. Therefore, it would be more accurate to consider the relative contribution of the number of molecules in relation with the weight of methionine and cysteine supplementation when formulating diets.

The dietary methionine requirement at a higher level of dietary cysteine (13.9 g kg^{-1}) in juvenile YTK was found to be optimum for FCR at 13.9 g kg^{-1}, SGR at 14.1 g kg^{-1}, and PRE 14.9 g kg^{-1} (average = 14.3 g kg^{-1}; **Figure 1.1**). The daily digestible methionine requirement at a higher level of digestible cysteine (0.37 g $kgBW^{-1}$ d^{-1}) was found to be optimum for FCR at 0.43 g $kgBW^{-1}$ d^{-1}, SGR at 0.41 g $kgBW^{-1}$ d^{-1}, and PRE 0.43 g $kgBW^{-1}$ d^{-1} (average = 0.42 g $kgBW^{-1}$ d^{-1}). The methionine requirements at relatively high levels of dietary cysteine (5.6 g kg^{-1}) are substantially lower than the methionine requirements measured at a lower level of dietary cysteine (13.9) and mark the 54.6% of methionine-specific TSAA requirement that cannot be spared by cysteine. Corollary, this indicates a finite sparing effect of cysteine on methionine in the TSAA (as Met or Met + Cys) requirement; therefore, an increase of cysteine intake would

proportionally decrease the methionine requirement until reaching the MOM requirement. The MOM requirement has been intensively studied in humans (Humayun et al., 2007, 2006) and in several agriculturally important animal models, such as pigs (Moehn et al., 2008), lambs (Wei et al., 2017), and turkeys (Behrends and Waibel, 1980); however, research on the MOM requirement in fish are missing. Measurements on the MOM requirement in animal models are conducted by feeding graded levels of methionine at excess dietary intake of cysteine (Ball et al., 2006).

Measured TSAA and MOM requirements in juvenile YTK can provide important data to calculate cysteine's approximate capacity to substitute for the TSAA portion that is used for its own metabolic biosynthesis from methionine. This study determined that cysteine could meet 40.4 - 49.2% of the TSAA (Met) requirement on an equimolar sulfur basis in YTK. At some point, the biosynthesis of methionine to cysteine is in all animals a "metabolic one-way street" and consequently, cysteine can only spare the proportion of methionine that would to be converted to cysteine (Ball et al., 2006; Brosnan and Brosnan, 2006). YTK appears to be no exception to this concept. YTK's methionine retention efficiency, regardless of being fed a diet low or high in dietary cysteine, was strikingly similar (**Figure 3.1**; **Figure 3.2**). YTK fed low levels of dietary methionine and low levels of cysteine exhibited the same MRE as YTK fed relatively low levels of dietary methionine and high levels of cysteine, indicating no sparing of dietary cysteine on methionine when it comes to MRE. These results confirm YTK's inability to convert cysteine to methionine. In contrast to MRE, the PRE response was influenced by the different concentrations of dietary cysteine, where the higher concentration of cysteine spared methionine. Similar results can be observed in fingerling rohu, where an increase of dietary

cysteine compensated for a decrease in dietary methionine in PRE (Abidi and Khan, 2011). Cysteine's sparing effect for methionine in the TSAA requirement and ultimately maintaining good PRE could be explained by cysteine's essential role in protein structures, building disulfide bonds. Several published studies confirm cysteine's sparing capacity for methionine in the TSAA requirement in fish, ranging from 40 - 60% such as in rainbow trout (42%; Kim et al., 1992), Nile Tilapia (47 - 49%; He et al., 2016; Nguyen and Davis, 2009b), yellow perch (*Perca flavescens*; 50 %; Twibell et al., 2000), red drum (Sciaenidae ocellatus [40%]; Moon and Gatlin, 1991), channel catfish (60%; Harding et al., 1977), Asian sea bass (40%; Poppi et al., 2017), and hybrid striped bass (*Morone saxatilis x M. Chrysops* [40%]; Griffin et al., 1994). Ball et al. (2006) describes that cysteine's sparing capacity is quantified by feeding graded levels of cysteine and methionine, which at least meet the MOM requirement and are below or at the TSAA (Met or Met + Cys) requirement. The above studies estimated cysteine's sparing capacity by feeding several diets, each formulated to contain a ratio of methionine and cysteine (Met : Cys) at either a fixed TSAA level, just below/at requirement, or a fixed methionine level, at MOM requirement. This would create a response that indicates the optimum Met : Cys ratio of a selected performance variable. An estimate on cysteine's sparing capacity can also be calculated by subtracting the MOM requirement value from the TSAA requirement value (Ball et al., 2006) as was done by the current study. Dietary methionine intake above or below the MOM requirement can potentially lead to an underestimation or rejection of cysteine's sparing capacity or compromise growth and feeding responses, respectively (Ball et al., 2006). Previous human nutrition studies observed a lack of cysteine sparing capacity for methionine in the TSAA requirement (Fukagawa et al., 1998; Raguso et al., 2000), using at that time the recommended level of methionine by FAO/WHO/UNO (1985). However, a later study, measuring the

methionine requirement, showed that the recommended level of dietary methionine was insufficient (di Buono et al., 2001b, 2001a). Subsequently, a study with the higher methionine content showed that cysteine reduced 64% of the TSAA related methionine requirement in humans (di Buono et al., 2003).

Dietary methionine and cysteine content (**Figure 3.1**) or digestible intake (**Figure 3.2**) exceeding the TSAA and MOM requirement were followed by a deterioration in FCR, SGR and PRE. However, levels of cysteine and methionine were not significantly higher in whole carcass at higher SAA intake, indicating that metabolic derivatives of methionine and/or cysteine are responsible for compromised growth and feed efficiency. Similar observations were made in other finfish species fed diets containing supraoptimal methionine levels (Cai and Burtle, 1996) e.g. in rohu, a methionine content above 12 g kg^{-1} diet caused significant growth depression (Abidi and Khan, 2011). Brosnan and Brosnan (2006) postulated that these responses are caused by the build-up of toxic end-products through methionine's transamination pathway.

Carcass composition of YTK was not affected by the methionine or cysteine content of the test diets. These results are in agreement with other studies that found dietary amino acid contents had no effect on carcass proximate composition of Tilapia (El-Sayed, 1990; Furuya et al., 2004; Michelato et al., 2016). Furthermore, Shearer (1994) points out that carcass protein and lipid contents do not depend on the dietary amino acid or protein content but are dependent on fish size and energy intake respectively. Results from this study indicate no significant effect of dietary methionine and cysteine on the digestibility of lipid, protein, energy, and ash contents; however, improved digestibility of methionine and cysteine was observed with an increase of

dietary methionine and cysteine in the lower cysteine series, respectively. A similar response was previously observed by Candebat et al. (2020), where an increase of dietary methionine led to improved digestibility of methionine. Cysteine's digestibility was statistically dependent on the methionine-cysteine interaction, improving cysteine's digestibility with increased dietary methionine content. However, taurine's digestibility was not significantly affected by methionine and cysteine, which again correlates with results from Candebat et al. (2020). The slightly improved digestibility of methionine and cysteine with an increase of each respective dietary SAA could be the result of the use of crystalline amino acids, which have shown to be more digestible than protein-bound amino acids (Nunes et al., 2014).

A great proportion of juvenile YTK fed the diet lowest in methionine and cysteine were afflicted with cloudy eye lenses, which may be indictive of cataracts. Similar symptoms have been recorded in rainbow trout, in which 95% of fish developed bilateral cataracts when fed methionine deficient diets (Cowey, 1994). The sulfhydryl, rather than the methyl group of methionine, is most important in preventing cataracts resulting from methionine deficiency (Poston et al., 1978). In the current study YTK fed the methionine-deficient diet supplemented with a relative high quantity of dietary cysteine did not exhibit cataracts, possibly due to the adequate supply of cysteine's sulfhydryl group. This may indicate that methionine is conditionally essential to prevent cataracts in YTK if dietary cysteine is sufficiently provided.

3.6 Conclusion

To conclude, our research provides new data on the SAA requirements and their interactions in juvenile YTK. YTK exhibited a lower and upper tolerable limit of dietary and digestible methionine and cysteine intake beyond which growth rate and feed efficiency are impaired. Furthermore, the methionine level, presently used by the Australian industry in YTK aquafeed, is below the average MOM and TSAA requirement as previously suggested by Candebat et al. (2020). We recommend that yellowtail kingfish should be provided with 24.5 g kg^{-1} diet of dietary SAA, of which 18.9 g kg^{-1} diet is methionine when cysteine is 5.6 g kg^{-1}diet, to cover TSAA requirement. Furthermore, YTK require a MOM content of at least 13.9 g methionine kg^{-1} diet when cysteine is 13.9 g kg^{-1} diet. These new data will facilitate aquafeed formulations tailored for optimized growth and feed efficiency in YTK. We further recommend extended investigations on the metabolic partitioning of SAA, methionine requirements in absence of methionine derivatives and abiotic-specific influences on TSAA requirements.

Chapter 4. Nutritional relevance of dietary methionine, cysteine and taurine for the hepatic, intestinal and circulatory systems in juvenile yellowtail kingfish (*Seriola lalandi*)

4.1 Abstract

Fishmeal in aquafeed formulations for carnivorous fish is progressively replaced by a nutrient-based approach in which dietary proteins that may lack essential nutrients are balanced with crystalline and synthetic nutrients to meet the dietary requirements of carnivores. Although sustainable and economically viable, the efficient production of healthy fish requires quantification of the obligatory dietary requirements and capacities for metabolic bioconversions in target species. Nutrient-based formulations carry the risk of over- or undersupplying essential dietary nutrients, especially when the species-specific nutrient requirements are unknown. The sulfur amino acids methionine and cysteine and their amino-sulfonic acid derivative taurine are all metabolically active molecules with interlinked nutritional requirements. Deficiencies in this group of nutrients have been linked to poor growth and health; however, the impacts of these deficiencies on organ structure and function are largely unknown. This study examines the effects of dietary methionine, cysteine, and taurine at different levels on juvenile yellowtail kingfish liver histology and surface color, plasma biochemistry, and posterior intestine histomorphology and histochemistry. Samples were collected from two previous dose-response feeding trials that quantified (1) the taurine requirement and sparing effect of methionine by feeding triplicate

groups of yellowtail kingfish for 45 days with one of seven taurine and one of two methionine levels (Candebat et al., 2020), and (2) the methionine requirement and sparing effects of cysteine by feeding triplicate groups of yellowtail kingfish for 54 days one of five methionine and one of two cysteine levels (Candebat et al., 2021). Dietary methionine, cysteine and taurine induced significant changes in liver histology and surface color and posterior intestine histology. Yellowtail kingfish fed inadequate levels of dietary methionine, cysteine, taurine exhibited bile ducts with thicker walls, livers that were less red, and acidic goblet cell mucus with protective properties, less absorptive surface area and an increased number of supranuclear vacuoles. Additionally, thicker bile duct walls correlated with reduced red surface liver coloration (a*, R) and increased hepatic fat correlated with a yellowing (b*) of the liver surface color. This study presents primary data on liver surface color, and the histology of the posterior intestine and liver in yellowtail kingfish fed suboptimal, optimal and supraoptimal combinations of methionine, cysteine, and taurine. Our results show a shift toward histological properties and functions indicative of improved posterior intestinal nutrient absorption, condition of the intrahepatic biliary system and maintenance of hepatic and intestinal homeostasis of yellowtail kingfish fed adequate amounts of methionine, cysteine, and taurine. These results may assist in formulating aquafeed for optimized gastrointestinal and liver functions and in maintaining good health and productivity in yellowtail kingfish.

4.2 Introduction

Over the past 20 years, global aquaculture has grown 65% and produced 82.1 million tons of seafood in 2018 (FAO, 2020). Concomitant with the expansion of aquaculture, 70% of the world's aquaculture now use pelleted feed for farmed fish (Béné et al., 2016), with a steady increase of proteins derived from sources other than from capture fisheries to provide nutrient-based and sustainable aquafeeds. However, these aquafeeds often lack essential nutrients that must be exogenously provided to the fish. Methionine (Met) is an essential sulfur amino acid and is often the first limiting essential amino acid in protein sources other than fishmeal (Li et al., 2011). In addition to its role as a component of structural proteins, Met plays important physiological roles in initiating protein synthesis (Brosnan & Brosnan, 2006), donating methyl groups in one-carbon metabolism (Espe et al., 2020, 2008), and in modulating lipid, glucose, and amino acid metabolism (Hasek et al., 2013; Skiba-Cassy et al., 2016). Varying levels of dietary Met can cause, aggravate or ameliorate various pathological conditions (Hasek et al., 2013; Li et al., 2021a; Serpa, 2020).

Deficiencies in essential amino acids are recognized to impair growth in fish, yet their physiological functions and metabolic interrelatedness are still under investigation. In fish, Met deficiencies are associated with an increase in retinal degeneration, bilateral cataract, non-infectious enteritis, inflammatory cell invasion, widening of the lamina propria, and hepatocyte necrosis and atrophy (Li et al., 2021a). Thus, balancing low- or non-fishmeal feeds with Met appears imperative to maintain the histological integrity and health in fish. However, dietary Met supplementation exceeding the physiological requirement leads to toxicity expressed by

histopathological lesions in the human intestine and liver (Anderson & Raiten, 1992), a decrease in intestinal microbiota richness and diversity in Nile Tilapia (Guo et al., 2020), and inferior specific growth rates, feed conversion efficiencies, and protein efficiency ratios in large yellow croaker (Mai et al., 2006). Overall, Met supplied below or above total sulfur amino acid (TSAA) requirements can disrupt physiological homeostasis and cause undesirable alterations in morphology and biochemistry. In fish, 39 to 60% of absorbed dietary Met is transsulfurated to endogenous cysteine (Cys) when fed adequate amounts of dietary Met; this is also the proportion of dietary Cys that can spare the TSAA requirement for the transsulfuration of Met to Cys in various fish species (Candebat et al., 2021; Griffin et al., 1994; Harding et al., 1977; Zehra & Khan, 2016).

Cys is a semi-essential amino acid in aquafeeds. However, the intracellular availability of Cys remains critical for the fitness of all eukaryotic animals by acting as a building block for protein synthesis and as a precursor for glutathione, pyruvate, inorganic sulfur, CoA, and taurine (Ball et al., 2006; Dominy et al., 2006; Serpa, 2020). Excess dietary Cys impairs growth, while excess injected Cys in fish can cause mortality in a manner that is similar to excess in Met (Benevenga & Steele, 1984; Yokoyama & Nakazoe, 1996). Impaired growth and health were also observed when rats, pigs and chickens were fed with Cys-supplemented diets that were already nutritionally complete, suggesting its toxicity (Dilger et al., 2007). In contrast to excess Met, Cys has also been shown to induce pathological liver conditions in rats (Dilger et al., 2007; Nath & Salahudeen, 1993). Yet if fed at optimum rates, dietary Cys has been shown to positively affect the liver by mobilizing excess lipid stores and decreasing mercury concentrations (Mok et al., 2014). Thus, measuring the effects of sulfur amino acids and taurine on liver may be

particularly important for YTK since feeds for carnivorous fish are increasingly supplemented with crystalline amino acids, for which the species-specific dietary requirements are often unknown.

In animals, the hepatic transsulfuration and decarboxylation of Met and Cys produce taurine (Tau), an amino sulfonic acid, which is considered semi-essential in fish (Candebat et al., 2020; Li et al., 2009b). The ability and efficiency of fish to synthesize Tau *de novo* depends on the substrate (Kwon & Stipanuk, 2001), species (Xuan Wang et al., 2016), life-stage (Wang et al., 2015), and enzymes cysteine dioxygenase (CDO), cysteine sulfinate decarboxylase (CSAD), cysteamine dioxygenase (ADO), and the taurine transporter (TauT; Liu et al., 2017). In fish, Tau contributes to the maintenance of physiological homeostasis by (1) conjugating with bile acid, emulsifying ingested lipids in the intestine (S. K. Kim et al., 2008); (2) conjugating with bilirubin to form ditaurobilirubin, eliminating the toxic by-products of heme breakdown through bile (Goto et al., 2001; Sakai et al., 1987); (3) regulating glucose metabolism (Zhang et al., 2019) and (4) osmoregulating and stabilizing the membranes of erythrocytes (Takagi et al., 2006b). Tau supplementation to a plant protein-based diet ameliorated gut inflammatory markers and apoptosis in the European seabass intestine (Martins et al., 2019) and reduced the signs of green liver syndrome in Japanese yellowtail (*Seriola quinqueradiata*) fed a zero-fishmeal diet (Takagi et al., 2005). Both studies indicate beneficial effects of Tau in fish; however, information on single and interactive effects of dietary Met, Cys, and Tau on fish intestinal and hepatic health is still limited (Krogdahl et al., 2020; Li et al., 2021a; Nordrum et al., 2000).

The intestinal tract forms an interface between the external environment and internal tissues and has myriad functions, including the absorption of fluids, digestion and transport of nutrients, immune response to nocive agents, removal of waste, osmoregulation, housing microbiota, and endocrine and nervous signaling (Al-Hussaini, 1949; Buddington et al., 1997; Grosell, 2006). The posterior intestine is particularly sensitive to the environment to which it is exposed, with factors such as life-stage (Chen et al., 2006), food ingredients (Bonaldo et al., 2008; Bowyer et al., 2012a; Ye et al., 2016), feeding habits (Day et al., 2014) and inadequate provision of amino acids and derivatives (Li et al., 2021a) shaping its morphology and function. This is exhibited in differences across histological measurements such as intestinal layer thicknesses, enterocyte structure, number and concentration of supranuclear vacuoles (Bansemer et al., 2015), and goblet cell mucus production and composition (Cerezuela et al., 2012).

Hepatic parenchyma is responsible for many metabolic reactions in animal intermediary metabolism, including the synthesis of molecules that are important to the correct functioning of all tissues within the body. Therefore, the correct functioning of the liver is critical for the growth and health of animals. An inadequate diet can often manifest by the accumulation of water, lipids, proteins, glycogen, and pigments that translate into changes in the macroscopic appearance of the liver (Brusle & Anadon, 1996; González-Reimers et al., 2013; Goto et al., 2001; Xinyu Li et al., 2020; Liu et al., 2019). In fish, imbalanced diets are associated with an acceleration of the normal synthesis and the insufficient removal of the natural, endogenous, green pigment biliverdin. Under normal conditions, biliverdin is converted into the yellow pigment bilirubin for excretion through bile (Takagi et al., 2010). However, nocive agents may induce homeostatic adaptation and inhibition, and the exhaustion of adaptive responses may

lead to cell lesion, fibrosis, necrosis, and hepatocytic hypertrophy and ballooning (Asaoka et al., 2013; Cordero-Espinoza & Huch, 2018). Macroscopic observations of the liver surface color of single fish from a cohort is a simple, primary diagnostic tool to assess health, feed quality and the surrounding environment (Brusle & Anadon, 1996; Hjeltnes et al., 1992; Schmitt & Dethloff, 2000). Diagnosing whether observations are adaptations within or outside the normal reference range is difficult because objective macroscopic and microscopic data on the livers of target species reared under aquaculture conditions are often limited.

Yellowtail kingfish is a carnivorous fish farmed for its high-quality white meat (Baldwin, 2003). Increased demands and a limited supply of marketable quantities have led to an increased interest in YTK feeds. Previous research has demonstrated inferior growth, feed efficiency and pathological conditions in YTK fed sub- and supraoptimal levels of Met, Cys, and Tau in low-fishmeal diets. Such conditions include small white spots and green patches on the liver, cataracts and enteritis (Candebat et al., 2021; Jirsa et al., 2011; Stephens et al., pers. com.). Candebat et al. (2020) established that the Tau requirement in YTK can be satisfied by sufficient dietary Met, categorizing Tau as conditionally dispensable. Follow-up experimentation further characterized the minimum obligatory methionine requirement, the TSAA and Cys sparing capacity on the TSAA requirement in juvenile YTK (Candebat et al., 2021). YTK fed sub-and supraoptimal levels of SAA exhibited poor feed conversion ratios and specific growth rates; yet, it was only at suboptimal levels of SAA that YTK developed clinical signs of cataracts (Candebat et al., 2021). The dietary Met, Cys, and Tau appear to play critical roles in the metabolic pathways of carnivorous fish to maintain good health, sustained growth, and resistance to environmental stressors, including pathogens (Li et al., 2009a). Nevertheless, knowledge of the

effects of dietary Met, Cys, and Tau on clinical pathology and adverse posterior intestinal and hepatic changes are, to our knowledge, limited in YTK.

Histomorphometric and histochemical measurements of fish organs and tissues can elucidate the modifying effects of dietary amino acids on inflammatory, immune, digestive, homeostatic stability, compositional and metabolic functions. A comprehensive quantitative histological and biochemical database providing reference values for fish would not only serve as a diagnostic tool for clinical symptoms but also as a biomarker to meet the nutritional requirements for ideal physiological performance. The aim of this study was to assess the histology, morphology, and biochemistry of liver and posterior intestine tissue samples from YTK, derived from Candebat et al. (2021, 2020), that were fed diets either replete, deficient or in excess of Met, Cys and Tau requirements, and to infer functional changes that relate to these nutritional imbalances.

