Gut structure and diet adaptability of yellowbelly flounder: A step towards integrating *Rhombosolea leporina* into New Zealand aquaculture

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Abstract

Aquaculture is a rapidly expanding industry as wild fish stocks decline due to overfishing, climate change, and increased seafood consumption. Flatfish, being highly prized, have faced significant declines in the wild, but several species are now being cultivated in land-based recirculating aquaculture systems to meet market demand. Flatfish are low-trophic feeders and thrive in relatively simple farming setups, making them viable candidates for sustainable aquaculture.

In New Zealand, the yellowbelly flounder (*Rhombosolea leporina)*, or pātiki, is a sought-after species in commercial and recreational fisheries. Introducing this species into aquaculture could support the local seafood market and aid in replenishing wild stocks. However, limited research exists on yellowbelly flounder compared to other flatfish, particularly regarding its digestive system and dietary needs. Feed costs are a significant challenge in aquaculture, especially when striving to reduce reliance on unsustainable ingredients like fishmeal and oil while maintaining low feed conversion ratios. Understanding the digestive tract structure and function of the yellowbelly flounder is critical for developing sustainable, species-specific diets.

This study examined the gut structure and function of wild-caught adult and juvenile yellowbelly flounder, as well as juveniles fed a mussel-based diet, a fishmeal-based pellet diet, or subjected to starvation. The histological analysis examined the muscularis externa, mucosal folds, and goblet cell distribution throughout the alimentary canal, while qPCR assessed the expression of the cholecystokinin hormone paralogs (*ccka* and *cckb*).

Unexpectedly, findings suggested that yellowbelly flounder may be agastric, differing from other flatfish. Consistent gut tissue patterns, goblet cell presence, and *ccka* and *cckb* expression throughout the canal supported this conclusion. The pellet diet did cause higher *ccka* expression compared to the mussel diet, but overall, the results suggested the yellowbelly flounder is adaptable to varying feeds. While limited by a small-scale study, these results provided a basis for further exploration of yellowbelly flounder digestive capabilities in hopes of facilitating this species into the New Zealand aquaculture industry.

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Now to begin a new chapter.

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Chapter 1. General Introduction

1.1 Flatfish aquaculture

Seafood remains an important component of the human diet due to its high protein content, rich supply of polyunsaturated fatty acids, and dense nutrient profile (Hosomi et al., 2012; Johnson, 2010). As of 2024, the global fish and seafood market is valued at approximately $676 billion USD, with continued growth projected (Statista, 2024). However, rising seafood demand, combined with the impacts of overfishing, has significantly strained wild fish stocks. In response, aquaculture production surpassed wild-caught seafood for human consumption in 2013 (Ritchie, 2019; WWF, 2018). That year, wild capture fisheries produced 90.87 million tonnes of seafood, while aquaculture generated 4.96 million tonnes more and that gap has continued to widen over time (World Bank, 2024).

Flatfish are highly appreciated as a seafood product as they are low in fat, have a versatile flavour and good nutritional qualities associated from their diets (Cerdà & Manchado, 2013). Unfortunately, this high demand has led to a decline in wild flatfish populations (Cerdà & Manchado, 2013). Additionally, climate change has accelerated this decline, prompting initiatives to farm flatfish as a sustainable alternative to alleviate the pressure on wild stocks (Cheung & Oyinlola, 2018). However, aquaculture presents significant challenges, especially regarding the cost of aquafeeds, which can constitute 50-80 percent of production expenses (Hasan & Soto, 2019). Therefore, optimizing feed sustainability and ensuring effective feed conversion ratios through high-quality feeds and appropriate farming practices are essential. Key species targeted for commercial production include the olive flounder (*Paralichthys olivaceus*), European turbot *(Scophthalmus maximus*)*,* Atlantic halibut (*Hippoglossus hippoglossus*), and common sole (*Solea solea*) (Cheung & Oyinlola, 2018). Global flatfish production is dominated by Southeast Asia with China and Korea contributing to over 90% of total global production in 2015 (Cheung & Oyinlola, 2018).

1.1.1 Flatfish ecology

Flatfish are bottom-dwelling species that prefer sandy-muddy topography and feed on organisms near the seabed. This feeding behaviour begins after their dramatic transformation during early metamorphosis, when they shift from a pelagic to a benthic lifestyle (Robledo et al., 2017). They are characterised by their asymmetrical body shape and the migration of both eyes to either the right or left dorsal side during the early stages of metamorphosis (Schreiber, 2013).

Flatfish play an important role in the benthic community due to their diverse diets. Some species primarily consume benthic polychaetes, crabs, and molluscs, while others target larger prey such as fish, squid, and crustaceans (Link et al., 2015). Their feeding strategies can be categorised into three main types: full visual feeders, predominantly visual feeders, and nonvisual feeders. Full visual feeders hunt moving or swimming prey, predominantly visual feeders focus on slow-moving benthic prey, and nonvisual nocturnal feeders consume sedentary benthic prey (Gibson et al., 2014). These feeding strategies are closely linked to the structure and function of the flatfish digestive tract.

1.1.2 Flatfish culture methods

Flatfish are cultured in both sea-based and land-based facilities, with the choice of method depending on local environmental conditions and the specific requirements of the species. Sea-based farming is only feasible when ambient conditions, such as temperature, remain within a suitable range for the species (Brown, 2002). However, the benthic nature of flatfish poses challenges in sea-based farming, as temperature fluctuations can significantly impact growth. Flatfish are more sensitive to environmental changes, which can result in extended grow-out periods, compared to pelagic or benthic fish with greater mobility, (Cheung & Oyinlola, 2018; Stieglitz et al., 2021).

Recently, there has been a shift toward land-based farming methods, particularly using recirculating aquaculture systems (RAS), which are known for their efficient water use and high productivity (Brown, 2002; Guan et al., 2018). Olive flounder in particular are increasingly cultured in land-based RAS facilities, where environmental parameters such as temperature can be tightly controlled. This is particularly important during early life stages where environmental fluctuations have a greater impact (Daniels & Watanabe, 2010). Systems such as RAS also allow for higher stocking densities, depending on the species. For example, olive flounder are naturally docile and tolerant of crowding, making them well suited for larger densities. In contrast, winter flounder (*Pseudopleuronectes americanus*) exhibits more aggressive behaviours, which can limit stocking density (Daniels & Watanabe, 2010). Both European turbot and olive flounder have successfully been cultured using stacked RAS systems, which further maximises stocking density and production efficiency (Daniels & Watanabe, 2010; Stieglitz et al., 2021). The ability to maintain consistent environmental conditions, particularly temperature, is critical for growth and spawning in flatfish. As such, land-based systems offer a more controlled environment, leading to consistent production, improved feed conversion ratios, and high-quality products (Guan et al., 2018; Stieglitz et al., 2021).