4.3 Methods

4.3.1 Ethics statement

All experiments were performed under the NSW DPI Fisheries Animal Care & Ethics Research Authority, known as 'Aquaculture Nutrition ACEC 93/5–Port Stephens' (ACEC, 2009) and were conducted at the Port Stephens Fisheries Institute, NSW, Australia.

4.3.2 Experimental design

Samples from two separately conducted feeding trials (Candebat et al., 2020, 2021) were collected to investigate the effects of different dietary Met, Cys, and Tau levels on liver histology, posterior intestine histology, blood plasma biochemistry, and liver surface color in juvenile YTK.

Throughout the present study, the Candebat et al. (2020) feeding trial will be referred to as the TauMet study, and diets are annotated with T+M. Briefly, the objective of the TauMet study was to quantify the Tau requirement in juvenile YTK at a Met content that either met or exceeded current industry practice (Candebat et al., 2020). For that purpose, a factorial dose-response approach was applied, using seven incremental levels of Tau, ranging from 1.6 to 20.4 g kg^{-1} diet, crossed with either one of two levels of dietary Met (10.9 g kg^{-1} diet or 17.2 g kg^{-1}) at one constant Cys level (5.9 ± 0.2 g kg^{-1}; **Appendix A** &**Appendix B**), resulting in 14 experimental diets (T+M 1–14). Each diet was randomly allocated to three replicated 200L tanks, each stocked with 14 fish (initial body weight; 53.3 ± 0.4 g $fish^{-1}$). After seven weeks, tissue samples from fish (n≥6) from all 14 feeding treatments were collected, all of which were analyzed for liver surface color and plasma biochemical analysis, and of which selected dietary treatments of YTK were analyzed for liver and posterior intestine histology. Please refer to **Table 4.1** for further information on selected dietary treatments and abbreviations.

Throughout the present study, the feeding trial by Candebat et al. (2021) is referred to as the MetCys study, and the diets are annotated with M+C, whereas the commercial diet is annotated as Com. The objective of the MetCys study was to quantify the dietary Met requirements at Cys levels that met or exceeded industry practice (Candebat et al., 2021). Again, a factorial dose-response approach was applied, using five levels of Met, ranging from 7.9 to 25.2 g kg^{-1}, combined with either one of two levels of dietary Cys (5.6 or 13.9 g kg^{-1}) at one constant Tau level (7.0 ± 0.03 g kg^{-1}; **Appendix C** & **Appendix D**), resulting in ten experimental diets (M+C 1–10). Each of the ten experimental diets and a commercial diet were randomly assigned

to three identical 200 L tanks, each stocked with 12 fish (initial body weight; 52.6 ± 1.0 g $fish^{-1}$). After eight weeks, the posterior intestines of six individual fish fed one of four diets, which contained the lowest or highest amount of Met and/or Cys, were collected (M+C 1: 8.8 g Met kg^{-1} & 5.8 g Cys kg^{-1}, M+C 2: 24.7 g Met kg^{-1} & 5.9 g Cys kg^{-1}, M+C3: 7.9 g Met kg^{-1} & 13.7g Cys kg^{-1}, M+C 4: 25.2 g Met kg^{-1} & 13.9 g Cys kg^{-1}). In addition, posterior intestines from six individual fish fed a commercial diet were collected for histological analysis (**Table 4.1**) at the start and end of the trial to provide baseline information on the relative effects on YTK health.

Fish used in this study were from the progeny of wild-caught YTK brood stock held at the PSFI hatchery. YTK were fed a commercial floating pellet (crude protein 50%, crude fat 14%, and crude fiber 4%) and maintained at 15 to 19°C before experiment stocking. Throughout the feeding trials, fixed photoperiods of 12 light: 12 dark (TauMet study) and 11 light: 13 dark (MetCys study) were maintained, using dimmable, overhead LED lamps and simulating natural photoperiods of the season. Water quality parameters were monitored daily (mean ± SE) for the TauMet study: temperature (23.3 ± 0.6°C), salinity (33.3 ± 4.6‰), dissolved oxygen (7.0 ± 1.0 mg L^{-1}), pH (8.3 ± 0.5), and TAN (0.7 ± 0.4 mg L^{-1}). For the MetCys investigation, the parameters were: temperature (21.2 ± 0.6°C), salinity (32.9 ± 3.2‰), dissolved oxygen (12.1 ± 3.0 mg L^{-1}), pH (7.4 ± 0.4), and TAN (≤ 0.25 mg L^{-1}).

Table 4.1 List of measured responses and calculated ratios from the TauMet and MetCys study to assess yellowtail kingfish (*Seriola lalandi*) vital and health status.

	Tissue	Study/diets	Measured response	Abb
Liver color	**Whole liver**	**TauMet/T+M** 1–14	L, a*, b* color space	CIE
			R, G, B color space	RGB
			H, S, B color space	HSB
Histomorphology & cytology	**Liver**	**TauMet/T+M** 1, 3, 4, 7, 8, 10, 11, 14	Bile duct wall thickness	BDW
			Fattiness	F
			Necrotic hepatocytes	NH
			Large nucleus	LN
			Marginated chromatin	MC
			Cytoplasm eosinophilia	E
Biochemistry	**Blood plasma**	**TauMet/T+M** 1–14	Cholesterol	
			Triglyceride	
			Alkaline Phosphatase	ALP
			Aspartate transaminase	AST
			Lactate dehydrogenase	LD
			Bicarbonate	
			Calcium	
			Phosphate	
			Urea	
			Glucose	
			Lactate	
Macro- & Histomorphology	**Posterior intestine**	**TauMet/T+M** 1, 4, 7, 8, 11, 14	Total fish weight	TW
			Viscera weight	VW
			Liver weight	LW
			Intraperitoneal fat weight	IPFW
			Posterior intestine circumference	PIC
			Total intestinal wall thickness	TIW
			Stratum compactum +granulosum	S
			Muscularis interna thickness	MI
		MetCys/M+C 1, 2, 3, 4 **Com** I, E	Villus area	VA
			Lamina propria area	LPA
			Lamina epithelial area	LEA
			Total villus height	TVH
			Villus length	VL
			Total villi count	TVC
			Supranuclear vacuoles	SV
Histochemistry	**Posterior intestine villi**	**TauMet/T+M** 1, 4, 7, 8, 11, 14	Total goblet cell mucus	TGC
			AB+ goblet cell mucus	AB+
		MetCys/M+C 1, 2, 3, 4 **Com** I, E	PAS+ goblet cell mucus	PAS+
			AB+PAS+ goblet cell mucus	AB+PAS+
			Small PAS+ bullet-shaped bodies	S-PAS+

4.3.3 Experimental diets and feeding regimes

All T+M and M+C diets were formulated to meet the protein and energy requirements of YTK (Booth et al., 2010) and comprised prime fishmeal, fisheries by-product meal, plant and terrestrial animal meals. The desired Met, Cys, and Tau specifications for the T+M diets were achieved by using a blend of raw ingredients that were supplemented at different rates with crystalline DL-Met and Tau (**Table 4.1**), and for the M+C diets by using a blend of raw ingredients, crystalline DL-Met, L-Cys, and Tau (**Table 4.3**). YTK were hand-fed to apparent satiation twice a day (at 9 AM and 3 PM) during weekdays and once per day at 9 AM on weekends for 45 days (TauMet study) and 54 days (MetCys study).

4.3.4 Sample collection

4.3.4.1 Study 1. TauMet

After the TauMet feeding trial, YTK were fasted for 24 hours, and all 14 YTK of each tank were individually measured for total length, whole body, fillet, viscera, liver, and intraperitoneal fat weight. Six randomly selected fish per feeding treatment (n = 84) were euthanized by a spike to the brain, followed by immediate blood sampling. Approximately 1 ml of blood was collected from the caudal vein, using a 5 mL syringe and 19 g × 38 mm gauge needle (Terumo, Tokyo, Japan). Collected blood was then rapidly transferred into lithium heparin-coated tubes (MiniCollect®Lithium Heparin, Austria) and centrifuged (LabCo®Mini Centrifuge) at 113 x 100 rpm for 14 minutes. Following centrifugation, the blood plasma was collected and pooled per experiment tank and frozen at -20°C until further biochemical analysis. The livers from the same six fish were removed and photographed under standardized light conditions for the digital liver

surface color analysis. Refer to **Section 2.6** for further information on image acquisition. Additionally, liver and posterior intestinal samples were collected from an additional six to eight fish fed diets T+M 1, 3, 4, 7, 8, 10, 11, and 14 (total n = 58), after euthanizing with the recommended dose of Aqui-S® (540 g L^{-1} isoeugenol; Aqui-S New Zealand Ltd.) and transferred to 10% buffered formalin for histological analysis.

4.3.4.2 Study 2. MetCys and Com

Before the sampling of the MetCys study, YTK were fasted for 24 hours and euthanized as above. Four initial fish, subsampled at the commencement of the trial and 12 YTK from each replicate tank at the conclusion of the trial, were individually measured for total length, whole body, viscera, and liver weight. The posterior intestines of the four initial fish and six of the 12 measured fish fed the diets M+C 1, 2, 3, 4, and Com (n = 30) were sampled for posterior intestines and transferred in 10% buffered formalin for histological analysis.

4.3.5 Histology preparation and data collection

Formalin-fixed organs, collected from 92 YTK from the TauMet and MetCys studies, were routinely dehydrated using increasing concentrations of ethanol from 50 to 100% (HistoCore Pearl Tissue Processor, Leica Microsystems Pty Ltd, Australia) and embedded in paraffin (HistoCore Arcadia C & H Embedding Center Leica Microsystems Pty Ltd, Australia) according to standard histological procedure. Subsequently, samples were cut in ~4 µM sections with a rotary microtome (~4.0 mm thickness, CUT 4060 model, microTec GmbH, Germany) and mounted onto glass slides for histological staining.

Slides were stained with either hematoxylin-eosin (H&E), a combination of Alcian blue (AB, pH 2.5) and periodic acid-Schiff (PAS) or Toluidine Blue (TB) and followed a slightly adjusted staining protocols by the James Cook University Veterinary Pathology Department (**Appendix E**). The pH of the AB stain was 2.5 to stain most of the acid goblet cell mucus. Livers were stained with Haematoxylin and Eosin (H&E) for semi-quantitative and quantitative morphometric and cytological evaluations. Posterior intestines were stained with AB-PAS for histomorphometric and histochemical evaluation of structures and the joint detection of neutral (PAS+; magenta), acid (AB+; blue), and mixed (AB+PAS+; purple) goblet cell mucus. Additionally, sections of posterior intestines were stained with Toluidine blue; however, mast cells were not detected (Reite & Evensen, 2006). Prior to the data collection, histology slides of the posterior intestine and liver were scanned at 40x magnification using an automated slide-scanning system (Aperio LV1 IVD, Leica Microsystems Pty Ltd, Australia). Aperio ImageScope software (Leica Biosystems, Nussloch, Germany) was used to visualize and measure the stained organs.

YTK livers collected from the TauMet study were scored for fattiness using a semi-quantitative scale (**Figure 4.2**, **Table 4.1**). Liver fattiness was ranked from one to four by the abundance of lipid vacuoles in 18 areas of interest (0.094 mm^2 $area^{-1}$) per liver section (**Figure 4.1**). The quantitative system included measurements and counts on bile duct wall thickness (BDW); including the fibrous wall and cholangiocytes), of which the five thickest bile ducts were selected for subsequent statistical analysis (**Figure 4.2**A), necrotic hepatocytes (NH), **Figure 4.2**B),

large nucleus (LN), cytoplasm eosinophilia (E), and marginated chromatin (MC) in hepatocytes in three 400x fields (0.094 mm^2 $area^{-1}$), respectively (**Figure 4.2**C).

Histological data of the YTK posterior intestine collected from the TauMet and MetCys studies were quantitatively measured, including measurements of the length, thickness, area and counting structures within the organs (**Figure 4.3**). Measured lengths included: total intestinal wall thickness (TIW), muscularis interna thickness (MI), stratum compactum + granulosum thickness (S) of 32 different areas along the intestine per individual, total villus height (TVH; **Figure 4.3**B) and villus length (VL) of 12 villi per individual (**Figure 4.3**A). Measured areas included: villus area (VA) and lamina propria area (LPA) of 12 villi per individual (**Figure 4.3**A). Measured circumferences included: the posterior intestine circumference (PIC) per individual. Quantified features of villi included: total villi count (TV) per individual, villus tip count (VT) of 12 villi per individual. Histochemical features that were assessed included: neutral, acidic, and mixed goblet cell mucus, small PAS + dense bullet-shaped bodies (S-PAS+; magenta) and supranuclear vacuoles of 12 villi per individual (**Figure 4.3**D). Prior to counting, a brightness/contrast filter was applied to digitized slides, enhancing the distinct colorations of acidic, neutral, mixed goblet cell mucus and supranuclear vacuoles.

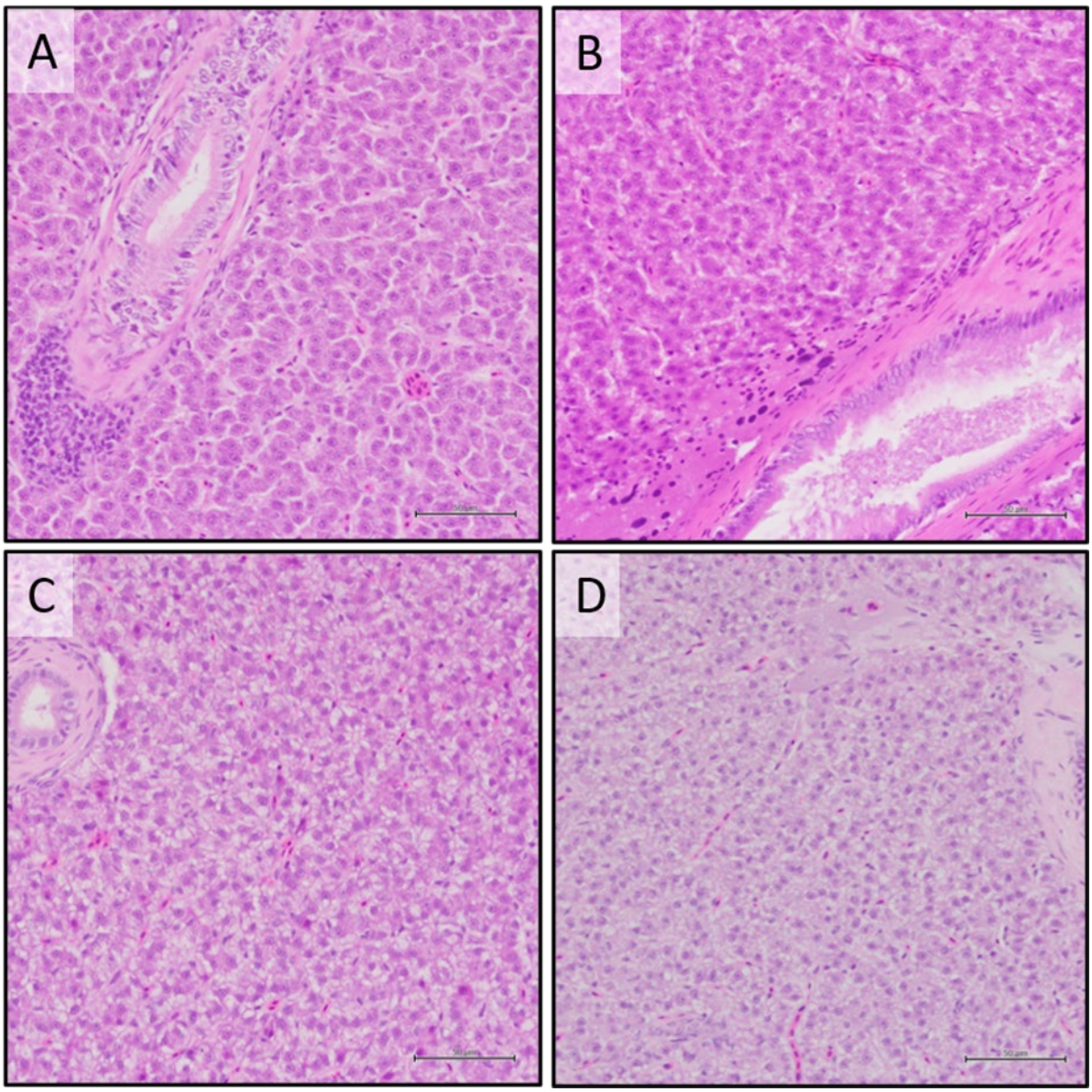

Figure 4.1 Semi-quantitative scoring of YTK liver collected from the TauMet study for the presence of lipid vacuoles, indicating fattiness/steatosis. The four levels are: (A) 0–normal; (B) 1–mild; (C) 2–moderate; (D) 3–severe. (H&E stain, scale bar = 50 µm).

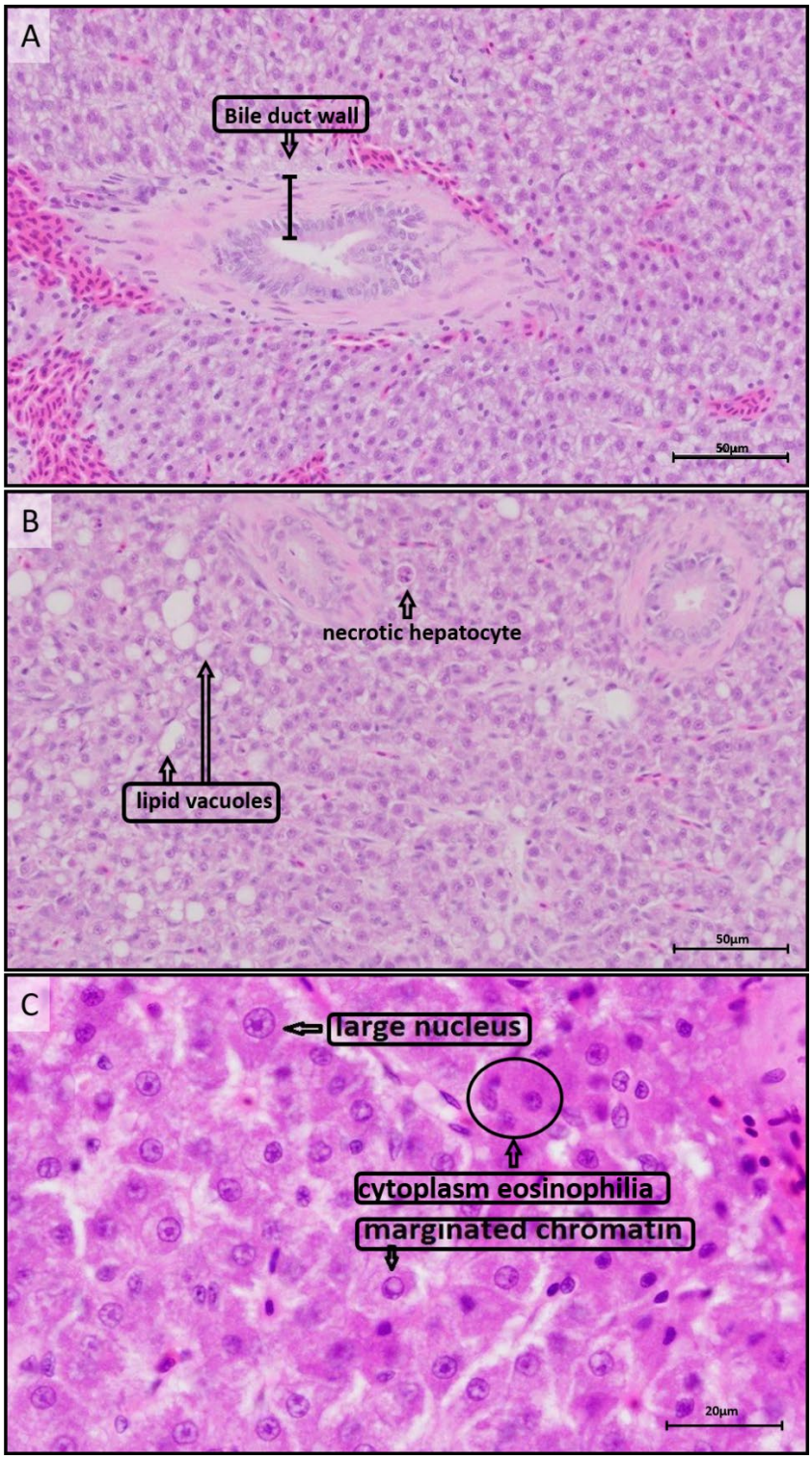

Figure 4.2 Histological features measured in YTK liver (TauMet study) fed one of seven dietary taurine and one of two methionine levels. YTK liver was measured and quantified for (A) bile duct wall thickness (fibrous wall + epithelium), (B) necrotic hepatocytes, lipid vacuoles (semi-quantitative, see Fig. 1), (C) large nucleus of hepatocytes, cytoplasm eosinophilia in hepatocytes, and marginated chromatin. (H&E stain, scale bar = 50 µm (A, B) & 20 µm (C)).

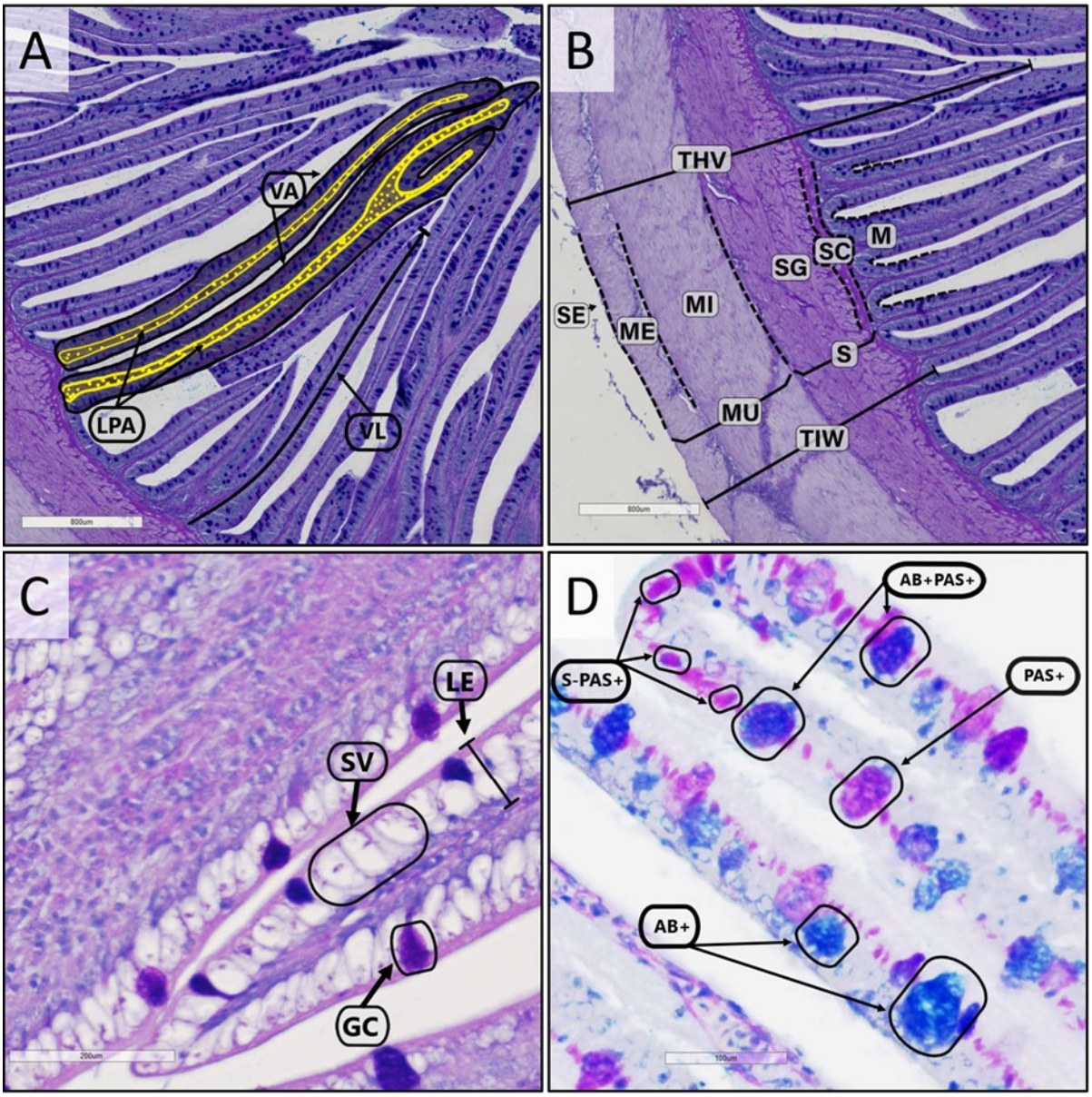

Figure 4.3 Posterior intestinal structures of juvenile yellowtail kingfish (*Seriola lalandi*). A [M] following descriptions indicates that structure was measured and statistically analyzed. (A): VA, Villus area; LPA, Lamina propria area (yellow area); VL, Villus length; (B): TVH, Total villus height [M]; MU, Muscularis; ME, Muscularis externa; MI, Muscularis interna [M]; SC, Stratum compactum; SG, Stratum granulosum; S, Submucosa [M]; M, Mucosa; TIW, Total intestinal wall thickness [M]; (C): LE, Lamina epithelial; SV, Supranuclear vacuoles [M]; GC, Goblet cells [M]; (D): AB+, Mucus that stained blue with Alcian blue (blue) [M]; PAS+, Mucus that stained with Periodic Acid-Schiff's (magenta) [M]; AB+PAS+, Alcian blue–Periodic Acid-Schiff's positive stain mucus (purple) [M]; S-PAS+, Small Periodic AcidSchiff's dense bullet-shaped

bodies (magenta and 18.6 ± 0.7 µm) [M]. (AB-PAS stain, Scale bar = 800 µm (A-B), 200 µm (C), 100 µm (D)).

4.3.6 Calculations and statistical analysis

Statistical analyses were performed using IBM SPSS statistics, the R software environment for statistical computing (2.13.) and R Studio v.4.0., using the R packages car, carData, ggplot2, ggpubr, multcompView, plyr and PMCMRplus. All dependent variables from the TauMet and MetCys study were subjected to two-way ANOVAs, examining the effect of seven dietary Tau levels at each of two dietary Met levels (TauMet study) or five dietary Met levels at each of two dietary Cys levels (MetCys study). Prior to statistical analysis dependent variables were validated for assumptions of (1) normality via Shapiro-Wilk test and (2) homogeneity of variance via Levene's test. If assumptions were not met dependent variables were log, sqrt, ln, and inverted transformed. Further, dependent variables were assessed for the assumptions of (3) linearity with covariates including body weight and villus area and (4) homogeneity of regression slopes between treatments. If assumption 3 and 4 were met, dependent variables were subjected to two-way ANCOVAS to control for the influence of the covariate (final body weight or villus area). All dietary treatment means within the feeding trial were compared via Tukey HSD post hoc test in the event of a significant interaction. Similarly, in the event of no significant interaction but significant main factor effects, the respective factor level means were compared using Tukey HSD post hoc test (Wei et al., 2011).

An abbreviation list on the measured responses can be found in **Table 4.1**. The lamina propria area and lamina epithelial indicated a strong and significant relationship with villus area and were therefore controlled through a two-way ANCOVA to examine the effects of dietary Met, Cys, and Taurine. Further, the density of AB + goblet cell mucus, PAS+ goblet cell mucus,

AB+PAS+ goblet cell mucus, total goblet cell mucus, small PAS+ dense bullet-shaped bodies, and supranuclear vacuoles within the respective villus area was calculated and statistically analyzed (LPA/VA; LEA/VA; AB+/VA; PAS+/VA; AB+PAS+/VA; TGC/VA; S-PAS+/VA; SV/VA). The semi-quantitative liver data were subject to a non-parametric Kruskal-Wallis test. Effects were considered significant at $P < .05$. Pearson's correlation coefficients between liver color values and bile duct wall thickness, fattiness, plasma cholesterol, and triglyceride were calculated using Excel.

4.3.7 *Ex vivo* whole liver color analysis

Image acquisition methods were adapted from Trampel et al. (2005). Before image acquisition, livers were visually assessed for whole or partial green discoloration. Images of 84 *ex vivo* YTK liver (TauMet study) were taken under standardized light and object orientation conditions. All images were captured using a digital SLR camera (Sony ILCE-6300, Japan) with a 16 mm lens and fixed settings of ISO-250, 1/13 second shutter speed, a focal length of 20 mm, and aperture f/6.3. Livers were carefully patted dry with paper towels before being placed individually on an 18% grey card background (8"x10", Delta 1, Texas, USA) inside a 290 x 450 mm closed photo box. The camera was placed in a slot at the top of the photo box, creating a perpendicular distance of 250 mm between the camera and liver samples. The photo box was made of white polypropylene, and the walls served as a diffuser to minimize the glare of lighting. The container was illuminated with 2 x 11 W white LED lights (Mirabella) placed 20 cm outside the longitudinal walls of the photo box. Additionally, the images were taken in a dark room, away

from daylight, to standardize the lighting conditions. The color assessment methodology applied to YTK liver was adapted from Weller & Westneat (2019) and van Belleghem et al. (2018).

Average liver color, liver color composition and color distance of individual YTK liver samples (TauMet study) were assessed. Image backgrounds, blood vessels on the liver, and glare were removed to reduce bias from these artefacts. Liver images were compared via RStudio v.4.0. (color distance package) by distinguishing color intensity and relative proportion of red, green, blue (RGB) metrics, followed by color distance calculations to quantify the degree of similarity or dissimilarity between two images (Weller & Westneat, 2019).

$$\text{Color distance} = \sum_{i=1}^{n} \sqrt{(R_i^a - R_i^b)^2 + (G_i^a - G_i^b)^2 + (B_i^a - B_i^b)^2} \qquad \text{Eq.1}$$

where *n,* number of bins; R, red value; G, green value; B, blue value

Color distance values were transferred into a principal coordinate analysis (PCoA) graph, visualizing dissimilarity by distance and similarity by clustering. This was achieved via RStudio v.4.0., using the R packages ggrepel, devtools, colordistance, ComplexHeatmap, splitstackshape, readxl, ggpubr, and cowplot.

The color composition of each liver was assessed using ImageJ software with the 3D Color Inspector/color histogram plugin (Schneider et al., 2012). Three areas of each liver were selected (46.441 ± 897 pixels area^{-1} and 51.2 ± 0.8 colors area^{-1}) to provide information on RGB and distribution. The average color of *ex vivo* livers was assessed via Adobe Photoshop 2021 by applying a blur filter on the extracted liver areas and calculating the average value in CIE Lab

(luminance, red-green, blue-yellow channels), RGB (red, green, and blue channels), HSB (hue, saturation, and brightness; Tapp et al., 2011). The average color was then sampled with the Photoshop eyedropper tool.

4.4 Results

4.4.1 TauMet study

4.4.1.1 Liver histology

Overall, the histological appearance of the livers was normal relative to that of farmed fish, with fatty livers and irregularities being minor in all treatments. Tau or Met did not affect our semi-quantitative measure of fattiness; however, quantitative measurements of the BDW thickness, necrotic hepatocytes, cytoplasmic eosinophilia, and large nuclei in hepatocytes were all modulated by diet (**Table 4.2**). YTK fed the highest levels of dietary Tau (T+M 7 & 14) had significantly thinner BDWs (**Figure 4.4**). Further, an increase of necrotic cells with dietary Met at 17.2 g kg^{-1} was observed. Yet, an incremental increase of Tau at 17.2 g Met kg^{-1} reduced the appearance of necrotic hepatocytes. YTK fed diets containing dietary Met at current industry practice had more large hepatocyte nuclei (7.45 ± 0.4; 94 μm^{-2}) than YTK fed Met above current industry practice (5.05 ± 1.5; 94 μm^{-2}). Cytoplasm eosinophilia in hepatocytes was most pronounced in YTK fed diet T+M 3 at 14.5 ± 1.3 $area^{-1}$ and was on average higher in YTK fed dietary Met at 10.9 g kg^{-1}; however, dietary Met and Tau did interact. Various hepatocytes also exhibited marginated chromatin, which was most pronounced in YTK fed diet T+M 4 (13.6 ± 1.6; 94 μm^{-2}); however, there were no significant differences among treatments.

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Table 4.2 Quantitative and semi-quantitative histological analysis of liver samples of juvenile yellowtail kingfish (*Seriola lalandi*) (TauMet), fed one of six different taurine-methionine levels. T+M 11, in bold, is closest to the average TSAA (Met+Cys) requirement of 24.5 g kg^{-1} and the methionine-dependent taurine requirement of 7.7 g kg^{-1} in YTK (Candebat et al., 2021, 2020).

	Experimental diets								p-value		
	T+M 1 (n = 9)	T+M 3 (n = 5)	T+M 4 (n = 9)	T+M 7 (n = 9)	T+M 8 (n = 6)	T+M 10 (n = 6)	T+M 11 (n = 6)	T+M 14 (n = 8)	T	M	T*M
Dietary taurine, methionine, and cysteine contents (g kg^{-1})											
Taurine	1.6	8.5	11.9	20.4	1.6	8.1	**11.7**	20.0	-	-	-
Methionine	12.0	11.1	10.8	11.1	16.7	16.4	**18.8**	15.0	-	-	-
Cysteine	6.8	5.6	5.4	5.7	5.9	5.5	**5.2**	5.8	-	-	-
Liver histology per 0.094mm^2 $area^{-1}$, unless stated otherwise											
Bile duct wall (µm)	15.5±0.8^{a}	21.7±0.9^{b}	16.2±0.8^{ab}	14.6±0.6^{a}	19.2±0.9^{ab}	19.7±0.7^{ab}	18.1±0.7^{ab}	14.5±0.5^{a}	***	NS	NS
Fattiness (semi-quantitative)	1.8±0.1	1.8±0.12	1.6±0.1	2.0±0.0	1.7±0.1	2.0±0.0	1.8±0.0	2.1±0.0	NS	NS	NS
Necrotic hepatocytes	0.8±0.1^{a}	0.4±0.1^{a}	1.0±0.1^{a}	0.7±0.1^{a}	4.2±0.5^{c}	3.6±0.3^{c}	2.0±0.3^{b}	1.2±0.2^{ab}	***	***	***
Large nucleus	7.3±0.3^{a}	7.6±0.6^{a}	9.7±0.5^{a}	5.2±0.2^{a}	5.3±0.5^{b}	8.2±0.5^{b}	2.8±0.2^{b}	3.9±0.3^{b}	NS	*	NS
Marginated chromatin	1.4±0.1	2.2±0.3	13.6±1.6	1.8±0.1	3.3±0.3	5.8±0.4	2.2±0.3	4.9±0.6	NS	NS	NS
Cytoplasm eosinophilia	2.9±0.5^{ab}	14.5±1.3^{a}	13.2±0.8^{a}	6.8±0.7^{ab}	2.7±0.3^{b}	4.8±0.8^{ab}	0.0±0.0^{b}	0.0±0.0^{b}	NS	***	**

[1] T, taurine; M, methionine. [2] Significant effects were determined by two-way ANOVA. Levels of significance are with respect to P< .05 (*), P< .01 (**), and P< .001 (***). Data with the same superscript letter within rows are statistically similar (P > .05). Data expressed as mean ± SE.

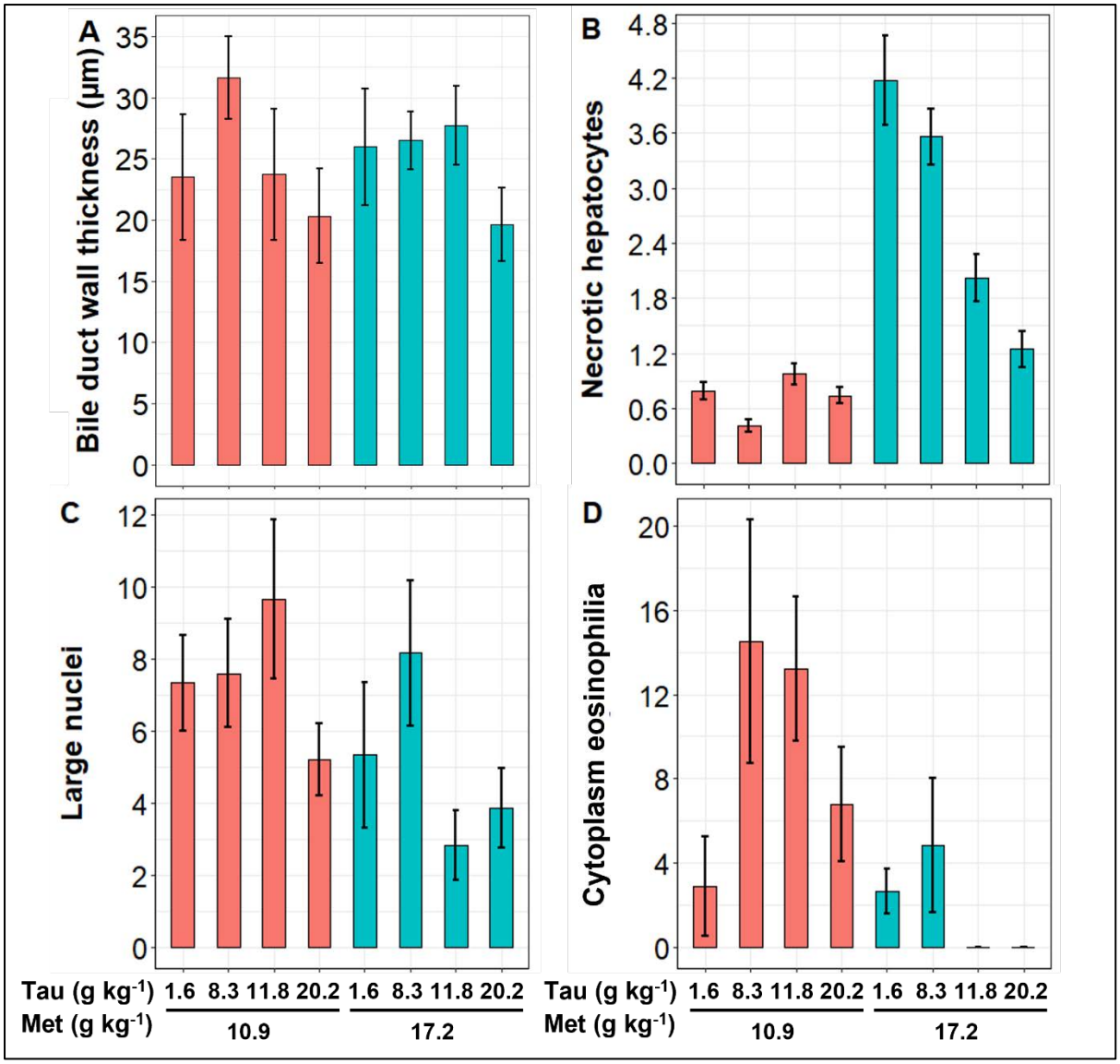

Figure 4.4 Liver histology of juvenile yellowtail kingfish (*Seriola lalandi*) (TauMet study), fed one of six taurine and one of two methionine levels. (A) bile duct wall thickness (µm), (B) number of necrotic cells per 0.094mm^2 area^{-1}, (C) count of large nuclei per 0.094mm^2 area^{-1}, (D) count of cytoplasm eosinophilia in hepatocytes per 0.094mm^2 area^{-1}. Data expressed as mean ± SE.

4.4.1.2 Plasma biochemistry

Blood plasma biochemistry was compared to Stephens et al. (2021 per. Comm.) reference ranges on sick, subclinical and healthy YTK plasma biochemistry. YTK from this study plasma had in comparison a mild elevation to depression of plasma biochemical parameters (**Figure 4.5**). Further, trends of biochemistry indicate Tau and Met dependent changes. Cholesterol and triglyceride contents in plasma were well below the suggested reference range of 5.4–8.5 mmol L^{-1} and 2.7–4.8 mmol L^{-1} and did not exceed 4.7 mmol L^{-1} and 1.6 mmol L^{-1}, respectively. However, lipid contents were generally higher in YTK fed 17.2 g Met kg^{-1}. The enzymatic activity of alkaline phosphatase (ALP), aspartate transaminase (AST), and lactate dehydrogenase (LD) appeared to be either responsive to dietary Tau or Met. YTK fed diets containing 17.2 g Met kg^{-1} exhibited higher AST and LD activity than YTK fed diets containing Met at current industry practice. Solute contents of bicarbonate, calcium, and phosphate indicated responsiveness to changes in dietary Tau concentrations, where bicarbonate decreased from 10 to 5 mmol L^{-1}, and phosphate and calcium increased from 1.85 to 3.07 mmol L^{-1} and 2.7 to 3.3 mmol L^{-1}, respectively, with incremental increases in dietary Tau.