1.2 New Zealand aquaculture and new candidate species

The New Zealand (NZ) aquaculture industry is primarily focused on the production of three key species; chinook salmon (*Oncorhynchus tshawytscha*), pacific oysters (*Crassostrea gigas*) and green-lipped mussel (*Perna canaliculus*) (Stenton-Dozey et al., 2020). These species combined brought a recorded revenue of $685 million NZD in 2022 (Fisheries NZ, 2023). For aquaculture to be profitable, cost-effective production and reliable, in-demand species that thrive under aquaculture conditions are necessary (Camara & Symonds, 2014). Therefore, there is potential in NZ for introducing a new species into the commercial aquaculture sector, to help diversify the industry and provide to the demanding market.

Despite eight flatfish species being included in New Zealand's commercial quota system, none are currently farmed domestically. In 2019, the commercial value of flatfish reached 96.6 million NZD, highlighting their economic importance (Stats NZ, 2020). However, the domestic market remains entirely reliant on wild-caught stocks, which are subject to natural population fluctuations and variable recruitment success (Open Seas NZ, 2024). Four of these species are flounder belonging to the family Rhombosoleidae; of these, three species, sand (*Rhombosolea plebeia*), black (*Rhombosolea retiaria*) and yellowbelly (*Rhombosolea leporina*) are endemic to NZ. The greenback flounder (*Rhombosolea tapirina*) is also found in Australia and has been considered as a potential aquaculture target in Southern Australia (Hart & Purser, 1996; Pankhurst & Fitzgibbon, 2006). In addition to commercial capture, yellowbelly flounder are commonly targeted in recreational and customary Māori fisheries. Pātiki (flounder) is considered a taonga species, meaning it holds significant cultural value to Māori, it has been reported however, that Māori communities have noticed a decline in abundance over time (NIWA, 2017).

Yellowbelly flounder is a desirable aquaculture candidate due to its desirable taste, favourable growth rate and market value (approximately NZD $25 - 35 / kg). They can typically be found in shallow, muddy estuarine environments along much of the NZ coast, with the highest concentration having been in the Hauraki Gulf region (Colman, 1972). They feed relatively low on the food chain sourcing a diet of polychaetes and molluscs on the benthic terrain (De Groot, 1971). This feeding ecology indicates the possibility for the successful use of alternative ingredients for improved diet sustainability. Despite a relatively short lifespan of three to four years, these fish can reach harvestable size within approximately two years (Colman, 1974; Fisheries NZ, 2018). Like other flatfish such as the olive flounder, yellowbelly may adapt well to raceway systems (Stieglitz et al., 2021). Consequentially, opportunities may exist to culturing this species in NZ to replenish wild stocks and support the commercial market demands for yellowbelly flounder. Key research avenues for developing a new aquaculture species typically focus on understanding reproduction/larval rearing and diet/nutrition. While recent studies have begun to examine reproduction in this species (Ellis-Smith 2022), a clear knowledge gap of their gut and digestive system still exists.

1.3 Gut structure and digestion in teleost fish

The digestive tract structure of teleost fish varies significantly across species due to their diverse habitats and feeding behaviours. Teleost's, or bony fish, include around 30,000 species, making up 50% of all vertebrates (Wootton & Smith, 2014). Their diverse shapes and sizes reflect a variety of digestive systems, each adapted to the feeding strategy of the fish and the environment they live in (Liu et al., 2016). Fish distribution is influenced by factors like seasonality, water quality, predation and food availability, with species inhabiting different ecosystems such as open oceans, coral reefs, kelp forests, and estuaries. This habitat diversity allows for varied diets, shaping the evolution of efficient digestive systems.

The digestive tract as a whole works to digest food so that nutrients can be absorbed and assimilated to support growth and metabolism (Cho et al., 2023; Ray & Ringø, 2014). To achieve this, teleosts have developed gut structures that are both morphologically and functionally adapted to their feeding ecology. The alimentary canal is generally divided into four main regions: the headgut, foregut, midgut, and hindgut (Grosell et al., 2011). The head gut includes the mouth and gill cavities, and the foregut includes the esophagus, stomach and pylorus (Smith, 1978). The midgut starts from where the intestines connect to the pylorus and then to where the gut diameter increases, as that is where the hindgut starts and continues to the anus (Smith, 1978). Further specialisation occurs within these segments. For instance, the stomach is often subdivided into cardiac, fundic, and pyloric regions, and the intestine can be classified into anterior, middle, and posterior sections (Cao & Wang, 2009).

A notable feature in many teleosts is the presence of pyloric caeca, which are small, finger-like projections located at the junction between the stomach and intestine. Present in approximately 60% of species, these structures enhance digestion by increasing surface area and compensating for shorter intestinal length (Borlongan et al., 2002; Cho et al., 2023; Farrag et al., 2020). However, morphology can vary among taxa. For example, flatfish in the genus *Pseudopleuronectes* exhibit prominent pyloric projections (Figure 1.1), while species in *Platichthyes* display reduced structures resembling small ridges within the intestinal wall (Farrag et al., 2020).

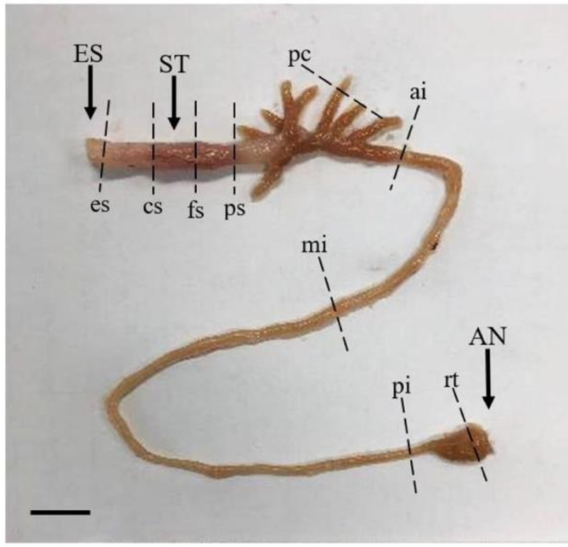


Figure 1.1. Dissected alimentary canal of a marbled flounder (Pseudopleuronectes yokohamae) displaying the different regions and features of the alimentary canal such as the pyloric caeca (pc) appendage (From Cho et al., 2023).