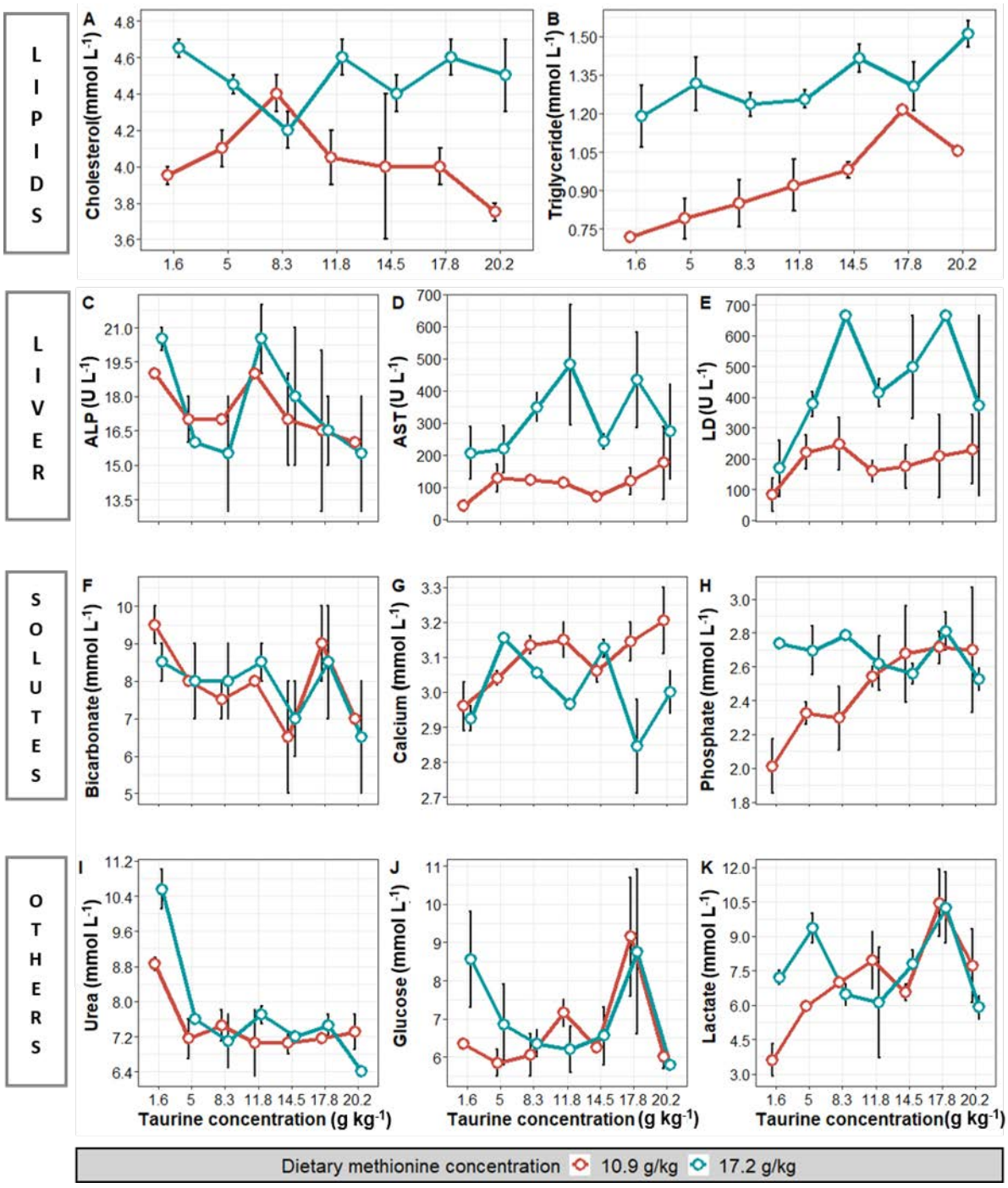

Figure 4.5 Plasma chemistry of juvenile yellowtail kingfish (*Seriola lalandi*) fed diets containing one of six different taurine levels and one of two methionine levels. Panel A–B show results on lipoproteins; Panel C–D show results on liver function tests; ALP, Alkaline phosphatase (C); AST, Aspartate transaminase (D); LD, Lactate dehydrogenase (E), Panel F–H show results on solutes that form electrolytes; and panel I–K show results on other plasma chemistry results. Red lines are the low met series (10.9 g met kg^{-1}), and blue lines are the high methionine series (17.2 g met kg^{-1}) at varying levels of taurine. The range bars indicate the two collected values.

4.4.1.3 Liver surface color

The visual and subjective liver surface color assessment indicated no green liver syndrome. In general, the liver surface color appeared pale and yellow. The digital assessment of the liver surface color demonstrated that the changes of green-red (a*), red (R), and brightness (B) values were dependent on dietary Tau and Met (**Table 4.3**). The a* value of liver surface color was at the highest (the reddest) when juvenile YTK were fed diet T+M 3 (8.5 g Tau kg^{-1} and 10.9 g Met kg^{-1}), which dietary Tau level was closest in meeting the Tau requirement at 10.9 g Met kg^{-1} (Candebat et al., 2020). Feeding dietary Met at 17.2 g kg^{-1} resulted in significantly higher red (R) and blue (B) values of liver surface color compared to feeding Met levels at 10.9 Met kg^{-1} (**Table 4.3**; **Figure 4.6**).

The average color of the liver surface, as indicated by the hex code, of each T+M diet was distinct (**Figure 4.6**). However, the liver surface color is composed of main colors, which constantly recurred in all dietary treatments, but at varying proportions (**Figure 4.6**).

The color distance between the individual liver surface colors ranged from 0 to 1 in a heatmap, and the conversion into a PCoA distribution indicate the color distance between the T+M diets (**Figure 4.7**), identifying a division into four color clusters. Livers of juvenile YTK fed the T+M diets that were both relatively low in dietary Tau were separated along PCoA 1 by relatively high dietary Met (**Figure 4.7**; l/h in blue) and low Met (**Figure 4.7**; l/l in purple) and did not fall within each other's 95% confidence ellipse. However, individuals fed relatively high Tau and Met (**Figure 4.7**; h/h in red) and relatively high in Tau and low in Met (**Figure 4.7**; l/h in

blue) are separated along the negative PCoA 2 axis and then mix along the positive PCoA2 axis, as shown by the overlap of the 95% confidence ellipse.

Table 4.3 Juvenile yellowtail kingfish (*Seriola lalandi*) liver surface color components from the TauMet study (n = 6) expressed in CIE, RGB, and HSB color model. T+M 11, in bold, is closest to the average TSAA (Met+Cys) requirement of 24.5 g kg^{-1} and the methionine-dependent taurine requirement of 7.7 g kg^{-1} (Candebat et al., 2021, 2020).

	Experimental diet														p-value		
	T+M 1	T+M 2	T+M 3	T+M 4	T+M 5	T+M 6	T+M 7	T+M 8	T+M 9	T+M 10	T+M 11	T+M 12	T+M 13	T+M 14	T	M	T*M
Dietary taurine, methionine, and cysteine contents (g kg^{-1})																	
T	1.6	4.8	8.5	11.9	15.0	17.3	20.4	1.6	5.1	8.1	**11.7**	13.9	18.3	20.0	-	-	-
M	12.0	10.5	11.1	10.8	10.9	10.1	11.1	16.7	19.3	16.4	**18.8**	16.0	17.9	15.0	-	-	-
C	6.8	6.1	5.6	5.4	5.6	5.8	5.7	5.9	5.4	5.5	**5.2**	5.6	5.6	5.8	-	-	-
CIE Lab (lightness, red-green, blue-yellow) color model																	
L	55.2±1.2	56.4±0.9	53.8±0.9	53.6±1.6	56.6±1.3	58.0±1.5	57.6±1.4	55.8±1.5	56.0±1.5	56.0±2.2	59.4±1.8	56.5±1.4	59.4 ±1.8	57.2±1.1	NS	NS	NS
a*	28.8±0.7^{ab}	31.0±1.2^{ab}	34.2±0.7^{a}	34.0±1.5^{ab}	30.2±1.0^{ab}	28.2±1.2^{b}	28.4±1.7^{ab}	31.2±1.3^{ab}	32.0±1.3^{ab}	31.2±2.0^{a}	29.4±2.1^{ab}	31.5±1.6^{ab}	28.6 ±1.2^{b}	31.0±1.7^{ab}	*	NS	NS
b*	33.6±1.4	32.8±0.6	31.6±1.6	30.8±1.1	33.8±1.2	33.4±0.2	32.4±0.9	32.0±0.8	33.0±0.5	33.0±1.5	33.4±1.4	33.2±1.5	33.2 ±1.0	33.2±1.2	NS	NS	NS
RGB (red, green, blue) color model																	
R	187.2±3.1^{a}	194.6±1.2^{a}	190.8±2.0^{a}	189.8±2.5^{a}	194.2±2.2^{a}	195.4±2.9^{a}	194.8±2.5^{a}	193.8±2.8^{b}	195.0±3.0^{b}	193.6±4.8^{b}	201.6±2.6^{b}	194.8±3.0^{b}	200.2±4.4^{b}	196.8±1.6^{b}	NS	**	NS
G	110.8±3.4	112.6±3.1	103.2±3.0	103.0±5.3	113.6±3.9	118.8±4.9	117.6±5.2	111.2±4.8	110.8±4.7	111.2±7.3	121.4±6.4	112.0±5.1	122.6±5.2	114.6±4.0	NS	NS	NS
B	74.2±3.4	79.6±2.0	75.4±1.6	77.0±3.0	78.2±4.0	82.0±3.0	82.8±2.9	80.2±2.5	78.4±2.7	78.2±5.0	86.0±4.4	78.8±3.4	87.0±5.1	81.0±2.7	NS	NS	NS
HSB (hue, saturation, brightness) color model																	
H	19.4±1.4	17.4±1.1	14.4±1.7	13.8±1.8	18.4±0.9	19.6±0.9	18.4±1.5	16.4±1.3	16.6±1.1	17.0±2.0	18.2±1.8	17.0±1.8	18.8±1.2	17.6±1.6	NS	NS	NS
S	60.6±1.4	59.0±0.9	60.6±1.1	59.6±1.2	59.2±1.5	58.0±0.9	57.6±1.1	58.8±0.9	60.0±0.8	59.8±1.9	57.2±1.8	59.8±1.4	57.2±1.8	58.8±1.1	NS	NS	NS
B	73.4±1.2^{a}	76.2±0.5^{a}	74.8±0.7^{a}	74.4±1.0^{a}	76.2±0.7^{a}	76.6±1.1^{a}	76.8±1.0^{a}	76.4±1.0^{b}	76.6±1.1^{b}	76.2±1.8^{b}	79.0±1.0^{b}	76.2±1.1^{b}	78.6±1.8^{b}	77.2±0.6^{b}	NS	**	NS

Levels of significance were assessed via two-way ANOVA and are with respect to P< .05 (*), P< .01 (**), and P< .001 (***). Data with the same superscript letter within rows are statistically similar (P < .05). Data expressed as mean ± SE.

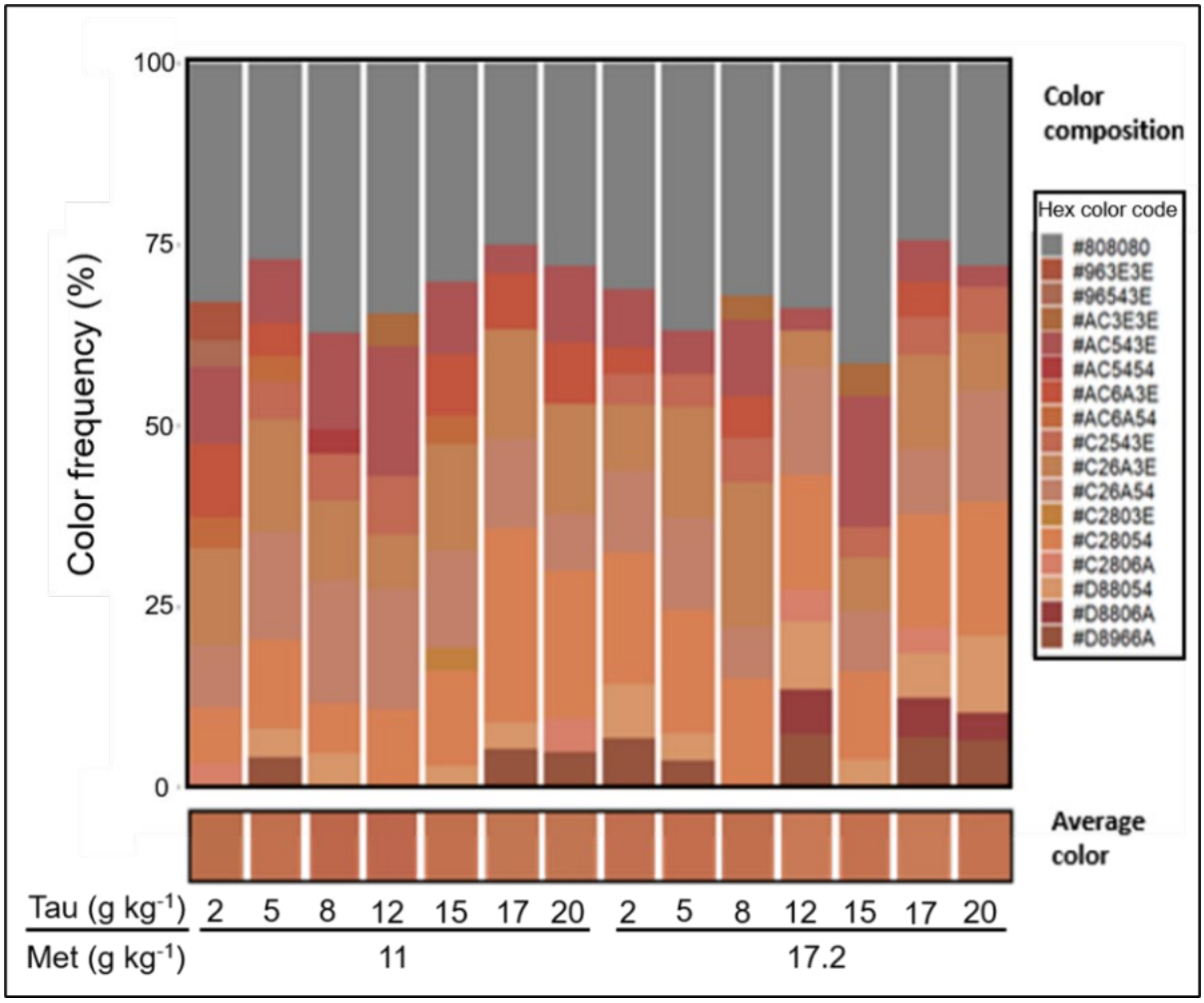

Figure 4.6 (**A**) Distribution of juvenile yellowtail kingfish (*Seriola lalandi*) liver surface colors across dietary treatments from the TauMet study. Grey sequences are the collection of colors that were each ≤ 3% present in the total liver surface color composition (n = 6) within a diet. Colored sequences are tinted in the respective hex color code and represented ≥ 3% of the total liver color composition (n = 6). (**B**) Average RGB liver surface color of each dietary treatment from the TauMet study. Average RGB values were converted to a single-color square that represents the average liver color of liver tissue (n = 6).

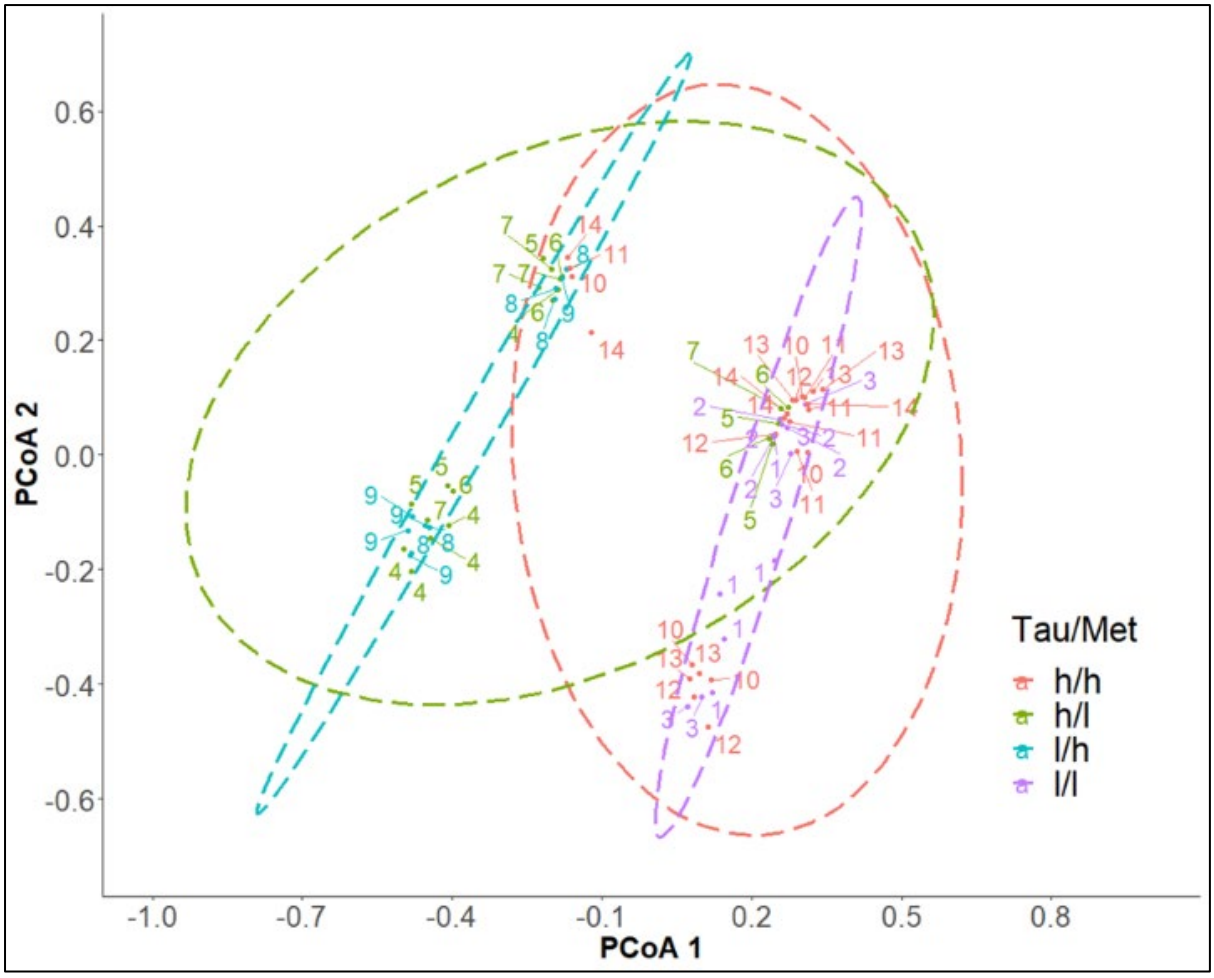

Figure 4.7 PCoA using the color distance method and clustered by similarity of individual juvenile yellowtail kingfish (*Seriola lalandi*) liver surface colors from the TauMet study. A dot represents an individual liver, and numbers correspond to the respective diet. Ellipses (dashed lines) indicate distribution at 95% confidence level of Tau/Met levels at high/high (h/h in red), high/low (h/l in green), low/high (l/h in blue), low/low (l/l in purple).

4.4.1.4 Relationship of liver surface color with biochemistry and histology

Weak to strong relationships between liver surface color and liver histology and plasma biochemistry were found (**Figure 4.4**). The BDW thickness correlated positive with the green-red channel (a*, CIE), and negative with the blue-yellow (b*, CIE Lab) and hue channel (H, HSB) of the measured liver surface color. The semi-quantitative fattiness scores had a positive

correlation with blue-yellow (b*; CIE Lab) and hue (H; HSB). Blood plasma cholesterol correlated positively with red and blue (R, B; RGB) and the brightness (B; HSB) of liver surface color. Blood plasma triglyceride contents correlated positively with the perceptual lightness (L; CIE Lab), red and blue (R, B; RGB), and brightness (B; HSB) of the liver surface color.

Table 4.4 Pearson correlation coefficients for liver coloration values and bile duct wall thickness, liver fattiness, blood plasma cholesterol, and triglyceride contents of juvenile yellowtail kingfish (*Seriola lalandi*).

	Bile duct wall thickness	Hepatic fattiness	Cholesterol (mmol/L)	Triglyceride (mmol/L)
CIE Lab color model (lightness, green-red, blue-yellow)				
L	-0.12	**0.48**	0.38	**0.54**
a*	**0.57**	**-0.45**	0.14	-0.19
b*	**-0.41**	**0.89**	-0.14	0.24
RGB (Red, Green, Blue) color model				
R	0.14	0.34	**0.57**	**0.60**
G	-0.23	**0.50**	0.28	**0.49**
B	0.05	0.16	**0.56**	**0.55**
HSB (hue, saturation, brightness) color model				
H	**-0.56**	**0.67**	-0.17	0.20
S	0.02	0.00	**-0.45**	**-0.42**
B	0.15	0.35	**0.55**	**0.60**

4.4.1.5 Posterior intestine

The posterior intestinal wall of YTK (250–327 g) ranged from 1 to 1.5 mm thick (**Table 4.5**). The intestinal wall was composed of a thin outer serosa layer, a muscularis layer consisting of a muscularis externa and interna (0.4–0.7 mm), a submucosa (0.3–0.4 mm) consisting of a stratum granulosum (2 longitudinal x 2 circular layers), stratum compactum, and a mucosa consisting of a lamina propria (149–355 mm^2) and lamina epithelial. Each 4 µm thick section of the intestine

had between 68 to 137 villi (TVC) that ranged from 3.9 to 5.6 mm in length (VL). The villi were complex, branching into several villus tips.

Histomorphometric measurements were tested for possible effects of body weight and villus area before being subjected to two-way ANOVAs. However, relationships violated linearity and were not statistically significant, thus data were not controlled. An exception were the measured lamina epithelial and lamina propria area, which strongly correlated with the villus area (VA) and met assumptions to adjust means for VA via two-way ANCOVA. YTK fed the diets T+M 8, 11, and 14 exhibited increased posterior intestinal circumference (PIC), total villi height (TVH), villus length (VL), villus area (VA), lamina epithelial surface, total intestinal wall (TIW) and muscularis interna (MI) thickness. Whereas YTK fed less dietary Tau and Met (e.g. diet T+M 1) had less intestinal surface area, for example, PIC, TVH, VL, TIW, and MI. However, YTK fed less dietary Tau had more villi, which number decreased when YTK were fed more dietary Tau. The intestinal mucosa did not exhibit necrosis or acute inflammation. Nevertheless, the villus area-controlled lamina propria areas (LA) were responsive to dietary Met and Tau. Overall, YTK fed dietary Met at 17.2 g kg^{-1} exhibited greater LPA, which may indicate increased lymphocytes, macrophages, and eosinophilic granule cell accumulations. The histochemical analysis of the posterior intestine revealed three types of mucus from goblet cells that stained: acidic, neutral, and mixed (**Figure 4.3**D). Goblet cell mucus predominately stained acidic AB+ (53–73 $villus^{-1}$) or mixed (18–60 $villus^{-1}$), whereas only a few mucus cells stained neutral (TauMet study: 3–14 $villus^{-1}$; MetCys study: 4–16 $villus^{-1}$; **Table 4.6**). The posterior intestines revealed a range of Tau and Met induced histochemical changes (**Table 4.6**).

Table 4.5 Macromorphometric and histomorphometric features of juvenile yellowtail kingfish (*Seriola lalandi*) posterior intestine, fed one of six different taurine-methionine levels from the TauMet feeding trial. T+M 11, in bold, is closest to the average TSAA (Met+Cys) requirement of 24.5 g kg^{-1} and the methionine-dependent taurine requirement of 7.7 g kg^{-1} in YTK (Candebat et al., 2021, 2020).

	Diets						Model	R^2	p-value		
	T+M 1	T+M 4	T+M 7	T+M 8	**T+M 11**	T+M 14			T	M	T*M
Dietary taurine and methionine contents (g kg^{-1})											
T[1]	1.6	11.9	20.4	1.6	**11.7**	20.0	-	-	-	-	-
M	12.0	10.8	11.1	16.7	**18.8**	15.0	-	-	-	-	-
C	6.8	5.4	5.7	5.9	**5.2**	5.8	-	-	-	-	-
Macromorphometrics (g, unless otherwise stated)											
BW	143.3 ± 1.7^{a}	176.8 ± 3.1^{ab}	173.4 ± 4.8^{a}	198.7 ± 4.8^{ab}	231.8 ± 3.9^{b}	230.7 ± 3.6^{b}	M1[2]	-	*	***	NS
VW	9.1 ± 0.1^{a}	10.9 ± 0.2^{ab}	10.4 ± 0.2^{a}	13.2 ± 0.3^{b}	13.3 ± 0.2^{b}	13.2 ± 0.2^{b}	M1	-	NS	***	NS
VSI (%)	6.4 ± 0.05^{a}	6.2 ± 0.03^{b}	6.1 ± 0.05^{b}	6.6 ± 0.03^{a}	5.8 ± 0.03^{b}	5.8 ± 0.04^{b}	M1	-	***	NS	NS
LW	1.2 ± 0.2^{a}	1.5 ± 0^{abc}	1.4 ± 0.0^{ab}	1.7 ± 0.0^{abc}	1.9 ± 0.0^{bc}	2.0 ± 0.0^{bc}	M1	-	*	***	NS
HSI (%)	0.82 ± 0.01	0.84 ± 0.01	0.83 ± 0.01	0.83 ± 0.01	0.83 ± 0.01	0.87 ± 0.01	M1	-	NS	NS	NS
IPFW	0.6 ± 0.0^{a}	0.8 ± 0.0^{a}	0.7 ± 0.0^{a}	1.2 ± 0.1^{b}	1.3 ± 0.1^{b}	1.4 ± 0.0^{b}	M1	-	NS	***	NS
IPFI (%)	0.45 ± 0.01^{a}	0.44 ± 0.02^{a}	0.38 ± 0.01^{a}	0.59 ± 0.02^{b}	0.53 ± 0.02^{b}	0.59 ± 0.02^{b}	M1	-	NS	**	NS
Histomorphometry of the posterior intestine (µm, unless otherwise stated)											
PIC	45655 ± 441^{a}	39599 ± 984^{a}	40820 ± 870^{a}	46387 ± 553^{b}	47538 ± 675^{b}	45662 ± 474^{b}	M1	0.04	NS	*	NS
TVH	4930 ± 107^{ab}	4598 ± 85^{a}	5293 ± 127^{bc}	5637 ± 185^{c}	5644 ± 127^{c}	5750 ± 124^{c}	M1	0.06	**	***	NS
VL	4493 ± 177^{ab}	3946 ± 152^{a}	5182 ± 220^{bc}	5626 ± 287^{c}	5057 ± 187^{bc}	5261 ± 207^{bc}	M1	0.01	**	***	*
TIW	1064 ± 14^{a}	1155 ± 17^{a}	1289 ± 27^{a}	1445 ± 26^{b}	1271 ± 26^{b}	1491 ± 27^{b}	M1	0.21	NS	**	NS
MI	443 ± 7^{a}	528 ± 16^{ab}	531 ± 7^{ab}	624 ± 9^{bc}	564 ± 9^{ab}	735 ± 6^{c}	M1	0.19	*	**	*
S	324 ± 2	367 ± 10	393 ± 10	401 ± 9	347 ± 3	420 ± 8	M1	0.14	NS	NS	NS
TV	125 ± 1^{a}	114 ± 2^{ab}	101 ± 2^{b}	118 ± 2^{a}	110 ± 1^{ab}	107 ± 2^{b}	M1	0.03	*	NS	NS
VA *(mm^2)*	1252 ± 65^{a}	975 ± 49^{ab}	1510 ± 86^{bc}	1608 ± 77^{c}	1706 ± 88^{c}	1821 ± 112^{c}	M1	0.05	***	***	*
LEA *(mm^2)*	1044 ± 53^{a}	826 ± 41^{a}	1260 ± 72^{a}	1311 ± 66^{ab}	1416 ± 74^{a}	1466 ± 90^{b}	M2[3]	0.99	NS	NS	***
LPA *(mm^2)*	209 ± 13^{ad}	149 ± 9^{abcd}	249 ± 16^{ad}	297 ± 15^{bcd}	290 ± 16^{ad}	355 ± 24^{bc}	M2[3]	0.80	***	***	NS

[1] T, taurine; M, methionine; C, cysteine; BW, total fish weight; VW, viscera weight; VSI, viscerosomatic index; LW, liver weight; HSI, hepatosomatic index; IPFW, intraperitoneal fat weight; IPFI, intraperitoneal fat index; PIC, posterior intestine circumference; TVH, total villus height; VL, villus length; TIW, total intestinal wall thickness; MI, muscularis interna thickness; S, submucosa thickness; TV, total villi count; VA, villus area; LPA, lamina propria area, LEA, lamina epithelial area. [2] The significant effects were determined by two-way ANOVA (M1) or ANCOVA (M2) and levels of significance are with respect to $P < .05$ (*), $P < .01$ (**), and $P < .001$ (***). Data with the same superscript letter within rows are statistically similar ($P > .05$). [3] LPA and LEA were controlled via two-way ANCOVA for villus area prior to the two-way ANOVA; however reported values are uncontrolled and expressed as mean ± SE.

Table 4.6 Histochemical analysis of intestinal mucus and supranuclear vacuoles in juvenile yellowtail kingfish (*Seriola lalandi*) fed one of six taurine-methionine levels (TauMet). T+M 11, in bold, is closest to the average TSAA (Met+Cys) requirement of 24.5 g kg^{-1} and the dependent taurine requirement of 7.7 g kg^{-1} in YTK (Candebat et al., 2021, 2020).

	Diets						Model	R^2	p-value		
	T+M 1	T+M 4	T+M 7	T+M 8	**T+M 11**	T+M 14			T	M	T*M
Dietary taurine and methionine contents (g kg^{-1})											
T[1]	1.6	11.9	20.4	1.6	**11.7**	20.0	-		-	-	-
M	12.0	10.8	11.1	16.7	**18.8**	15.0	-		-	-	-
C	6.8	5.4	5.7	5.9	**5.2**	5.8	-		-	-	-
Goblet cell and vacuole count per villus											
TGC	93.8 ± 5.1^{a}	79.8 ± 4.8^{a}	117.4 ± 6.5^{ab}	130.9 ± 7^{ab}	108.5 ± 6.3^{ac}	132.9 ± 10.2^{a}	M2[2,3]	0.55	***	NS	**
AB+	72.8 ± 4.1^{a}	54.2 ± 3.1^{a}	58.5 ± 3.9^{b}	55.2 ± 3.5^{b}	52.7 ± 3.3^{b}	62.2 ± 5^{b}	M2	0.39	***	***	***
PAS+	3.1 ± 0.4^{a}	4.8 ± 0.6^{ab}	14.1 ± 1.7^{d}	11.5 ± 0.9^{cd}	7.7 ± 0.9^{bc}	10.5 ± 0.9^{cd}	M1	0.11	***	***	***
AB+PAS+	17.8 ± 1.6^{a}	50.7 ± 2.9^{ab}	44.8 ± 4.0^{b}	64.3 ± 3.5^{c}	48.1 ± 4^{b}	60.1 ± 6.5^{bc}	M1	0.32	**	***	***
S-PAS+	106.2 ± 11.5^{a}	118.0 ± 7.4^{a}	107.4 ± 8.5^{a}	224.0 ± 12.4^{c}	185.4 ± 11.9^{bc}	169.1 ± 13.4^{bc}	M2	0.27	***	***	***
SV	66.8 ± 3.9^{a}	23.7 ± 3.9^{bc}	15.8 ± 3.9^{c}	21.2 ± 3.9^{c}	18.7 ± 3.9^{c}	37.2 ± 4.2^{b}	M1	0.06	***	***	***
Density of goblet cell mucin and vacuoles per villus mm^2											
AB:PAS ratio	23:1	11:1	4:1	5:1	7:1	6:1	-	-	-	-	-
AB+/ VA	0.059 ± 0.002^{a}	0.058 ± 0.003^{a}	0.038 ± 0.001^{b}	0.035 ± 0.002^{b}	0.031 ± 0.001^{b}	0.035 ± 0.002^{b}	M1	-	**	**	***
PAS+/ VA	0.002 ± 0.001^{a}	0.005 ± 0.001^{ab}	0.013 ± 0.002^{c}	0.007 ± 0.000^{b}	0.004 ± 0.000^{ab}	0.006 ± 0.00^{ab}	M1	-	***	***	***
AB+PAS+/ VA	0.014 ± 0.001^{a}	0.022 ± 0.005^{ab}	0.039 ± 0.005^{cd}	0.041 ± 0.002^{d}	0.027 ± 0.002^{abc}	0.033 ± 0.002^{bcd}	M1	-	***	***	***
TGC/ VA	0.076 ± 0.002^{ab}	0.085 ± 0.006^{b}	0.091 ± 0.007^{b}	0.084 ± 0.003^{b}	0.063 ± 0.002^{a}	0.074 ± 0.003^{ab}	M1	-	NS	**	**
S-PAS+/ VA	0.080 ± 0.005^{a}	0.125 ± 0.005^{bc}	0.070 ± 0.005^{a}	0.150 ± 0.007^{c}	0.113 ± 0.005^{b}	0.107 ± 0.010^{b}	M1	-	***	NS	***
SV/ VA	0.055 ± 0.004^{a}	0.021 ± 0.003^{b}	0.010 ± 0.002^{c}	0.013 ± 0.002^{bc}	0.011 ± 0.002^{bc}	0.018 ± 0.002^{bc}	M1	-	***	***	***

[1] T, taurine; M, methionine; C, cysteine; AB+, AB+ goblet cell mucus count; PAS+, PAS+ goblet cell mucus count; AB+PAS+, AB+PAS+ goblet cell mucus count; TGC, Total goblet cell mucus count, S-PAS+, Small PAS+ bullet-shaped bodies; SV, Supranuclear vacuoles; VA, Villus area. [2] The significant effects were determined by two-way ANOVA (M1) or ANCOVA (M2) and levels of significance are with respect to P< .05 (*), P< .01 (**), and P< .001 (***). Data with the same superscript letter within the T+M rows are statistically similar (P < 0.05). [3] For the two-way ANOVA TGC, AB+, and S-PAS+ in section 'Goblet cell and vacuole count per villus' were controlled for villus area after meeting assumptions; however reported values are expressed as uncorrected mean ± SE.

The total goblet cell mucus production was most pronounced in fish fed the T+M 7 diet, exhibiting the greatest total goblet cell density (TGC/VA; **Table 4.6**; **Figure 4.8**). Dietary Tau and Met induced significant changes in the composition of goblet cell mucus types, where increasing levels of dietary Tau and Met decreased the density of acidic (AB+) goblet cell mucus. Increasing levels of dietary Tau at 10.9 g Met kg^{-1} increased mixed and neutral goblet cell mucus; however, at Met levels above current industry practice, increasing dietary Tau led to a decrease in mixed and neutral goblet cell mucus (**Figure 4.6**).

Besides larger goblet cells that stained PAS+ (41.4 ± 1.45 µm), smaller bullet-shaped S-PAS+ bodies situated in the apical areas of the enterocytes were observed with an average size of 18.36 ± 0.68 µm (**Figure 4.3**D). The density of S-PAS+ was significantly affected by dietary Tau, which interacted with dietary Met and was the highest in YTK fed the T+M 8 diet (0.15 0 ± 0.007 $µm^{-2}$). YTK had supranuclear vacuoles that were also situated above the enterocytes in the lamina epithelial and were on average 21.4 ± 1.08 µm. The supranuclear vacuoles density in YTK fed the T+M diets was significantly affected by interacting dietary Tau and Met, where fish fed the T+M 1 diet exhibited an exceptionally higher supranuclear vacuoles density (0.055 ± 0.004 per $µm^2$) than other T+M diets.

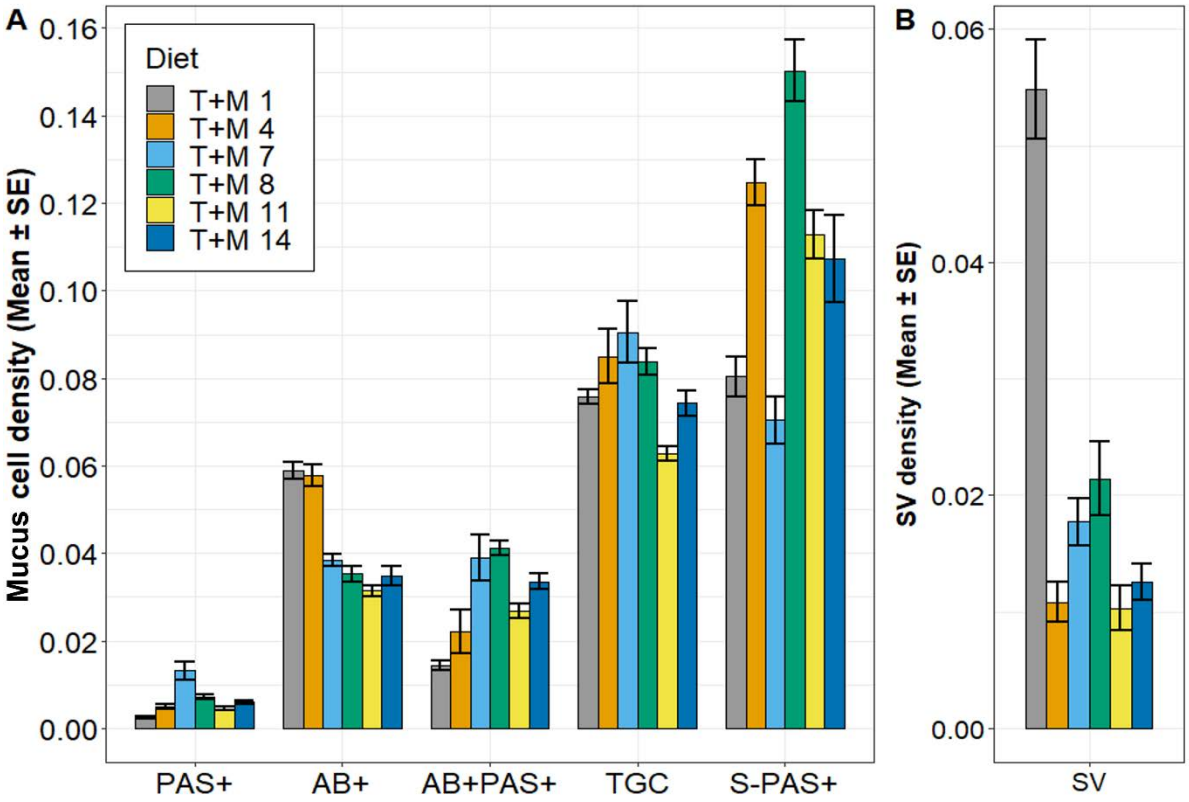

Figure 4.8 Barplots on the histochemical analysis per villus area of the PI of juvenile yellowtail kingfish (*Seriola lalandi*) fed one of six taurine-methionine combinations (TauMet). (A) neutral goblet cell mucus (PAS+ villus area^{-1}), acid goblet cell mucus (AB+ per villus area), or mixed goblet cell mucus (AB+PAS+ villus area^{-1}), total goblet cell mucus (TGC villus area^{-1}), and bullet shaped PAS+ mucus (S-PAS+ per villus area^{-1}). (B) supranuclear vacuole density (SV per villus area^{-1}). Error bars indicate SE.

Besides larger goblet cells that stained PAS+ (41.4 ± 1.45 µm), smaller bullet-shaped S-PAS+ bodies situated in the apical areas of the enterocytes were observed with an average size of 18.36 ± 0.68 µm (**Figure 4.3**D). The density of S-PAS+ was significantly affected by dietary Tau, which interacted with dietary Met and was the highest in YTK fed the T+M 8 diet (0.150 ± 0.007 µm^{-2}). YTK had supranuclear vacuoles that were also situated above the enterocytes in the lamina epithelial and were on average 21.4 ± 1.08 µm. The supranuclear

vacuoles density in YTK fed the T+M diets was significantly affected by interacting dietary Tau and Met, where fish fed the T+M 1 diet exhibited an exceptionally higher supranuclear vacuoles density (0.055 ± 0.004 per µm^2) than other T+M diets.

4.4.2 MetCys study & Commercial

4.4.2.1 Posterior intestine

Overall, the posterior intestines appeared clinically normal. The posterior intestinal wall of YTK (140–230 g) was between 1 and 1.3 mm thick (**Table 4.7**). The microscopic observations and measurements revealed that the intestinal wall composition and thickness were similar to the samples collected from the TauMet study (MI: 0.4–0.5 mm; SC: 0.3–0.4 mm; LPA: 184–261 mm^2). Each intestinal cross-section (4 µm) contained between 64 and 124 villi (TVC) that ranged from 4.1 to 4.9 mm in length (VL) and exhibited multiple tips, which contributes to a greater complexity and surface area.

Histomorphometric measurements from the MetCys study were tested for possible effects of body weight and villus area before being subjected to two-way ANOVAs to thest for Met and Cys effects on the dependent variables. However, relationships mostly violated linearity and were not statistically significant, thus data were not controlled. An exception were the measured lamina epithelial and lamina propria area, which strongly correlated with the villus area (VA) and met assumptions to adjust means for VA via two-way ANCOVA.

YTK fed the M+C 2 diet, which was closest in meeting the average MOM (14.3 g kg^{-1}) and the TSAA (Met) requirement (26.2 g kg^{-1}) at 5.9 g cysteine kg^{-1} in YTK (Candebat et al., 2021), had the longest villi, thickest muscularis interna, more villi in the posterior intestine and increased lamina epithelial area, indicating more complex villi and greater absorptive surface area. In comparison, YTK fed diet M+C 1, which Met and Cys content were below that of the TSAA and MOM requirement, had the least villi in the posterior intestine, less lamina epithelial area, shorter villi, yet the thickest intestinal wall. Whereas YTK that were fed the high dietary cysteine series, M+C 3 and 4, had significantly shorter villi, decreased villi height, thinner intestinal walls, the least lamina epithelial surface and villus area.

YTK sampled at the start of the MetCys feeding trial had substantially thinner PIC (28551 ± 654 µm), TIW (716 ± 13 µm), MI (261 ± 5 µm), and S (275± 5 µm). YTK fed the Com or M+C diets and sampled after eight weeks had comparatively thicker PIC, TIW, MI, stratum granulosum & stratum compactum indicating histomorphometric changes in the posterior intestine that may have been induced substantially through growth and ontogenic changes.

Table 4.7 Macro- and histomorphometric features of juvenile yellowtail kingfish (*Seriola lalandi*) posterior intestine fed one of four different methionine-cysteine levels from the MetCys study and the commercial diet. M+C 2, in bold, was closest in meeting the average MOM (14.3 g kg^{-1}) a and the TSAA (Met) requirement (26.2 g kg^{-1}) at 5.9 g cysteine kg^{-1} in YTK (Candebat et al., 2021).