In addition to the intestine, the liver and gall bladder play vital roles in digestion. The liver processes digested nutrients and should be monitored for changes in size, shape, or colour, as these may signal dietary imbalances (Smith, 1978). The gall bladder, situated near the liver, stores and releases bile in response to hormonal signals and its green colouration can vary, typically darkening during fasting and lightening after feeding (Chao, 1973; Grosell et al., 2011). Digestive processes are also closely regulated by cellular and hormonal factors, such as the distribution of goblet cells and the secretion of hormones like cholecystokinin, which are key regulators of digestion (Hur et al., 2013).

Understanding the structural adaptations of the digestive system provides insight into how diet and habitat influence gut morphology and function. Detailed histological examination of the alimentary canal such as the analysis of tissue layers, pyloric caeca, goblet cells, and hormone expression, is essential for assessing digestive capability and function in both wild and cultured fish species.

1.3.1 Teleost stomach morphology

The morphology of fish stomachs varies widely across species, with structural differences typically emerging post-hatch or upon the initiation of exogenous feeding (Cyrino et al., 2008; Grosell et al., 2011). Stomach development is closely influenced by dietary intake, often requiring several weeks to months to fully mature (Rønnestad et al., 2013). While certain species possess a functional stomach at the onset of exogenous feeding, others initially rely on endogenous reserves and only develop a differentiated stomach during metamorphosis (Rønnestad et al., 2013).

Stomach morphology ranges from straight or curved tubular forms to pouch-like structures, all adapted to facilitate the digestion of ingested material before it enters the intestine (Weitzman & Parenti, 2025). Four primary stomach morphologies are commonly described: J or U-shaped, as seen in species such as the African catfish (*Clarias gariepinus*) and Atlantic salmon (*Salmo salar*); straight or channel-shaped, characteristic of pike (*Esox* lucius) and halibut; Y-shaped forms, observed in tilapia (*Oreochromis sp.*); and the complete absence of a stomach, as found in agastric species like the ballan wrasse (*Labrus bergylta*) (Borlongan et al., 2002; Moawad et al., 2016) (Figure 1.2).

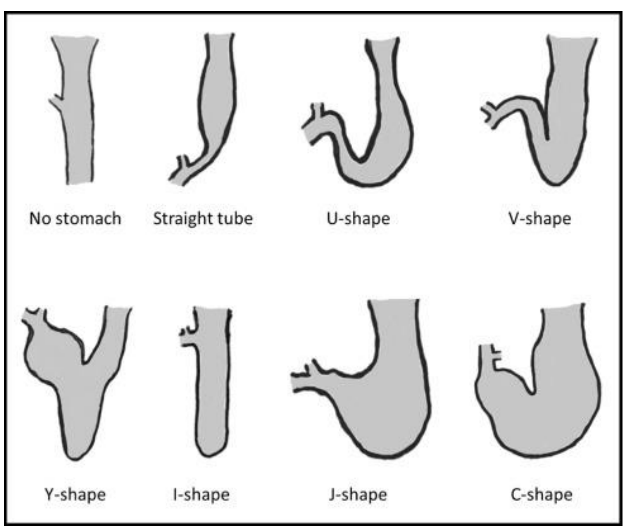


Figure 1.2. Example of various stomach shapes in teleost fish (From Small, 2022).

Functionally, the stomach serves as a dilated portion of the gastrointestinal tract, playing a central role in protein digestion. It contains gastric glands that secrete hydrochloric acid and pepsin, facilitating the enzymatic breakdown of food (Gomes et al., 2014; Koelz, 1992; Smith, 1978). Additionally, the stomach is involved in the temporary storage and mechanical mixing of ingested material, initiating the early stages of digestion and food processing (Day et al., 2011; Horn, 1998; Koelz, 1992). In herbivorous species, acidic gastric conditions assist in the breakdown of plant cell walls, particularly cellulose in algae (Horn, 1998). In contrast, agastric fish lack a stomach entirely and rely on the anterior portion of the intestine to perform some of the functions typically carried out by the stomach, such as enzymatic digestion and nutrient processing (Ray & Ringø, 2014). Overall, the variation in stomach morphology and function across species highlights the diverse adaptations fish have evolved to meet their dietary needs and ecological niches.

1.3.2 Teleost intestine morphology

Intestinal morphology in teleost fish varies considerably across species and holds a primary role in the absorption of nutrients (Khojasteh, 2012). Structures ranging from short, smooth tubes to highly folded and complex arrangements. Some species possess villi to increase absorptive surface area, while others exhibit elongated, coiled, or looped intestines to maximise nutrient absorption within limited body space (Khojasteh, 2012). These morphological differences are strongly influenced by dietary composition and environmental conditions, which also shape related anatomical features such as mouth structure, gill raker number, the presence of a stomach, pyloric caeca, and overall intestinal length (Noguera et al., 2015).

At early developmental stages, fish possess a simple, unsegmented digestive tract. Differentiation into specialised gut regions typically occurs toward the end of the larval phase and during juvenile metamorphosis, resulting in a fully functional alimentary canal capable of exogenous feeding (Cyrino et al., 2008). Digestive tract morphology can vary not only across species but also throughout the life cycle of an individual, independent of chronological age. For instance, many species such as pinfish (*Lagodon rhomboides*), tilapia, and butterfish (*Odax pullus*) undergo ontogenetic dietary shifts from planktivory in early stages to carnivory and, eventually, herbivory in adulthood (Beveridge & McAndrew, 2000; Givens et al., 2015; Johnson et al., 2017). These shifts are linked to physiological changes in gut morphology as larval fish typically require protein-rich diets to support rapid growth and thus possess shorter, simpler intestines (German & Horn, 2006). Alternatively, adult herbivores develop longer digestive tracts to facilitate cellulose breakdown (Becker et al., 2010). Even in maturity, intestinal morphology may undergo minor changes in response to seasonal dietary shifts, environmental variation, or key life history events such as reproduction (Rønnestad et al., 2013; Johnson et al., 2017).