	Commercial diet (Com)		Experimental diets				Model	R^2	p-value		
	Initial	Final	M+C 1	**M+C 2**	M+C 3	M+C 4			M	C	M*C
Dietary methionine and cysteine contents (g kg^{-1})											
M[1]	12.6	12.6	8.8	**24.7**	7.9	25.2	-	-	-	-	-
C	6.5	6.5	5.8	**5.9**	13.7	13.9	-	-	-	-	-
T	7.5	7.5	7.0	**6.9**	7.0	6.9	-	-	-	-	-
Macromorphometrics (g, unless otherwise stated)											
BW	52.7 ± 0	327.2 ± 4.3	246.3 ± 7.7	254.1 ± 8.9	257.6 ± 3.4	256.2 ± 8.3	-	-	NS	NS	NS
VW	NA ± NA	NA ± NA	14.3 ± 0.5	14.6 ± 0.6	14.2 ± 0.1	15.1 ± 0.6	-	-	NS	NS	NS
VSI (%)	NA ± NA	NA ± NA	6.1 ± 0.1[a]	6.0 ± 0.1[a]	5.5 ± 0.0[b]	5.1 ± 0.1[b]	-	-	NS	***	NS
LW	NA ± NA	NA ± NA	2.05 ± 0.08	2.04 ± 0.08	2.04 ± 0.02	2.41 ± 0.14	-	-	NS	NS	NS
HSI (%)	NA ± NA	NA ± NA	0.85±0.01	0.93±0.04	0.77±0.01	0.80±0.01	-	-	NS	NS	NS
Histomorphometry of posterior intestine (µm, unless otherwise stated)											
PIC	28551 ± 654	45361 ± 803	39769 ± 831	47620 ± 826	4173 ± 1544	44131 ± 871	M1	0.06	NS	NS	NS
TVH	2924 ± 97	4755 ± 174	5092 ± 160[a]	4910 ± 146[a]	4722 ± 178[b]	4416 ± 106[b]	M1	0.04	NS	**	NS
VL	2670 ± 173	4106 ± 184	4153 ± 156[a]	4912 ± 243[b]	4114 ± 179[ab]	4134 ± 194[ab]	M1	0.01	*	*	NS
TIW	716 ± 13	1310 ± 48	1320 ± 37	1190 ± 19	1159 ± 42	1025 ± 14	M1	0.01	NS	NS	NS
MI	261 ± 5	472 ± 23	516 ± 15	540 ± 24	439 ± 20	387 ± 4	M1	0.01	NS	NS	NS
S	275 ± 4	357 ± 20	450 ± 11	337 ± 8	388 ± 16	422 ± 12	M1	0.04	NS	NS	NS
TV	66 ± 1	102 ± 1	83 ± 1	110 ± 1	98 ± 3	103 ± 2	M1	0.02	*	NS	NS
VA (mm^2)	693 ± 50	1464 ± 109	1316 ± 78[a]	1461 ± 107[b]	1143 ± 72[b]	1155 ± 71[c]	M1	0.04	***	***	NS
LEA (mm^2)	567 ± 43	1216 ± 93	1094 ± 65	1200 ± 88	924 ± 59	971 ± 61	M2	0.99	NS	NS	NS
LPA (mm^2)	126 ± 9	248 ± 19	222 ± 16	261 ± 20	219 ± 15	184 ± 12	M2	0.81	NS	NS	NS

[1] M, methionine; C, cysteine; T, taurine; BW, total fish weight; VW, viscera weight; VSI, viscerosomatic index; LW, liver weight; HSI, hepatosomatic index; PIC, posterior intestine circumference; TVH, total villus height; VL, villus length; TIW, total intestinal wall thickness; MI, muscularis interna thickness; S, submucosa. [2] The significant effects were determined by two-way ANOVA (M1) or ANCOVA (M2) and levels of significance are with respect to P< .05 (*), P< .01 (**), and P< .001 (***). Data with the same superscript letter within the M+C rows are statistically similar (P < 0.05). [3] For the two-way ANOVA LPA and LEA were controlled via ANCOVA for villus area after meeting assumptions; however reported values were reported as uncontrolled mean ± SE.

The total goblet cell mucus production was most pronounced in fish fed the M+C 4 diet, exhibiting elevated goblet cell mucus densities of total goblet cells (TGC/VA). The histochemical analysis of the posterior intestine indicated three types of goblet cell mucus: acidic, neutral and mixed (**Table 4.8**). Goblet cell mucus cells predominately stained acidic (14–44 villus^{-1}) or mixed (56–78 villus^{-1}), whereas only a few goblet cell mucus cells stained neutral (TauMet study: 3–14 villus^{-1}; MetCys study: 4–16 villus^{-1}). YTK fed more dietary Met, and Cys exhibited more mixed and less acidic goblet cell mucus cells. YTK fed M+C, and Com diets also had supranuclear vacuoles and S-PAS+ bodies. YTK fed Cys below 13.9 g kg^{-1} but with higher Met concentrations had the highest supranuclear vacuoles density of YTK (diet M+C 2) fed the M+C diets (0.101 ± 0.012). However, the initial and final supranuclear vacuoles densities of YTK fed the Com diet were even more pronounced (0.280 ± 0.022 µm^{-2} and 0.264 ± 0.041 µm^{-2}). Overall, YTK fed M+C and commercial diets exhibited considerably lower S-PAS+ and higher supranuclear vacuoles densities than YTK fed the T+M diets (**Figure 4.9**).

Table 4.8 Histochemical analysis of mucus and supranuclear vacuoles in juvenile yellowtail kingfish (YTK; *Seriola lalandi*) intestine, fed one of six methionine-cysteine combinations (MetCys study) or a commercial diet. M+C 2, in bold, was closest to meeting the average MOM (14.3 g kg^{-1}) and the TSAA (Met) requirement (26.2 g kg^{-1}) at 5.9 g cysteine kg^{-1} in YTK (Candebat et al., 2021).

	Commercial diet		Experimental diets				Model	R^2	p-value		
	Initial	Final	M+C 1	**M+C 2**	M+C 3	M+C 4			M	C	M*C
Dietary methionine and cysteine contents (g kg^{-1})											
M[1]	12.6	12.6	8.8	**24.7**	7.9	25.2	-	-	-	-	-
C	6.5	6.5	5.8	**5.9**	13.7	13.9	-	-	-	-	-
T	7.5	7.5	7.0	**6.9**	7.0	6.9	-	-	-	-	-
Goblet cell and vacuole count per villus											
TGC	63.1 ± 5.9	82.2 ± 5.2	109.7 ± 7.3^{a}	102.2 ± 10.1^{a}	93.4 ± 5.7^{a}	121.2±7.5^{b}	M2	0.47	NS	**	***
AB+	23.9 ± 3.3	0.0 ± 0.0	44.3 ± 4.1^{a}	14.0±1.5^{a}	23.8±2.2^{a}	27.5±4.2^{b}	M1	0.05	*	NS	NS
PAS+	3.0 ± 0.4	27.2 ± 2.5	9.7 ± 1.8^{a}	22.7±4.0^{b}	4.3 ± 0.4^{a}	15.9±2.2^{b}	M2	0.40	***	NS	NS
AB+PAS+	23.9 ± 3.3	55.0 ± 3.8	55.8 ± 5.9^{a}	65.5 ± 6.3^{ab}	65.3 ± 5.4^{bc}	77.7±5.1^{c}	M2	0.39	**	***	NS
S-PAS+	0.0 ± 0.0	18.2 ± 4.8	54.5 ± 10.1^{a}	65.3 ± 11.1^{b}	49.8 ± 8.1^{ab}	17.5±4.1^{ab}	M2	0.35	NS	NS	**
SV	176.7 ± 19.3	276.8 ± 29.9	48.6 ± 12.0^{a}	165.8 ± 26.6^{b}	53.5 ± 10.1^{a}	56.1±15.0^{a}	M1	0.27	***	**	***
Density of goblet cell mucin and vacuoles per villus mm^2											
AB: PAS ratio	*8:1*	*0:1*	*5:1*	*1: 1.6*	*6:1*	*2:1*	-	-	-	-	-
TGC	0.095 ± 0.005	0.074 ± 0.006	0.087 ± 0.003^{a}	0.069 ± 0.005^{b}	0.087 ± 0.003^{a}	0.115 ± 0.006^{c}	M1	-	NS	**	**
AB+	0.054 ± 0.007	0.000 ± 0.000	0.041 ± 0.040^{a}	0.009 ± 0.001^{c}	0.028 ± 0.003^{b}	0.024 ± 0.003^{b}	M1	-	***	NS	***
PAS+	0.005 ± 0.000	0.027 ± 0.004	0.006 ± 0.001^{a}	0.014 ± 0.002^{b}	0.004 ± 0.000^{a}	0.017 ± 0.002^{b}	M1	-	***	NS	NS
AB+PAS+	0.036 ± 0.004	0.046 ± 0.004	0.041 ± 0.004^{a}	0.045 ± 0.003^{ab}	0.056 ± 0.003^{b}	0.074 ± 0.004^{c}	M1	-	**	***	NS
S-PAS+	0.000 ± 0.000	0.012 ± 0.003	0.034 ± 0.005^{a}	0.041 ± 0.006^{a}	0.038 ± 0.006^{a}	0.011 ± 0.002^{b}	M1	-	NS	**	***
SV	0.280 ± 0.022	0.264 ±0.041	0.039 ± 0.010^{a}	0.101 ± 0.012^{b}	0.036 ± 0.007^{a}	0.040 ± 0.010^{a}	M1	-	**	**	**

[1] M, methionine; C, cysteine; T, taurine; AB+, AB+ goblet cell mucus count; PAS+, PAS+ goblet cell mucus count; AB+PAS+, AB+PAS+ goblet cell mucus count; TGC, Total goblet cell mucus count, S-PAS+, Small PAS+ bullet-shaped bodies; SV, Supranuclear vacuoles; VA, Villus area. [2] The significant effects were determined by two-way ANOVA (M1) or ANCOVA (M2). Levels of significance are with respect to P< .05 (*), P< .01 (**), and P< .001 (***). Data with the same superscript letter within the M+C rows are statistically similar (P < 0.05). [3] For the two-way ANOVA TGC, PAS+, AB+PAS+, and S-PAS+ were priorly controlled via ANCOVA for villus area after meeting assumptions; however values were reported asuncontrolled mean ± SE (n = 6).

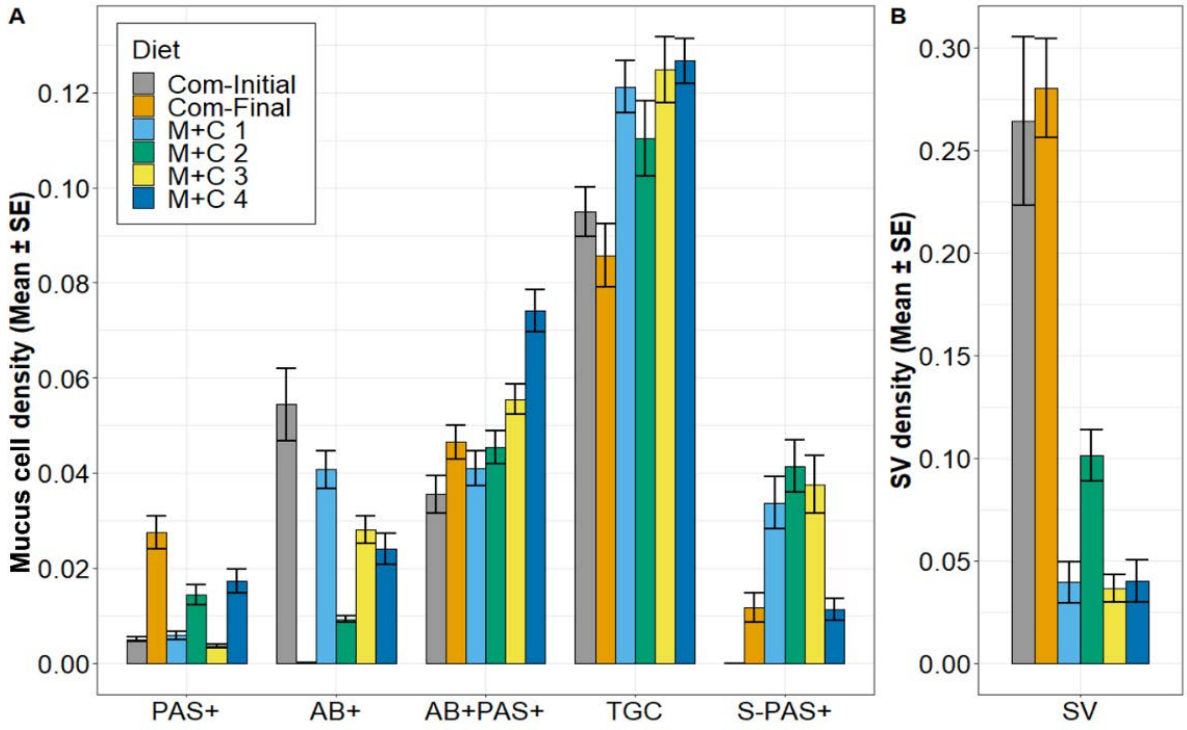

Figure 4.9 Barplots on the histochemical analysis of juvenile yellowtail kingfish (*Seriola lalandi*) posterior intestine, fed one of five methionine-cysteine combination (MetCys). (A) neutral goblet cell mucus (PAS+ villus area^{-1}), acid goblet cell mucus (AB+ villus area^{-1}), or mixed goblet cell mucus (AB+PAS+ villus area^{-1}), total goblet cell mucus (TGC villus area^{-1}), and bullet shaped PAS+ mucus (S-PAS+ per villus area^{-1}). **(B)** supranuclear vacuole density (SV per villus area^{-1}). Error bars indicate SE.

4.5 Discussion

The TSAA, minimum obligatory methionine and Tau requirements of juvenile YTK have recently been quantified based on optimized growth, feed efficiency and protein retention (Candebat et al., 2020, 2021). However, information on the liver and posterior intestine condition of juvenile YTK fed suboptimal, optimal and supraoptimal combinations of Met, Cys and Tau remain unknown. This study provides primary data on the Met, Cys and Tau altering effects on the functional and structural properties of hepatic and intestinal tissues and demonstrates that

adequate intakes of Met, Cys and Tau can improve nutrient assimilation capacity and help to maintain homeostasis. Diets T+M 11 (Candebat et al., 2020) and M+C 2 (Candebat et al., 2021) came closest to the Met, Cys and Tau specifications to meet YTK requirements for optimized growth, feed efficiency and protein retention (Candebat et al., 2021, 2020). As a reference, a level of 24.5 g Met+Cys kg^{-1} was recommended for YTK diets, provided that 7.7 g Tau kg^{1}, at least 14.3 g Me kg^{-1} and no more than 13.9 g Cys kg^{-1} were supplied.

4.5.1 Liver histology, plasma biochemistry, and liver surface color

In YTK high levels of dietary Tau led to significantly thinner bile duct walls. To our knowledge, this is the first study to observe this in fish. In mice and humans, intrahepatic bile duct wall thickening and proliferation is a negative property that is linked to bile acid-induced inflammation, fibrosis and necrosis of cholangiocytes and hepatocytes with consequential reductions in biliary flow (Cai & Boyer, 2021; Fickert & Wagner, 2017). Tau may have alleviated the symptoms of BDW thickening by conjugating with hydrophilic bile acids that are less cytotoxic, and that stimulate secretin hydrocholeresis (Hofmann & Hagey, 2008; Hohenester et al., 2012; Kortner et al., 2016; Úriz et al., 2011). Dietary Met supplementation may have increased the production of S-adenosyl methionine, known to improve conditions of chronic liver diseases, and Tau (Lu & Mato, 2012; Wen et al., 2019). Overall, blood plasma lipid concentrations were up to four times lower than suggested reference ranges for YTK (Cholesterol: 5.4–8.5 $mmoL^{-1}$, triglyceride: 2.7–4.8 $mmol\ L^{-1}$; Stephens 2021 per. Communication) and Japanese yellowtail (cholesterol > 6.3 mmol L^{-1}, triglyceride > 3.7 mmol L^{-1}; Maita 2012 pers. com.). However, the deviation from the latter communications may be due to differences in fat contents

of fed diets, fish size or other factors. YTK fed dietary Met at 17.2 g kg^{-1} showed increases in plasma triglyceride contents. Similar results are reported for rats, where Met restriction altered lipogenesis resulting in decreased circulating lipid levels (Hasek et al., 2013). Additionally, YTK fed diets containing Met levels at 17.2 g kg^{-1} exhibited higher AST and LD activity. Elevated AST and LD activities are pathological markers indicative of cell damage when they exceed reference range thresholds. However, in the absence of reference ranges or any indication of pathology, elevated levels of these enzymes may indicate their activity level in the plasma rather than liberation from cell lysis. This is consistent with Liu et al. (2020), who found elevated LD for the fastest growing kingfish, consistent with its role in energetics. Likewise, the elevated AST levels for fish fed higher levels of Met found in our study are likely due to altered amino acid metabolism rather than pathology. LD is a glycolytic enzyme critical for fish burst swimming capacity and directly linked to metabolic rate (Dahlhoff, 2004). Met is metabolized to cystathionine, which can form α-ketoglutarate, which is transaminated by AST to glutamate (Wu, 2020).

On subjective, macroscopic assessment, liver surface colors were mostly red-orange-yellow-peach. On objective, digital assessment liver surface colors were close to the hex color "copper-red; #CB6D51". Schmitt and Dethloff (2000) subjectively described the color of "normal" liver as red to light red and fatty liver as "coffee with cream". In contrast Brusle and Anadon (1996) described the color of "normal" liver as red-brown and fatty liver as yellow. The liver surface color of YTK fed the T+M diets was bluer, greener and 16% lighter than the hex color "dark red" (#AB2328), which is subjectively classified as a "normal" liver by Brusle and Anadon (1996) and Schmitt and Dethloff (2000). The subjective assessment of zebrafish linked liver

surface color lightness to hepatic triglyceride accumulations (Asaoka et al., 2013). The digital assessment of broiler liver surface color linked lightness to a carbohydrate-rich diet, whereas a darkening was linked to feeding less fat, more Met, or fasting animals for 12 hours (Trampel et al., 2005). YTK from this study had more plasma cholesterol and triglycerides when fed the higher Met diets (17.2 g kg^{-1}) yet not more hepatic fat. The subjective and semi-quantitative hepatic fattiness scoring may not have been sensitive enough to capture dietary Met effects (Silva et al., 2015); however, hepatic fat positively correlated with the yellow-blue channel (b*), indicating that increased hepatic fat made the liver surface color appear more yellow.

Interestingly, the intrahepatic BDW thickness correlated with the liver surface color, which thickness was significantly affected by dietary Tau. YTK fed T+M 7 and 14, which contained the maximum rate of dietary Tau (20.4 & 20.0 g kg^{-1}) and YTK fed T+M 1, which contained the least amount of dietary Tau, had the thinnest BDWs. Yet only YTK fed T+M indicated the least red and bright livers (hex color: B86948). Bile ducts are responsible for the transport of bile, which is comprised of water, bile salt, conjugated bilirubin (yellow), cholesterol, lecithin, and inorganic substances from the hepatocytes into the intestine for digestion and waste removal (Brusle & Anadon, 1996; Faccioli et al., 2014). A thickening of the intrahepatic bile duct walls may indicate a compromised export of breakdown and bile products and may consequently alter the liver surface color. Further research is warranted, yet to our knowledge, the present study formed primary data linking the liver surface color to Tau-dependent BDW thickness. The liver surface colors of YTK fed diets T+M 3 and T+M 4, which contained sufficient dietary Tau to meet the requirement at a Met content of 11 g kg^{-1}, were up to 21% more red (a* = 34.2 and 34.0) than YTK fed any other T+M diet. YTK fed the T+M 11 diet, which was closest to

meeting the total sulfur amino acid requirement and met the Tau requirement, had the reddest yet brightest liver surface color throughout all treatments. This suggests that YTK fed dietary Tau and Met close to the requirement had better liver conditions, assuming that redder livers are necessarily healthier livers. The accuracy of a subjective color scoring system for fish liver remains questionable; firstly, because relationships between objective, quantitative histology and macroscopic color scores have not been established, and secondly because the judgement relies on the observer's perception of color. At this stage, it may serve as a simple clinical tool when underlying health conditions present in strong color variations. More subtle color changes may not be visible to the naked eye.