Teleosts are generally categorised into broad functional feeding groups of carnivores/piscivores, herbivores, omnivores, and benthic or detritus feeders, based on the relationship between gut morphology and dietary preference (Ray & Ringø, 2014). Carnivorous species often possess relatively short digestive tracts due to the ease of protein digestion, whereas herbivorous fish tend to have longer, more coiled intestines to allow extended retention time for the breakdown of plant material (Borlongan et al., 2002; Weitzman & Parenti, 2025). This trend is often quantified using relative gut length (RGL), a metric first proposed by Al-Hussaini (1949), with further refinement by Dasgupta (2004), who established a correlation between RGL and dietary habits among freshwater teleosts. This metric is calculated as the ratio of intestinal length to standard body length and is used to compare digestive morphology across species. Dasgupta reported that carnivorous fish typically exhibit shorter RGLs, around 0.7, while herbivorous species possess longer RGLs, often exceeding 4.7. However, these patterns are not universal, as many species exhibit dietary flexibility, and digestive tract length may not always correlate directly with trophic level (Smith, 1978). Overall, stomach and intestine morphology reflect functional adaptations that optimise nutrient acquisition through appropriate digestive retention times, surface area expansion, and enzyme production tailored to dietary composition (Holt, 2011).

1.3.3 Benthic fish alimentary canal

Benthic feeders remain close to the seabed, consuming organisms found in bottom sediments, such as crabs, worms, and other invertebrates (Gerking, 1994). They may also ingest small fish, molluscs, and algae opportunistically (Plante et al., 1990). Species like flatfish, catfish, groupers, and carp exhibit this feeding strategy. Due to the often low nutritional quality of benthic prey, some species require almost continuous feeding to meet energy and nutrient demands (Plante et al., 1990). This feeding strategy leads to the ingestion of sand, silt, and mud, necessitating a digestive system adapted to processing large amounts of indigestible material, typically reflected in an extended gut tract (Link et al., 2002).

In addition to an elongated digestive tract, some benthic species exhibit further anatomical adaptations to handle sediment-rich diets. Some benthic species have a well-developed gizzard-shaped pyloric stomach (thick and muscular) to break down and process the indigestible material consumed (Fugi et al., 2000). Other species, however, can have simple gut structures with no stomach. This can also be effective for a benthic diet, as digestive enzymes and nutrient absorption can occur along the entire length of the digestive tract (Bowen, 1983; Plante et al., 1990). Benthic feeders, particularly those that consume mud and silt, typically possess the longest and most complex digestive tracts compared to other dietary categories. Their intestines are often long and coiled, resembling the gut structure of herbivores, which facilitates the prolonged breakdown of sedimentary components and maximises nutrient absorption (German et al., 2010; Plante et al., 1990; Borlongan et al., 2002). In contrast, species that prey on larger benthic organisms generally have shorter, less complex intestines, which are better suited for processing larger prey more efficiently (Borlongan et al., 2002).

The development and structure of the digestive tract in benthic feeders is closely linked to dietary habits, which vary across species and life stages. Like many fish, benthic feeders begin their larval stage with a simple, undifferentiated digestive tract, which gradually develops into specialized regions as the diet diversifies (Khoa et al., 2021). For example, Atlantic and Californian halibut (*Paralichthys californicus*) initially possess a simple gut, but during metamorphosis, their digestive system differentiates to accommodate a more complex diet (Gisbert et al., 2004; Gomes et al., 2014). However, adult digestive anatomy varies widely among benthic species. Some flatfish, such as certain flounder, develop large stomachs with relatively short intestines, whereas sole species may exhibit smaller stomachs and longer intestines (Ladle, 2016). Given the diversity among flatfish, there is considerable variation in digestive tract structure, especially in the length of the intestine and the presence or absence of pyloric appendices (De Groot, 1971, Figure 1.3). These differences highlight the strong relationship between digestive morphology and dietary composition in benthic feeders.

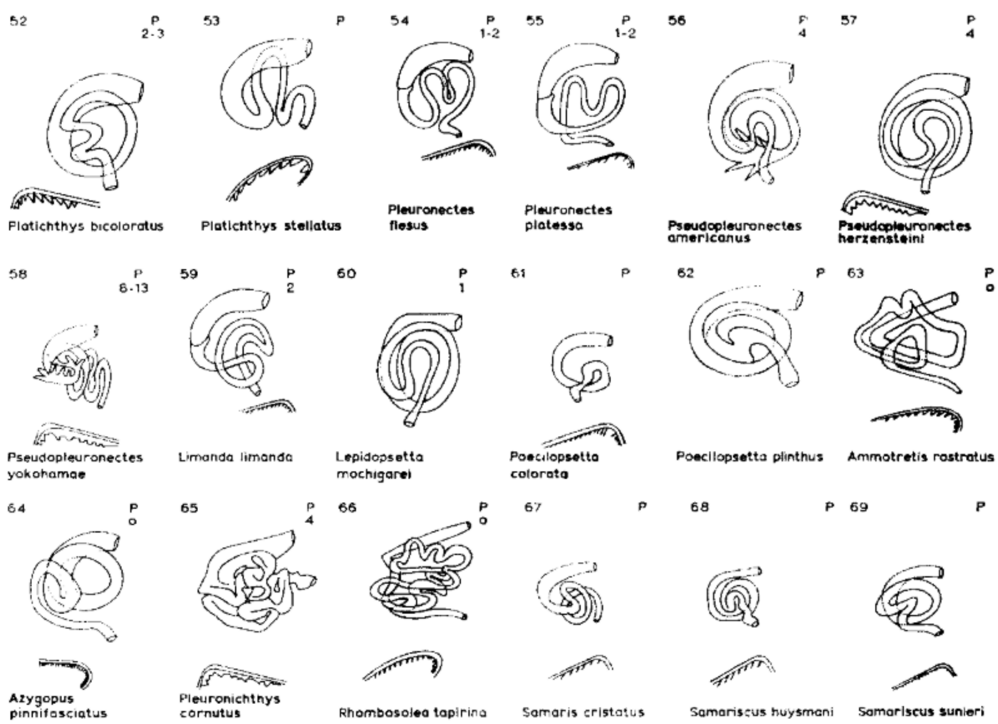


Figure 1.3. Sections of alimentary tract from different flatfishes demonstrating the diversity of gut structure amongst species (From De Groot, 1971)

1.4 Diet development for sustainable aquaculture

Developing nutritionally balanced diets is fundamental to sustainable aquaculture. However, optimal formulations must account for species-specific requirements, life stage, sex, and environmental factors, making diet design a complex but critical task (Manam, 2023). Effective feed formulation requires a balanced profile of essential macronutrients (protein, lipids and carbohydrates) and attractants. However, physical characteristics such as pellet size and buoyancy should also align with the cultured species feeding strategies and preferences (Manam, 2023).