4.5.2 Green liver syndrome in YTK

In our study, YTK livers did not exhibit segmental or diffuse green discolorations as previously described by Bowyer et al. (2012b) in YTK and Takagi et al. (2011) in red seabream. Japanese Yellowtail, red seabream (*Pagus major*), and Totoaba (*Totoaba macdonaldi*) fed low-fishmeal diets deficient in Tau exhibited accumulated biliverdin and bilirubin in the liver due to increased hemolysis and insufficient conjugation with Tau for the excretion into the biliary system (Goto et al., 2001; Sakai et al., 1987; Satriyo et al., 2017; Takagi et al., 2011, 2010, 2005). However, YTK fed a Tau and Met deficient diet (T+M 1 diet) had no signs of green liver discolorations, indicating that Tau deficiencies may not cause green liver syndrome but might exacerbate previous conditions. This finding is similar to YTK that were fed soy protein concentrate (SPC) based diets low in Tau, Met, and lysine, yet had no green liver (Bowyer et al., 2013), whereas YTK fed a 100% rapeseed oil-based diet and reared at 18°C had green livers. However, dietary

Tau content in the experimental diets was not presented in that study (Bowyer et al., 2012b). Interestingly, in the cases where low Tau correlated with green liver, the fish were often exposed to low temperatures (12.3–18.0°C) and/or fed alternative protein sources, indicating that low water temperature may contribute to green liver (Takagi et al., 2006a), including in Japanese yellowtail (Takagi et al., 2005), red seabream (Goto et al., 2001), and YTK (Bowyer et al., 2012b). In poikilothermic frogs and salmon, hyperthermy increases hemolysis, increasing the biliverdin production, yet reduces the biliverdin reductase, decreasing biliverdin (green) removal (van den Hurk, 2006). This may lead to an accumulation of hepatic biliverdin and, therefore, a green discoloration. YTK from this study were held at 23.3 ± 0.6°C, which may be within the thermal tolerance to maintain a healthy balance between production and removal of hematogenous pigments. Japanese Yellowtail that was held at 23.9 ± 2.0°C and fed non-fishmeal diets had green liver due to mucosporozoa infestations that blocked the biliary system, indicating that the diet made the fish susceptible to infestation (Watanabe et al., 1998). Overall, it appears that the green liver condition in fish may be induced by distinct physiological mechanisms that are triggered by a single or an interplay of factors such as imbalanced diet, toxicity, pathogens, and temperature.

4.5.3 Structural layers and connective tissue of the posterior intestinal wall

Although it is widely recognized that dietary Tau and Met supplementation may be of benefit for fish health and growth (Andersen et al., 2016; Li et al., 2021b; Salze and Davis, 2015), in this study the submucosa of YTK were not affected by either dietary Met or Tau. Adequate dietary Met can downregulate pro-inflammatory and upregulate anti-inflammatory cytokine

mRNA levels (Pan et al., 2016). In salmon soy protein-based diets induced inflammatory responses and consequently increased stratum compactum/submucosal thickness (Knudsen et al., 2008). Interestingly, with an incremental increase in both dietary Met and Tau, the unaffected submucosa was reflected by a marked expansion rather than a reduction in the lamina propria. This observation is different from what one would expect. Expansion of the submucosa and lamina propria by immune cell infiltration usually occurs simultaneously (Jutfelt, 2011; Rombout et al., 2011; Urán et al., 2009), but the expansion may also be the result of the lamina propria's and stratum granulosum's division by the stratum compactum, about which immune cell exchange is little known. Moreover, it is possible that the observed expansion of the lamina propria is not the product of an increased immune cell infiltration but may also be related to its additional role as circulatory system for nutrients and blood (Olsson, 2011). Overall, adequate dietary Tau and Met left the submucosa unaffected, yet simultaneously not the lamina propria. The reason behind this effect is unclear, and further research is warranted to understand the intestinal immune cell exchange and infiltration.

4.5.4 Posterior intestinal mucus production and composition

Goblet cell mucus is divided into two pH types. Acidic mucus (AB+/blue) is suggested to form a barrier against pathogens, resisting degradation of bacterial glycosidase activity and limiting bacterial translocation by increasing viscoelasticity. Neutral mucus (PAS+/magenta) forms a physical barrier that promotes digestion and neutralizes digestive juices, protecting the lamina epithelial from autodigestion (Deplancke & Gaskins, 2001; Leal et al., 2017; Machado-Neto et al., 2013). The posterior intestine is often exposed to a higher bacterial load and has been shown

to contain more acidic goblet cell mucus that is protective against pathogens (Abd-Elhafez et al., 2015; Deplancke & Gaskins, 2001). However, lipids and proteinogenic amino acids may be unavailable for absorption until reaching the posterior intestine (Krogdahl et al., 2011) and therefore, the posterior intestine may, to a certain extent, have goblet cell mucus that assists with the nutrient assimilation.

YTK from our study had predominantly acidic and mixed posterior intestine goblet cell mucus and fewer neutral goblet cells, indicating that properties of the goblet cell mucus were protecting the system from pathogens. However, an increase of dietary Met, Cys, or Tau decreased the secretion of acid goblet cell mucus and simultaneously stimulated the production of mixed and neutral goblet cell mucus, shifting the properties toward more digestive and absorptive functions. In a previous study, YTK had more neutral goblet cell mucus, which composition was unaffected by soy protein inclusions and water temperature (Bansemer et al., 2015). However, unlike our study, Bansemer's study defined neutral and mixed goblet cells mucus as neutral. The anterior intestine of uninfected brown trout had predominately neutral goblet cell mucus; whereas *Cyathocephalus truncates*-infected brown trout had four times more acid goblet cell mucus (43 per villus^{-1}) than neutral goblet cell mucus (10 per villus^{-1}), which is similar to the YTK from our study exhibiting 2 to 27 times more acid than neutral goblet cell mucus. This highlights the importance of acid goblet cell mucus as an immune response to pathogens in the posterior intestine of fish.

Apart from shifting the pH of the goblet cell mucus composition into the acidic range, the posterior intestine can also temporarily increase mucus secretion upon bacterial recognition

(Sicard et al., 2017), acting as a lubricant and attacking pathogens (Bucke, 1971; Kamphuis et al., 2017; Purushothaman et al., 2016). Yet, it has been suggested that less goblet cell mucus improves nutrient absorption capacity (Machado-Neto et al., 2013). YTK fed the T+M diets had 94 to 133 TGC villus^{-1}, which is similar to the TGC of the anterior intestine of *Pomphorhynchus laevis* infected brown trout (110 TGC villus^{-1})(Bosi et al., 2005). YTK fed the T+M 11 and M+C 2 diet, which nearly met dietary Met, Cys, and Tau requirements (Candebat et al., 2021, 2020), had the least number of total goblet cells indicating that Met, Cys, and Tau complete diets lessen the total goblet cell mucus production and improve absorption capacity. Overall, YTK indicated a shift toward absorptive properties when fed diets that contained adequate dietary Met, Cys, and Tau levels.

4.5.5 Posterior intestinal surface area in YTK - Nutrient absorption

Enterocytes within the lamina epithelial are cells that absorb amino acids, simple peptides, lipids, and monosaccharides via endocytosis, catabolize nutrients, and release them into the circulation (Sundell & Rønnestad, 2011). In YTK, amino acid retention was shown to depend on the water temperature and dissolved oxygen concentrations (Pirozzi et al., 2019). Intestinal characteristics associated with an increase in the absorptive area include enterocyte density, total villus height, villus length, lamina epithelial area, villus density, and intestinal circumference. YTK fed increasing dietary Met and Tau exhibited an increase in absorptive surface area. Further, YTK exhibited a decreasing absorptive surface area when fed Cys at 13.9 g kg^{-1}, marking an upper Cys threshold, which agrees with the suppressed growth and feed efficiency observed in YTK fed the same dietary Cys contents (Candebat et al., 2021). Overall,

YTK fed dietary Cys below 13.9 g kg^{-1} had more villi in the posterior intestine and an increased lamina epithelial area thus larger surface areas for the absorption of nutrients.

YTK had large, transparent supranuclear vacuoles in the cytoplasm of enterocytes. These vacuoles are presumed to have contained lipids that were washed out during histological processing as they did not stain positive for glycogen (PAS+) or protein (eosin). Deficiency in both Tau and Met (T+M diet 1) caused increased vacuolization in the posterior intestine tissue and may be caused by increased lipid absorption or decreased clearance from enterocytes into circulation (Caballero et al., 2003). Similar results have been attributed to lipid-rich diets (Kowalska et al., 2011), deficiencies of some fatty acids (Bou et al., 2017) or diets rich in soybean proteins (Ostaszewska et al., 2005; Urán et al., 2009). Since plasma triglyceride levels were particularly low for fish fed the low Met and low Tau diet, this result indicates that diets low in Tau and Met concurrently decrease the clearance of lipids from intestinal enterocytes and thereby decrease energy available for metabolism. YTK also had S-PAS+ bodies, indicative of glycogen or glycogen-rich accumulations such as lysosomes (Schwake et al., 2013). YTK fed the T+M diets, which contained 23.8% carbohydrate, had substantially more 185 S-PAS+ bodies $villus^{-1}$ in comparison to YTK fed the M+C diets, which contained only 12.3% carbohydrates and induced only half as many S-PAS+. Thus, increased dietary starch may increase dietary glycogen deposition.

4.6 Conclusion

To conclude, dietary Met, Cys and Tau-induced alterations in the liver and posterior intestine suggest shifted functional and structural properties. YTK fed insufficient dietary Met, Cys or Tau exhibited decreased red coloration in their livers, acidic goblet cell mucus with immune responsive properties, reduced absorptive surface area and increased accumulation of lipid supranuclear vacuoles. However, when YTK were fed diets containing Met, Cys, and Tau close to physiological requirements, bile duct walls were relatively thinner, livers appeared redder and brighter, goblet cell mucus shifted toward absorptive properties, the intestinal absorptive surface area increased, and YTK had less supranuclear vacuoles in villi enterocytes. These findings form a coherent overall picture with our previous findings of YTK fed dietary SAA and Tau at suboptimal or supraoptimal levels, exhibiting inferior growth, feed efficiency, protein retention and eye health. This contrasts with YTK fed adequate levels and which subsequently exhibited a relatively superior response. Therefore, hepatic and intestinal histological responses that were statistically different to YTK fed adequate levels of SAA and Tau may reflect nutritionally incomplete diets. With increasing commercial YTK aquaculture production, optimized gastrointestinal and liver function form critical components for good health and productivity. This study has shown that dietary SAA and Tau concentrations alter nutrient absorption and protective properties and provide insight into the macroscopy, histomorphology and histochemistry of the liver and posterior intestine. Nevertheless, further research is needed to investigate the relationships between histology and function, particularly regarding intestinal goblet cell mucus, stratum granulosum and immune responses.

Chapter 5. General discussion

5.1 Introduction

Protein-bound methionine, cysteine and unbound taurine were traditionally supplied to farmed fish in quantities via fishmeal that naturally satisfied the requirements. However, in the last two decades and since the increasing substitution of aquafeed with plant proteins, these three metabolically linked nutrients have gained interest. Plant proteins often lack dietary methionine and are generally free of dietary taurine and thus, if not corrected, pose the risk of inducing nutritional deficiencies (**Table 1.2**). Although the nutritional essentiality of methionine and cysteine in fish was classified as early as the 1987s (Wilson and Halver, 1986), their versatile metabolic and regulatory functions are only slowly being revealed in the last two decades. Taurine, in particular, has gained interest as marine carnivorous fish fed low-fishmeal diets revealed a dietary essentiality for taurine due to the limited ability to synthesize taurine *de novo* from its precursor cysteine (Salze and Davis, 2015). Early reports of taurine deficiency have linked the nutrient as an important component for liver system health, leading to increased cases of green liver syndrome when deficient (Goto et al., 2001; Takagi et al., 2011, 2006a, 2005). Initial trials with suboptimal dietary methionine levels have resulted not only in poorer growth of fish, as methionine is a building block of proteins, but also in bilateral cataracts, underscoring its importance in eye health (Cowey et al., 1992). These deficiencies previously hampered the use of alternative proteins; however, improved amino acid production processes now allow the large-scale supplementation with crystalline and synthetic unbound forms of methionine, cysteine, and taurine (Nunes et al., 2014; Selle et al., 2020). Although the digestibility of unbound

amino acids require further investigations methionine and taurine are already used in aquafeed formulations for target species with established dietary sulfur amino acid and taurine requirements.

It has become increasingly important for the growing production of carnivorous fish species to measure accurate nutritional requirements for taurine and the sulfur amino acids. Thus, this research was motivated by the broad knowledge gaps regarding these requirements in YTK. Nutrient specification for the formulation of YTK aquafeed are not tailored to the species-specific requirements, thereby potentially affecting the large-scale mariculture and profitability of the Australian YTK industry. Research outcomes were designed to address and overcome production bottlenecks by quantifying the requirements for essential nutrients and, thus, provide data toward the formulation of sulfur amino acid and taurine complete aquafeed for YTK.

Prior to this work, there were no published data on the digestible taurine (Tau) and sulfur amino acid (SAA) requirements of YTK. The initial objective of this thesis was to investigate and quantify the dietary requirement for Tau in YTK, for which the ability to endogenously metabolize Tau from its precursor methionine (Met) and cysteine (Cys) was considered limited. This assumption was built on incidences of green liver syndrome in YTK, a pathology also observed in Japanese yellowtail fed low-fishmeal, low Tau diets, and findings on the limited ability of other species from the genus *Seriola* to synthesize Tau *de novo* (D'Antignana and Bubner, 2008; Takagi et al., 2005). Furthermore, Tau has gained renewed interest in recent years largely due to its diverse functions, physiological significance and nutritional essentiality for many carnivorous fish species (Salze and Davis, 2015). Yet, the Tau requirement in YTK

has so far not been assessed, although Salze et al. (2018) present data suggestive of a dietary Tau requirement in the closely related California yellowtail. The data obtained from the first feeding trial clearly indicate that Tau only needs to be exogenously supplied to YTK if the diet is deficient in both Tau and Met. Yet, if dietary Met is supplied at levels above that used by industry, then the dietary Tau deficiency can be fully met by Met and thus, spare the requirement for dietary Tau in YTK. This highlighted that the formulation of YTK aquafeed for SAAs was based on the requirements of other species and motivated the quantification of SAA requirements in YTK in a second feeding trial with particular emphasis on investigating the capacity of Cys to spare dietary Met.

The second feeding trial indicated that the dietary Met level used by the industry to formulate YTK aquafeed was too low to meet the minimum obligatory methionine and total sulfur amino acid levels that are required for optimal growth. In addition to inferior growth responses in YTK fed dietary SAAs below and above the requirement, the SAA deficiency also resulted in bilateral cataracts. The observations that SAAs are vital in the maintenance of ocular health, irrespective of their functional role in protein retention, and that Tau deficiencies may cause green liver syndrome led to our investigation of YTK's nutritional pathological and histological profile forming the third part of this thesis. Histological investigations of the liver and posterior intestine provided data on optimized gastrointestinal and liver function, which forms critical components for optimal health and productivity. Dietary SAAs and Tau have been shown to alter nutrient absorption and protective properties, thus structural measurements provide insights into the hepatic and intestinal macroscopy, blood plasma biochemistry, histomorphology and histochemistry of YTK.

The major findings of this study and their potential applications are outlined below and, while acknowledging potential overlap, are described within four broad themes:

1. YTK Tau requirements
2. YTK SAA requirements
3. YTK's capacity for metabolic bioconversion of the SAAs and Tau
4. The significance of suboptimal, optimal and supraoptimal levels of SAAs and Tau on YTK histology and ultimate health.

Research outcomes from this Ph.D. contribute towards overcoming production barriers by implementing improved feed formulations for the emerging large-scale production of YTK, engaging and challenging current academic understanding of amino acid requirements, and building on the knowledgebase around concepts of utilization and metabolism in high protein demanding fish species of commercial interest.

5.2 Overview of major findings

Since commercial aquafeed for YTK have not necessarily been tailored to meet species-specific requirements, a series of feeding trials were conducted that provide quantitative recommendations for optimizing YTK aquafeed formulations with respect to dietary SAAs and Tau. YTK's SAA and Tau requirements were quantified by evaluating dose-response curves that derived from a series of regression models filtered and compared for best fit using R^2, sum of squares, AICc, and Sy.x. The studies examined the effect of dietary Met, Cys, and Tau on the growth, feed efficiency and other physiological variables to assess requirements.

5.2.1 Chapter 2: Dietary methionine spares the requirement for taurine in juvenile yellowtail kingfish (*Seriola lalandi*)

- Tau supplementation is conditionally essential in YTK aquafeed
- Adequate dietary Met spares the Tau requirement in low Tau diets for YTK
- Inadequate dietary Met intake provokes a dietary Tau requirement in YTK
- YTK aquafeed containing ~10.9 g Met kg^{-1} should provide at least 7.7 g Tau kg^{-1} to optimize growth
- The industry standard for dietary Met for YTK aquafeed may require reassessment if growth is to be optimized.

5.2.1.1 Implications for industry and fish nutrition research:

Juvenile YTK require a daily digestible Tau intake of 0.16 g $kgBW^{-1}$ d^{-1} at an average daily digestible Met intake of 0.25 g $kgBW^{-1}$ d^{-1}. Therefore, YTK aquafeed should contain at least 7.7 g kg^{-1} dietary Tau if the feed only contains 10.9 g Met kg^{-1}. Additionally, dietary Met at 10.9 g kg^{-1} is not sufficient to meet requirements for the *de novo* synthesis of Tau. Juvenile YTK showed improved performance when fed a 17.2 g Met kg^{-1} diet than when fed the standard inclusion of 10.9 g kg^{-1}, indicating that current industry practice is likely to be inadequate. However, further research is required to precisely quantify the dietary Met requirement of YTK by measuring the optimum response at given methionine contents in the context of low and high dietary Cys levels. This may provide information on the total sulfur amino acid and minimum obligatory methionine requirements.

In considering these data, our findings shift Tau's classification from "essential" in YTK aquafeed to "conditionally essential" and allows for the inclusion of ingredients in YTK aquafeed that were previously considered unsuitable due to a lack of Tau. However, the direct supplementation of dietary Tau might potentially be required to improve performance or ameliorate pathological conditions because this study determined Tau conversion indirectly via dietary Met. In so doing, this study did not consider the energetic cost of *de novo* synthesis of Tau and the fact that excess Met was found toxic in some fish species (Tacon & FAO, 1992). The results from this chapter stand in contrast to those found in Japanese yellowtail, California yellowtail, and in other carnivorous species, which demonstrate a dietary Tau requirement due to a limited ability to synthesize Tau *de novo* (Salze et al., 2018; Takagi et al., 2008). However, for both species, experiments were not designed to account for interactions between Tau and its metabolic precursors Met and Cys. The Tau requirements were assessed at constant levels of Met and Cys in low-fishmeal diets. If Met and Cys were not supplied at adequate levels to meet the SAA requirements, a Tau requirement might have been induced. This highlights the importance of understanding the metabolic relationship between nutrients and that the recommended requirement must always be interpreted in the context of amino acids whose essentiality is undisputed such as Met. Additionally, the results of this research clearly demonstrate the need to determine essential nutrient requirements for each species before investigating metabolite requirements when formulating experimental diets and aquafeeds. Our findings suggest that Tau may have a wide variety of physiological functions ranging from maintaining hepatic homeostasis to serving as an osmolyte and that this end-product of Met's transsulfuration appears to be of conditional essentiality in aquafeeds for juvenile YTK.

5.2.2 Chapter 3: The sulfur amino acid requirements of juvenile yellowtail kingfish (*Seriola lalandi*)

- The total sulfur amino acid requirement was met at an average of 24.5 g kg^{-1} (18.9 g Met kg^{-1} + 5.6 g Cys kg^{-1})
- The minimum obligatory Met requirement was met at an average of 14.3 g Met kg^{-1}
- 40.4–49.2% of the total sulfur amino requirement can be met by dietary Cys
- Relatively low dietary Met (8.8 g kg^{-1}) and Cys (5.8 g kg^{-1}) induced bilateral cataracts
- YTK exhibited a lower and upper tolerable limit of dietary Met and Cys; levels below and above the total sulfur amino acid requirement (23.4–26.1 g kg^{-1}) induced inferior feed conversion ratio, specific growth rate, and protein retention efficiency
- The current Met specification for the formulation of YTK aquafeed is below the average minimum obligatory Met and total sulfur amino acid requirement.

5.2.2.1 Implications for industry and fish nutrition research:

Juvenile YTK require a daily digestible SAA intake of at least 0.70 g $kgBW^{-1}$ d^{-1} (0.56 g Met $kgBW^{-1}$ d^{-1}+ 0.14 g Cys $kgBW^{-1}$ d^{-1}). Therefore, YTK aquafeed should contain at least 24.5 g kg^{-1} of dietary SAA, of which 77% should be provided by dietary Met and 23% by dietary Cys to satisfy the total sulfur amino and minimum obligatory Met requirements at their respective digestibilities. It is not recommended to include dietary Cys at or above 13.9 g kg^{-1}, as such levels may depress growth. In addition, bilateral cataract formation was mediated by a Met and Cys deficiency in YTK; however, sufficient dietary Cys prevented such effects.

In conclusion, YTK's requirements for SAAs appear to be quite narrow, and deviations from the requirements can quickly lead to inferior performance and alterations of internal microscopic structures and properties in YTK. Findings that dietary SAAs above the requirement can quickly lead to the accumulation of noxious agents were made across different finfish and mammalian species (Osman et al., 1997; Yokoyama and Nakazoe, 1996). Dietary SAA level above 17.0 g Met kg^{-1} at 3.1 g Cys kg^{-1} and 11.9 g Met kg^{-1} at 6.7 g Cys kg^{-1} induced inferior specific growth rates in black sea bream (*Sparus macrocephalus*) and in juvenile Cobia (Rachycentron canadum), respectively.

The SAA requirements in YTK were previously unknown. Thus, aquafeed formulations for YTK were based on the Met requirement of Japanese yellowtail as determined by Ruchimat et al. (1997) using a broken-line regression analysis. A broken-line regression analysis assumes that the responses to the nutrient of interest are linear. An assumption of linearity may not be accurate because the data often tend to be curvilinear, leading to the underestimation of nutritional requirements (Robbins et al., 2006). Thus, it is questionable if the estimates provided by Ruchimat et al. (1997) are sufficient to meet the Met requirement in Japanese yellowtail, much less in YTK.

In tandem with the recent renewed interest in Tau, there has been a growing interest in Tau's precursors due to their metabolic involvement in protein retention, inflammation, and oxidative processes. Met and Cys together constitute the requirement for total sulfur amino acids in aquafeeds, yet their capacity to interact metabolically within a species (e.g., Cys's sparing capacity, SAA toxicity, and the minimum obligatory methionine requirement) has been largely overlooked in fish nutrition. Estimates on Cys's sparing capacity, SAA toxicity, and the

minimum obligatory methionine requirement are of importance due to the increase of nutrient-based aquafeed formulations in which nutrient imbalances are adjusted through crystalline amino acids supplementation.

Although the methods to assess the SAAs requirements are well established in livestock and human nutrition, in fish nutrition estimates are inconsistent in their methodological approach. Thus, this chapter provides clear and systematic information on the formulation of experimental diets to quantify accurate SAA requirements, which include the minimum obligatory methionine requirement, total sulfur amino acid requirement, and calculations on Cys's sparing capacity. It is important to emphasize that Cys's sparing capacity of 40.4–49.2% of the total sulfur amino requirement was measured indirectly by calculation. This method assumes that the proportion of dietary Cys that can spare Met in the total sulfur amino acid requirement is similar to the difference between the total sulfur amino acid and the obligatory minimum requirement (Ball et al., 2006). This is a highly theoretical approach that may have limited applicability to practical feed formulation as it is only an approximate estimate. A feeding trial using graded contents of Cys at a constant Met content which can meet the minimum obligatory Met requirement, could provide more detailed and practical findings.

5.2.3 Chapter 4: Nutritional relevance of adequate methionine, cysteine, and taurine for hepatic, intestinal, and circulatory conditions and functions in juvenile yellowtail kingfish (*Seriola lalandi*)

- Dietary SAA and Tau-induced alterations in liver and posterior intestine suggest shifted functional and structural properties
- YTK fed adequate dietary Met, Cys and Tau exhibited thinner bile duct walls, redder livers, globally reduced, yet more absorptive goblet cell mucus, more absorptive surface area, and fewer lipid supranuclear vacuoles.
- Dietary Cys above 13.9 g kg^{-1} led to a reduction in absorptive surface area of the YTK posterior intestine
- YTK did not exhibit green liver syndrome, even under Met and Tau deficiency
- Increased hepatic fat correlated with a yellowing of the liver surface color
- The industry standard for dietary SAA for YTK aquafeeds requires adjusting to optimize hepatic and intestinal performance.

5.2.3.1 Implications for industry and fish nutrition research:

For optimized gastrointestinal and liver functions and to maintain good health and productivity in YTK, it is important that feed for YTK contain adequate amounts of dietary Met, Cys, and Tau. Further, an increased incidence of supranuclear vacuoles might not always be indicative of well-fed fish or a lipid-rich diet but could also indicate a deficiency of dietary Tau or Met.

Met, Cys, and Tau have a variety of metabolic functions, which are the focus of current research. Therefore, it is not unexpected that they are involved in the regulation of metabolic and physiological homeostasis in fish. However, the extent of these effects and their underlying mechanisms are still largely unknown. The experimental diets that were fed to YTK in this project were low in fishmeal and contained increased proportions of ingredients from plant and terrestrial livestock origin. The general consensus is that some plant proteins can potentially cause pathology in carnivorous fish due to their high concentrations of noxious agents such as antinutrients and fiber. Thus, hepatic and intestinal histology and appearance may not be that of wild YTK, yet measurements are representative of farmed carnivorous fish that were fed mixed proteins from plants, livestock, and fishmeal. In addition, new observations were made on the submucosa of the intestinal wall, indicating a subdivision into a stratum compactum and a stratum granulosum. Further, YTK had bullet-shaped structures within villi enterocytes that stained positive to Periodic acid-Schiff and correlated with the carbohydrate content in the diet. However, the exact function and significance of these structures remains unknown, and further investigations are recommended.

5.3 General considerations

The findings and data presented in this thesis contribute toward understanding the importance of SAAs and Tau inclusion and interaction in YTK nutrition. In addition, the data presented provide preliminary insights into the effects of SAAs and Tau on YTK health and physiological function. Many functions and effects are in line with those found in mammals. However, there are several factors that must be considered when reviewing the material, methods, and data of this thesis and which may ultimately limit their practical application.

- This thesis quantified the SAA and Tau requirements on a digestible basis which will facilitate the formulation of feeds, utilizing other proteins of known digestibility. Nevertheless, the experimental diets included 12.5 to 20% fishmeal, whereas the industry is aiming to develop and establish zero-fishmeal diets that contain larger amounts of proteins from plant and terrestrial livestock. High levels of plant proteins can sometimes adversely affect the utilization and intake of proteins, growth efficiency, and health of fish (Fournier et al., 2003; Kaushik et al., 1995). In this study, YTK were fed proteins predominately from plant (35–36%) and livestock (30–33%) origin. This indicates that there may be some flexibility in diet formulations while achieving appropriate SAA and taurine specifications for YTK aquafeed using alternative proteins.

- Requirements were assessed on growth, feed efficiency, and protein retention of YTK. Intestinal, hepatic, circulatory, and ocular physiology were used as supplementary indicators to determine whether established requirements are satisfactory for the maintenance and health of internal organs in YTK. It may be possible that the requirement for growth may have been lower relative to the metabolic requirement to maintain a healthy system, as previously observed in salmon for histidine. Salmon require 8 g histidine kg^{-1} diet for growth, whereas 14.4 g histidine kg^{-1} diet is required to reduce the risk of cataract (Remo et al., 2014).

- Recommendations and estimates on SAA and Tau requirements from this research highlight the physical and physiological mechanisms that are relevant for the size and culture

conditions undertaken in this study. However, life-stage, genetics, environmental factors, and stress can alter the metabolism toward increased or decreased requirements for methionine, cysteine, and taurine.

- While histological deviations from the norm were detected in this study, the length of the feeding trials of seven to eight weeks may not have been sufficient for YTK to develop histological conditions that can occur over the whole production cycle (larval to broodstock) or time to harvest. Thus, this study captured the metabolic and physiological requirements of juvenile YTK (initial weight 53 g) whose weight at least tripled over the duration of the experiments, ensuring sufficient tissue turnover and measurable effects of sulfur amino acids and taurine on the histology and pathology.

5.4 Future directions

Fish nutrition is evolving toward fishmeal and fish oil free diets, formulated based on nutrient contents that are expected to sufficiently match the nutrient requirements of the target species. However, the suite of essential amino acid requirements and specifications for most farmed fish species are still yet to be determined. As seen in YTK from this thesis, an imbalance of dietary sulfur amino acids can lead to serious health problems (e.g., cataract) or directly lead to growth depression. It should therefore be of primary interest to industry and academia to continue the conduct of requirement studies, particularly on nutrients known to be essential for fish. Furthermore, key nutrients such as methionine and cysteine have been shown to be base units and metabolic anchor points for the production of several other nutrients, where deficiencies not

only limit the production of the derivates but also regulate enzyme activities and genes involved in other macronutrient metabolism e.g. dietary methionine can affect the lipid metabolism and shift amino acid flux (Skiba-Cassy et al., 2016). This thesis has shown that it is imperative in the future to classify amino acid significance in feed, metabolism, and physiological maintenance. Thus, experimental designs should focus on understanding the holistic metabolic web of amino acid functions, which often include the regulation and shifting of metabolic bioconversions from one substrate to another. A good example is cysteine, which may only be a conditionally essential amino acid, yet it's metabolic connection with methionine enables the dietary sparing of methionine to meet the total sulfur amino acid requirement. Similar relationships are observed in other amino acid groups, e.g., between the nonessential amino acid tyrosine, which can spare the dietary requirement of the essential amino acid phenylalanine. Investigative tools, including nutrigenomics and metabolomics, may assist in developing a more detailed understanding of the largely unknown amino acid fluxes in fish. Further research will also assist in determining how amino acid availability affects the final partitioning of amino acids for protein synthesis, oxidation and substrate catabolism to produce metabolites. The continued use of fishmeal in special feeds for YTK broodstock and fingerlings highlight the importance of establishing the SAA and Tau requirements at different life-stages of YTK. Investigations on other life-stages than the grow-out may be difficult and expensive to conduct yet are necessary to ensure the formulation of sustainable aquafeeds for YTK. Moreover, it is important to continue to measure relevant biological responses additional or supplemental to growth and survival, as the requirements for growth can differ from those for optimal immune response, organ function and physiological homeostasis. Additionally, investigations examining the interactions between fish nutrition and the environment may contribute towards the sustainable and efficient growth of

the aquaculture industry as carnivorous fish in mariculture systems are often exposed to extreme changes in abiotic factors, especially at times of climate change.

5.5 Conclusion

This thesis studied the SAA and Tau requirements in YTK for optimized performance and health. This was achieved by conducting feeding trials that estimated the sulfur amino acid and taurine requirements with an emphasis on metabolic interactions and effects on the species' health. The findings of this thesis can be used by the aquaculture industry to optimize feed formulations and provide further information to help improve our understanding of SAA requirements and knowledge on SAA metabolites in carnivorous fish that are of commercial interest. Further, this thesis presents a clear and systematic quantification of SAA requirements for YTK.

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Appendices

Appendix A Diet formulation of the TauMet experimental diets (Candebat et al., 2020).

Ingredient (%)	Low methionine series							High methionine series						
	T+M 1	T+M 2	T+M 3	T+M 4	T+M 5	T+M 6	T+M 7	T+M 8	T+M 9	T+M 10	T+M 11	T+M 12	T+M 13	T+M 14
Wheat flour	17.9	16.2	15.4	15.4	15.4	15.4	15.4	15.4	15.4	15.4	15.4	15.4	15.4	15.4
Poultry meal	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0
Fish oil	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0
Dehulled lupin	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Fishmeal Prime	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Fishmeal by-product	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Meat meal	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Corn gluten	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Blood meal	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Diatomaceous earth	1.7	3.1	3.56	3.23	2.91	2.59	2.27	3.29	2.97	2.65	2.32	2	1.68	1.36
NaH_2PO_4	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vit/min premix	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Rovimix Stay-C (35)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Choline chloride (70%)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Methionine	0.2	0.2	0.2	0.2	0.2	0.2	0.2	1.11	1.11	1.11	1.11	1.11	1.11	1.11
Taurine	0.0	0.32	0.64	0.97	1.29	1.61	1.93	0.0	0.32	0.64	0.97	1.29	1.61	1.93
Y_2O_3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Appendix B Compositional analysis of the TauMet experimental diets (g kg^{-1} dry matter basis, unless indicated otherwise; Candebat et al., 2020).

	Low methionine series							High methionine series						
	T+M 1	T+M 2	T+M 3	T+M 4	T+M 5	T+M 6	T+M 7	T+M 8	T+M 9	T+M 10	T+M 11	T+M 12	T+M 13	T+M 14
Proximate composition														
Ash	117.0	115.8	119.3	118.6	117.4	116.2	115.4	116.1	116.6	117.2	114.9	113.5	111.0	110.0
Total lipid	144.5	149.0	158.4	154.1	152.8	151.5	157.7	178.3	161.3	155.8	160.6	139.9	144.6	154.3
Total protein	485.9	494.3	498.3	485.4	500.4	488.7	495.1	482.7	494.5	487.5	493.1	503.1	488.4	484.4
GE (MJ kg^{-1})	21.9	22.0	22.2	22.2	22.1	21.9	22.9	21.8	22.0	22.1	22.3	22.2	22.2	22.7
Amino Acid composition														
Ala	30.0	29.3	29.3	28.0	28.3	28.9	28.0	29.4	28.3	29.0	27.6	27.9	28.6	30.2
Arg	56.5	54.0	56.4	52.2	51.9	52.2	51.3	53.0	52.1	52.7	51.8	50.6	51.7	53.9
Asx	43.4	42.1	43.2	40.6	40.2	40.8	40.1	41.6	40.6	40.6	39.0	38.8	40.1	40.3
Cys	6.8	6.1	5.6	5.4	5.6	5.8	5.7	5.9	5.4	5.5	5.2	5.6	5.6	5.8
Glx	70.5	67.1	68.0	64.5	62.6	64.0	62.4	64.2	62.4	61.5	60.0	59.7	61.4	61.9
Gly	27.6	27.2	27.1	25.7	26.4	26.2	26.4	30.1	26.1	27.8	26.0	25.6	26.7	28.4
His	14.6	18.2	15.6	14.3	16.1	13.8	15.0	14.1	13.6	13.2	14.0	12.5	13.9	12.2
Ile	17.0	16.1	16.5	15.9	16.2	16.5	16.4	16.4	16.2	16.3	15.8	15.6	16.0	16.5
Leu	42.9	41.4	42.2	40.9	41.4	42.7	42.1	42.3	41.0	41.8	40.3	40.0	41.1	42.1
Lys	29.2	26.6	30.2	27.0	26.6	26.0	27.6	26.9	27.0	24.2	25.4	23.3	23.6	23.4
Met	12.0	10.5	11.1	10.8	10.9	10.1	11.1	16.7	19.3	16.4	18.8	16.0	17.9	15.0
Phe	23.8	23.4	23.3	23.2	23.5	24.2	24.0	24.6	23.6	24.7	23.0	23.0	23.8	24.2
Pro	25.9	25.2	24.9	23.9	24.0	24.5	24.4	25.4	24.1	24.0	23.5	23.2	24.1	24.3
Ser	25.0	25.2	25.1	22.9	23.9	22.8	23.1	23.4	22.6	25.5	23.2	22.5	23.5	28.4
Tau	1.6	4.8	8.5	11.9	15.0	17.3	20.4	1.6	5.1	8.1	11.7	13.9	18.3	20.0
Thr	20.7	20.0	20.6	19.4	19.4	19.5	19.0	19.9	19.3	19.9	18.7	18.7	19.2	20.4
Tyr	14.5	15.1	16.1	14.4	15.8	15.0	14.5	14.3	14.9	15.2	14.8	14.5	15.0	15.2
Val	26.6	25.8	26.3	25.5	26.0	26.8	26.3	26.8	25.9	26.8	26.1	25.9	26.6	27.3
SUM AA	488.6	477.9	490.0	466.8	473.9	477.1	477.7	476.6	467.4	473.2	464.8	457.3	477.1	489.6

Appendix C Diet formulations of the MetCys study. Please note that M+C 1 refers to Diet 1, M+C 2 refers to Diet 5, M+C 3 refers to Diet 6, M+C 4 refers to Diet 10 (Candebat et al., 2021).

Ingredient (%)	Low cysteine series		High cysteine series	
	M+C 1	M+C 2	M+C 3	M+C 4
Dehulled Lupins	19.91	19.91	19.91	19.91
Soy protein concentrate	15.20	15.20	15.14	15.20
Fishmeal	12.60	12.97	12.97	12.97
Gelatine	14.33	12.73	13.45	11.86
Sodium Caseinate	9.78	10.10	9.78	10.10
Fish Oil	10.00	10.00	10.00	10.00
Blood Meal	5.00	5.00	5.00	5.00
Diatom. Earth	5.17	5.13	4.40	4.54
Feather Meal	2.87	2.85	3.00	3.20
Maize starch	2.50	1.33	2.60	1.33
NaH2PO4	0.50	0.50	0.50	0.50
Vit-min premix	0.50	0.50	0.50	0.50
Choline Chloride (70%)	0.30	0.30	0.30	0.30
Rovimix Stay-C	0.30	0.30	0.30	0.30
Lysine	0.29	0.29	0.29	0.29
Methionine	0.00	2.14	0.00	2.15
Cysteine	0.00	0.00	1.11	1.10
Taurine	0.65	0.65	0.65	0.65
Y_2O_3	0.10	0.10	0.10	0.10

Appendix D Compositional analysis of the diets from the MetCys study (g kg^{-1} dry matter basis, unless indicated otherwise). Please note that M+C 1 refers to Diet 1, M+C 2 refers to Diet 5, M+C 3 refers to Diet 6, M+C 4 refers to Diet 10 (Candebat et al., 2021).

	Commercial diet	Low cysteine series		High cysteine series	
		M+C 1	M+C 2	M+C 3	M+C 4
Proximate composition					
Ash	40	107.3	108.2	100	104.3
Total lipid	140	135.8	135.2	130.2	138.1
Total protein	500	638.8	619.5	637.9	651.1
GE (MJ/Kg)	NA	21.8	22.2	22.4	22.4
Amino acid composition					
Ala	27.9	30.6	28.7	29	28
Arg	26.8	39	37.4	37.5	36.6
Asp	39.8	44.7	42.2	42.1	41.4
Cys	6.5	5.8	5.9	13.7	13.9
Glu	84.6	84.4	80.6	80.3	79
Gly	24.8	53.1	48.2	50.2	47.2
His	17.4	13.1	13.1	12.6	12.6
Hyp	NA	18.5	16	17.3	15.7
Ile	20.8	21.8	21.8	21	21.1
Leu	44.8	39.8	39.5	38.4	38.3
Lys	31.1	33.9	32.9	32.6	32.2
Met	12.6	8.8	24.7	7.9	25.2
Phe	24.6	23	22.8	22.2	22.1
Pro	29.9	45.8	43.1	43.8	42.1
Ser	22.2	26.9	26	26.2	25.6
Tau	7.50	7.0	6.9	7.0	6.9
Thr	20.4	20.2	19.8	19.6	19.3
Try	NA	4.9	4.7	5.1	4.4
Tyr	14.5	15.2	15	14.5	14.7
Val	27.0	26.4	26.3	25.4	25.5
Sum AA	477.2	562.9	555.6	546.4	551.8

Appendix E Staining protocols of yellowtail kingfish liver and posterior intestine collected from chapter 2 and 3 for chapter 4.

Protocol 1: A combination of Alcian blue-Periodic acid-Schiff stain (AB-PAS) was applied to demonstrate nuclei, acid (blue) and neutral (neutral) mucus in specimen in the posterior intestine of yellowtail kingfish.

Reagents were as followed:

(1) Alcian Blue solution (0.5% Alcian Blue 8GX in 3% acetic acid)
(2) 1% Periodic acid
(3) Schiff's reagent
(4) Mayer's Haematoxylin
(5) Scott's tap water substitute

Staining steps:

1.	Xylene bath	2 min
2.	Xylene bath	2 min
3.	Ethanol bath	2 min
4.	Ethanol bath	1 min
5.	Ethanol bath	1 min
6.	Water wash	1 min
7.	Apply Alcian blue (pH 2.5)	20 min
8.	Running tap water bath	5 min
9.	Rinse in distilled water	
10.	Apply period acid	2 min
11.	Running tap water bath	5 min
12.	Remove excess water	
13.	Apply Schiff's reagent	8 min
14.	Running tap water bath	5 min
15.	Mayer's haematoxylin batch	30 sec
16.	Thorough rinse in water	
17.	Apply Scott's tap water substitute	30 sec
18.	Thorough rinse in water	
19.	Ethanol bath	10 dips
20.	Ethanol bath	1 min
21.	Xylene bath	2 min
22.	Xylene bath	1 min
23.	Keep in xylene bath until cover slipped	

Protocol 2: H&E demonstrate nuclei in dark blue, and cytoplasm, connective tissue muscles, and red blood cells in various shades in liver of yellowtail kingfish.

Reagents were as followed:

(1) Mayer's Haematoxylin
(2) Young's Eosin
(3) Scott's tap water substitute

Staining steps:

1.	Xylene bath	2 min
2.	Xylene bath	2 min
3.	Ethanol bath	2 min
4.	Ethanol bath	1 min
5.	Ethanol bath	1 min
6.	Water wash	1 min
7.	Mayer's Haematoxylin	8 min
8.	Water bath	30 sec
9.	Scotts's Tap water Substitute	30 sec
10.	Water wash	2 min
11.	Young's Eosin	4 min
12.	Water wash until red-purple	~20 sec
13.	Ethanol bath	10 dips
14.	Ethanol bath	1 min
15.	Xylene bath	2 min
16.	Xylene bath	1 min
17.	Keep in xylene bath until cover slipped	

Protocol 3: Toluidine Blue to demonstrate metachromatic substances including mast cells (reddish-purple) in posterior intestine of yellowtail kingfish (was not successful in YTK-either fixation or staining requires adjustment).

Reagents were as followed:

(1) 0.2% Toluidine Blue O in 60% ethanol

Staining steps:

1.	Xylene bath	2 min
2.	Xylene bath	2 min
3.	Ethanol bath	2 min
4.	Ethanol bath	1 min
5.	Ethanol bath	1 min
6.	Water wash	1 min
7.	Apply Toluidine blue	1 min
8.	Water bath	1 min
9.	Ethanol bath	10 dips
10.	Ethanol bath	1 min
11.	Xylene bath	2 min
12.	Xylene bath	1 min
13.	Keep in xylene bath until cover slipped	

Appendix F A single case of green discoloration of liver in a yellowtail kingfish fed a choline deficient diet and held at 16°C (water temperature). Courtesy of Angela Liu (Liu et al., 2021).