Feed efficiency is essential to both economic viability and environmental sustainability. A key metric used to evaluate feed efficiency is the feed conversion ratio (FCR), which quantifies the amount of feed required to generate one kilogram of animal biomass (Fry et al., 2018). Aquaculture is often regarded as a favourable industry in terms of protein production because many aquatic species exhibit relatively low FCRs compared to terrestrial livestock. This is partly due to the reduced energy expenditure associated with living in the aquatic environment (Fry et al., 2018). However, despite this efficiency, aquatic animals cannot segregate themselves from the waste they generate. Accumulated organic matter, uneaten feed, and nitrogenous waste can degrade water quality and adversely affect animal health and growth performance (Amirkolaie, 2011). Consequently, reducing FCR is not only beneficial for improving feed utilisation but also crucial for mitigating environmental impacts. This highlights the importance of developing tailored diets to the digestive physiology and ecological needs of each cultured species.

1.4.1 Feeds in the aquaculture industry

In fish aquaculture, two main types of feeds are utilised: live feeds and formulated diets. Larval fish are typically fed live options, such as artemia or rotifers, due to their small size and ease of digestion, while mature fish are transitioned to fresh/frozen feeds or formulated pellet diets (Walsh et al., 2015). Both live and formulated feeds offer distinct advantages and disadvantages. Live feeds support natural foraging behaviour, particularly in species intended for release into the wild, but are labour-intensive and costly to produce (Hart & Purser, 1996). In contrast, well-formulated pellets provide a consistent, balanced nutrient profile, with a lower environmental impact due to their low FCR, and they require significantly less labour (Manam, 2023; Robb & Crampton, 2013; Walsh et al., 2015). However, pellet digestibility varies by life stage, as larvae often lack the enzymatic capacity to process harder feeds, meaning pellets cannot always be used throughout the entire lifecycle of a species (Hart & Purser, 1996).

One challenge in aquaculture nutrition is the development of sustainable feed formulations that maintain optimal growth and health while reducing reliance on traditional marine-derived ingredients. Fishmeal and fish oil have traditionally been the primary ingredients in aquafeeds, constituting 50-70% of the overall ingredients (Glencross et al., 2007; Han et al., 2016; Macusi et al., 2023). These ingredients are rich in protein and lipids and provide a balanced essential amino acid profile, which is crucial for growth, palatability, and digestion (Back et al., 2020; Han et al., 2016; Hodar et al., 2020; Tacon, 1995). However, the expanding aquaculture industry has increased demand, driving up costs and raising sustainability concerns (Huntington & Hasan, 2009; Li et al., 2022). Consequently, alternative protein sources have been developed and utilised to reduce the reliance on fishmeal in feeds, creating a more affordable, sustainable, and nutrient rich option. Any alternative ingredient considered for an aquafeed must be easily digestible, palatable, possess a suitable nutrient profile to meet the species' requirements and be sustainable and readily available (Glencross et al., 2007; Hodar et al., 2020).

1.4.2 Alternative aquafeed ingredients

The development of alternative aquafeed ingredients aims to balance performance, cost, and sustainability. Since fishmeal alternatives differ in nutrient composition and digestibility, they are often used in combination with fishmeal to replicate its nutritional value (Macusi et al., 2023). While complete fishmeal replacement remains challenging, reducing its inclusion remains beneficial. Common alternatives include poultry, mussel, insect, and plant meals. Animal-derived ingredients often have superior protein quality and amino acid profiles, though some plant sources are also viable when used strategically (Macusi et al., 2023).

Alternative plant sources like soybeans are appealing due to their cost-effectiveness, sustainable production, and high protein content (Chakraborty et al., 2019). However, they can also contain anti-nutritional factors (ANF), which limit their use in aquafeeds. ANF are natural chemicals produced by plants as a defence mechanism that hinder the absorption of nutrients like proteins and vitamins, potentially affecting the consumer (Francis et al., 2001; Prabu et al., 2017). Inclusion of ANF in aquafeeds can lead to adverse health and physiological effects, such as hindered growth due to reduced nutrient absorption (Francis et al., 2001; Hajra et al., 2013). The impact of ANF can be neutralised through processing methods, such as heat treatment or by limiting the amounts included in the feed (Hajra et al., 2013).

Insect meal alternatives have gained popularity due to their high protein content and comparable amino acid profiles to fishmeal, particularly with the black soldier fly larvae (BSFL) (Khieokhajonkhet et al., 2022; Perry et al., 2020). However, insect meal like BSFL, when used in aquafeeds, are highly species-specific due to the presence of chitin, a structural polysaccharide polymer found in the shells of crustaceans (Cha et al., 2008; Perry et al., 2020). While chitin ingestion is natural for fish that feed on crustaceans, its digestibility varies among species. Although it can promote growth, it can also have adverse effects (Hasan et al., 2023). Exploring alternatives to fishmeal is crucial for the future of sustainable aquaculture and should be continually trialled on various species where possible. While some amount of fishmeal may still be necessary in aquafeeds, reducing this proportion would be highly advantageous.

1.5 Thesis aims and objectives

Considering the global demand for flatfish and the high market value of yellowbelly flounder, this species is an interesting candidate for aquaculture. Understanding their gut structure and function is an important step toward developing a sustainable diet for this species. This thesis aims to provide an initial description of the morphological and functional gut structure in wild and captive yellowbelly flounder as a preliminary knowledge base for further research into diet development. This was achieved through the following objectives:

1.) Histomorphometric description of the general gut structure in wild and captive yellowbelly flounder (Chapter 2)
2.) Quantitative characterisation of the gene expression of the digestive hormone, cholecystokinin (*cck*) in wild and captive yellowbelly flounder (Chapter 3).

1.5.1 Thesis structure

- Chapter 1: General Introduction
- Chapter 2: Classification of the digestive tract of the yellowbelly flounder at an adult and juvenile life stage and juveniles fed either a mussel diet or a formulated pellet diet
- Chapter 3: Assessment of *cck* expression in the digestive tract in adult and juvenile yellowbelly flounder and in juveniles fed either a mussel diet or a formulated pellet diet
- Chapter 4: General discussion and conclusion

1.6 Ethics statement

Juvenile flounder were caught under the Ministry for Primary Industries Special Permit 698-3 (project M7) and handled according to the requirements of the NZ animal ethics standards and UoW AEC protocol 1172.

Chapter 2. Gut Structure of *Rhombosolea leporina*

2.1 Introduction

Teleost alimentary canal structure is a functional adaptation to their species-specific feeding ecology (Rønnestad et al., 2013). Therefore, to develop yellowbelly flounder aquaculture, it is crucial to first understand their gut structure. This knowledge is essential for achieving a sustainable, easily digestible and well-assimilated diet that supports low feed conversion ratios.

2.1.1 Yellowbelly flounder alimentary canal

As benthic feeders, flatfish often exhibit gut structures that differ from pelagic species, reflecting their unique dietary habits. De Groot (1971) classified flatfish into three primary feeding groups: (1) piscivores, (2) crustacean feeders, and (3) polychaete and mollusc feeders. These feeding strategies influence the morphology of the alimentary canal, particularly the length of the esophagus, stomach, and intestine, which adapt to the digestive demands of their diet (Figure 2.1).

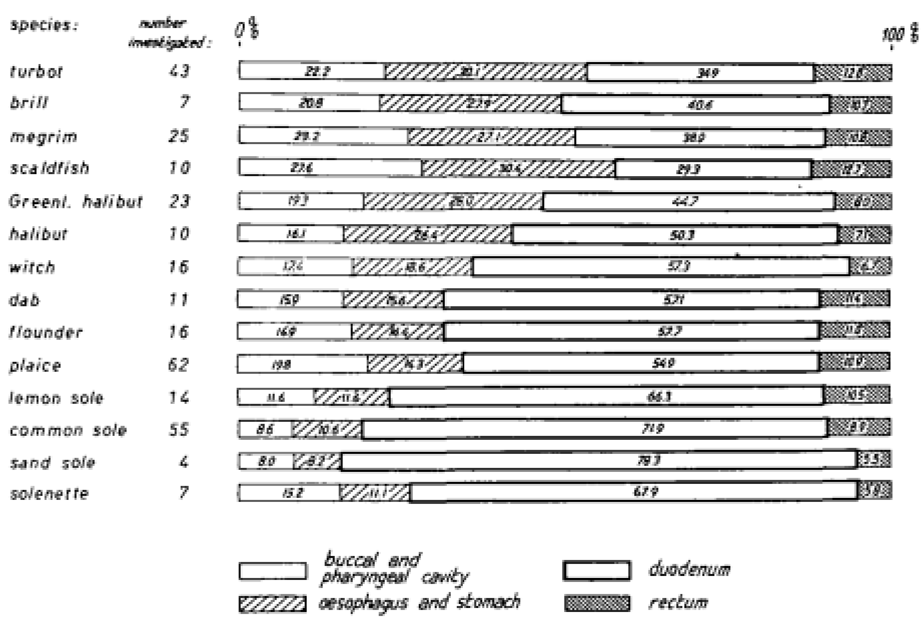


Figure 2.1. Relative lengths of the different parts of the alimentary canal in certain flatfish species (De Groot, 1969).

New Zealand yellowbelly flounder are classified as type 3 feeders and typically possess a small esophagus and stomach, along with an extensive and complex intestinal loop to process the large amounts of indigestible material in their diet. Members of the *Pleuronectidae* family, including the yellowbelly flounder, display considerable variation in stomach size (De Groot, 1971). Research on the greenback flounder, a closely related species within the same family, suggests that stomach loss occurs at the conclusion of the larval phase (Grosell et al., 2011). This finding may have implications for understanding digestive development in *Rhombosolea* species, which include the yellowbelly, sand, and black flounder.

2.1.2 Tissue layers

To better understand the structure of the fish gut, it is essential to examine the different tissue layers throughout the digestive tract. The basic structure of the digestive system in fish consists of four main tissue layers: the serosa, muscularis externa, submucosa, and mucosa, which is the innermost layer (Figure 2.2). These layers can undergo changes influenced by factors such as feeding habits, diet, age, weight, and sex (Cao & Wang, 2009; Cho et al., 2023; Mokhtar, 2021; Noguera et al., 2015; Purushothaman et al., 2016).

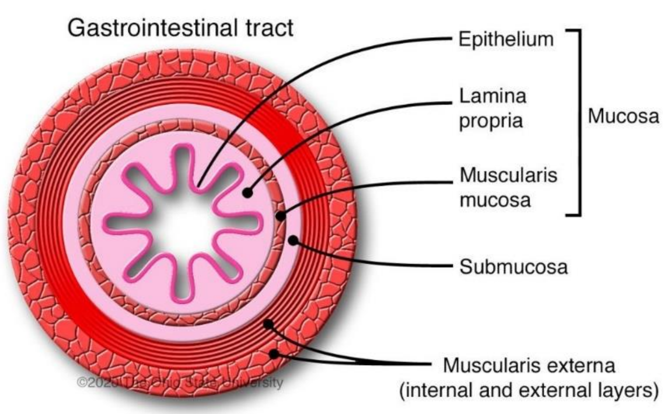


Figure 2.2. Cross section of a digestive tract showing the various tissue layers (From Jennings & Premanandan, 2017)

The mucosa, as the innermost layer comprises of three components, the epithelium, lamina propria, and muscularis mucosa, a thin longitudinal muscle layer (Noguera et al., 2015). The epithelium is primarily responsible for absorption and secretion and is the site of mucous secretory cells such as goblet cells and gastric pits (Noguera et al., 2015). In the esophagus, the epithelium is typically a stratified squamous layer, while a simple columnar epithelium is found throughout the rest of the gut tract (Chao, 1973; Ekele et al., 2014; Islam, 1951; Machado et al., 2013). The lamina propria beneath the epithelium contains blood capillaries (Noguera et al., 2015).

Moving outward, the submucosa acts as a connective tissue layer, providing structural support and connecting the mucosa to the muscularis externa (Chao, 1973; Noguera et al., 2015). The muscularis externa is made up of two muscle layers, the inner circular and outer longitudinal, which work together to contract and propel food through the digestive tract (Kasozi et al., 2024; Nasruddin et al., 2014; Noguera et al., 2015; Purushothaman et al., 2016). This enables peristalsis which is a combination of oral and anal contractions and relaxations to propel food down the length of the gut (Olsson & Holmgren, 2001). The thickness of the muscularis externa varies along different regions of the gut depending on the mechanical demands of digestion in those areas (Nasruddin et al., 2014). The serosa, the outermost layer, provides an additional protective covering for the digestive tract.

These tissue layers vary not only across species but also throughout the gut of individual species. The presence of gastric glands and pits in the stomach are in the mucosa layer with the pits within the epithelium secreting acid into the stomach to aid in digestion (Palladino et al., 2023). For example, in species with a stomach, such as yellow catfish (*Pelteobagrus fulvidraco*), gastric glands are present in the cardiac and fundic regions of the stomach, similar to those observed in marbled flounder (*Pseudopleuronectes yokohamae*) and Asian seabass (*Lates calcarifer*) (Cao & Wang, 2009; Cho et al., 2023; Purushothaman et al., 2016). Conversely, species that lack a stomach do not have gastric glands or pits but may instead possess an intestinal bulb that is histologically similar to the anterior intestine (Chao, 1973; Gabaudan, 1986). Additionally, goblet cells, which are responsible for secreting mucus, are typically absent from the stomach and are primarily found in the post-gastric mucosa layer (Murray et al., 1996).

Mucosal folds, which serve to increase the surface area for digestion and accommodate gut expansion during feeding, are found throughout the digestive tract (Back et al., 2020). The length of these folds varies across gut regions, with some species, such as the marbled flounder, exhibiting the longest folds in the esophagus and stomach, while others, like the Northern pike, display longer folds in the intestine (Cho et al., 2023; Bosi et al., 2020). The stomach is typically divided into three regions: the cardiac, fundic, and pyloric regions, while the intestine is segmented into anterior, middle, and posterior portions (Cao & Wang, 2009). While these tissue layers may appear similar in certain species, particularly in channel-shaped stomachs, histological methods are necessary to differentiate the layers and identify specialized structures such as goblet cells.

2.1.3 Goblet cells

Goblet cells play a crucial role in facilitating the movement of food and waste through mucus secretion, while also providing protection to the intestinal epithelium (Back et al., 2020; Hur et al., 2016). These cells are located in the epithelial layer throughout the digestive tract, with higher concentrations typically found in the esophagus, intestine, and rectum (Baglole et al., 1997; Chao., 1973; Murray et al., 1996; Ray & Ringø, 2014). A study by Murray et al. (1996) found goblet cells to be most abundant in the rectum of Atlantic halibut, yellowtail flounder (*Pleuronectes ferruginea*), and winter flounder. The researchers observed an increase in goblet cell numbers moving away from the pyloric caeca, likely due to the increased need for mucus to assist in the expulsion of faecal matter. In contrast, Hur et al. (2013) reported a decrease in goblet cell numbers toward the rectum in blacktip grouper (*Epinephelus fasciatus*), a species that retains a stomach. Goblet cell abundance is also influenced by nutritional status. For example, starvation has been shown to increase goblet cell numbers in certain species as a stress response to protect the epithelial layer (Berillis & Mente, 2017; Zhao et al., 2024). Together, these findings suggest that goblet cell distribution and density vary depending on species, the presence or absence of a stomach, and dietary factors. Measuring goblet cell abundance throughout the digestive tract can therefore provide insight into both gut morphology and mucosal function.

2.1.4 Dietary effects on gut morphology in fish

As outlined in Chapter One, the morphology of a fish's alimentary canal is strongly influenced by its diet. For example, fish that consume tougher or lower-quality diets such as herbivores or benthic feeders ingesting mud-like material often develop longer intestinal tracts (Duque-Correa et al., 2024; Leigh et al., 2018). These dietary influences can also lead to changes in gut tissue layers, including variations in the thickness of the muscularis and lamina propria, as well as the length and thickness of villi and mucosal folds (Aidos et al., 2023; Jiao et al., 2023).

Starvation, while often naturally occurring in wild populations due to fluctuations in food availability, temperature, or population density, can also affect farmed fish during feed management issues (Rahmati et al., 2016; Sun et al., 2020; Wang et al., 2006). It can significantly impact growth, energy allocation, immune function, and gut morphology (Rahmati et al., 2016). For instance, Sun et al. (2020) reported extended lamina propria and shortened villi in juvenile blunt snout bream subjected to starvation, indicating clear histological changes in the intestine.

Alternative diets to fishmeal pose a challenge not only in matching protein and amino acid profiles but some, such as soybean, can cause inflammation in the gut. This was evidenced by histological signs of inflammation in Atlantic Salmon fed a soybean-based diet (Krogdahl et al., 2023). Such inflammation may lead to immune dysfunction and the downregulation of digestive enzymes, ultimately affecting growth and health. Understanding how different diets affect intestinal structure is therefore essential for promoting health and performance in aquaculture systems (Norvdi et al., 2023).

2.2 Aim for Chapter 2

This study set out to better understand the gut structure of yellowbelly flounder with a view of diet development for aquaculture. Specifically, this chapter aims to characterise the morphological structure of the gut in adult and juvenile yellowbelly flounder and examine the possible effects of formulated diets on juvenile gut development. This was achieved through the following objectives:

1.) A histomorphometric analysis of muscularis externa thickness and mucosal fold length across different regions of the gut tract

2.) Quantification of goblet cells across different regions of the gut

2.3 Methods

To provide a baseline for the effects of a natural diet and environment on yellowbelly flounder gut structure, three wild-caught adult (Waiaua river, Bay of Plenty; -37.985716, 177.386728) and three juvenile (Pahoia Domain, BOP; -37.630785, 176.002803) yellowbelly flounder were caught using either set nets or dragnets, respectively. The adult fish were humanely dispatched by spiking of the brain (Iki jime method) and the juveniles were euthanised using an overdose of 2-phenoxyethanol and the gut tract was dissected out immediately. These were further cut into nine key regions, the esophagus, cardiac, fundic and pyloric stomach, pyloric caeca, anterior, middle and posterior intestine and rectum.

In an attempt to explore the possible effects of formulated diets on the yellowbelly flounder gut structure, 14 wild-caught (Miranda, Hauraki Gulf; -37.151927, 175.309110) juveniles were divided into two dietary treatment groups ('natural' and 'formulated'). Fish were sampled between 6 to 10 weeks after being fed their diet. A third 'pseudo' treatment became evident in that some fish did not adapt to the diets and starved themselves. Fish that showed no interest in eating were transferred to a separate tank and were continued to be offered food during the study and were observed to confirm that they had not eaten before the food was removed. Fish were fed to satiation 5 days a week. Tanks were siphoned clean 20 – 30 min after feeding.

2.3.1 Flounder husbandry

Following capture fish were placed into sealed polythene bags with 1/3 seawater and 2/3 pure oxygen and transported back to the University of Waikato Coastal Marine Field Station in Tauranga. The fish were prophylactically treated with 0.1mL/L of 5% formalin for 30 min to reduce infection risk, before being moved into the experimental tank systems.

The experimental tanks were recirculating seawater systems comprised of six 53 L glass tanks (flow rate of 1L/min). Two groups of three tanks (system 1 and system 2) connected to individual 100 L sumps. The glass tanks from both systems were semi-submerged in a larger plastic tray which acted as a temperature-controlled water bath (Figure 2.3). The photoperiod was kept at a 12:12 light: dark cycle, using fluorescent light tubes covered in shade cloth to simulate the dim light that flounder experience in the muddy estuarine environment. Water quality was regularly measured, and temperature was kept between 17-18.5°C with water changes performed when appropriate to maintain the following nitrogen levels; ammonia (< 0.05 ppm), nitrite (< 0.05 ppm) and nitrate (< 20 ppm).

Figure 2.3. System set up with glass tanks allocated to different sumps and submerged within a controlled water bath for temperature control

2.3.2 Experimental diets and allocation

The 14 juveniles used in the diet experiment were randomly allocated throughout system 1 (formulated gel-pellet diet) or system 2 (mussel diet). Two diets were used, the first representing a more natural diet being a fresh, green-lipped mussel diet. These were bought fresh from a local supermarket and were removed from their shell and frozen. When being fed to the juveniles, they were thinly sliced and diced into small mouthpiece sizes and then dispersed throughout the tanks, sinking to the bottom. The formulated gel-pellet diet was a fishmeal-based pellet supplied from Plant and Food Research that was ~49% protein sourced from a mixture of fishmeal, soy protein and poultry meal which is typical for a commercial fish pellet. The pellets were initially crushed into mouth sized pieces and soaked in water before being added to the tanks however, the juveniles didn't appear to be successful in ingesting the pellets. Because of this, the pellets were made into a gel-pellet to create a softer consistency to aid in the consumption of the firm pellet feed. The gel-pellet feed was formulated using the recipe in **Appendix A**.

From the two diets being fed there were four juveniles which didn't eat either diet when offered and so they were classified as a starved group. Overall, this resulted in three experimental groups; 1.) mussel diet (MD) of 5 juveniles (system 1) 2.) formulated gel-pellet diet (PD) of 5 juveniles (system 2) 3.) starved group (S) of 4 juveniles (system 1).

2.3.3 Dissection process

All 14 of the juveniles were euthanised using an overdose of 2-phenoxyethanol a day after being fed (withheld from feed for ~12hrs). Once euthanised (adults and juveniles), they were weighed, and their body length was photographed with a ruler (later to be accurately measured on ImageJ software, measuring from the mouth to the end of the body tissue) (Figure 2.4).

Figure 2.4. Example of a juvenile flounder body length measurement from mouth to end of the body tissue

Once measurements were taken, the fish gut cavity was cut open under microscope and the entire gut tract from the esophagus to the rectum was carefully cut out as a whole tract. The tract was photographed with a ruler (later to be measured on ImageJ) and used to calculate the relative gut length by dividing the length of the gut tract by the body length. The gut tract was further divided into nine regions (Figure 2.5). These regions were challenging to distinguish due to the gut tract being very uniform, so the regions were taken as estimates of their location based off knowledge from other fish. It was clear that no pyloric caeca region was present in this fish species however, a pyloric caeca region cutting was still taken for this study. Each region had three thin cuttings removed, if possible, to have a cutting from each region in different solutions for further analysis. Each cutting from the nine regions was placed in a 2 mL Eppendorf tube filled with either 10% neutral buffered formalin fixative (NBF), bouin's fixative or ribonucleic acid (RNA) Later solution. The liver was also removed from the gut tract and weighed after the gallbladder was removed as best as possible from the liver.

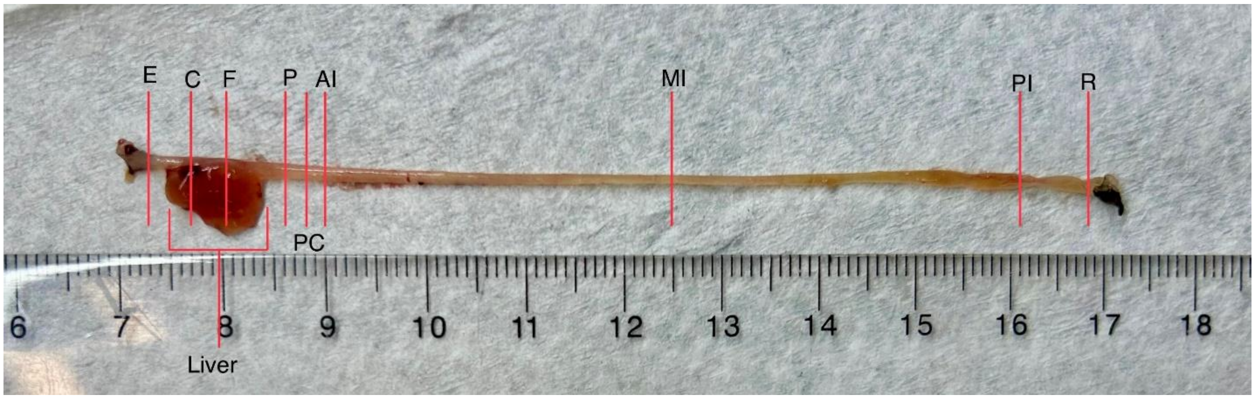


Figure 2.5. Example of a juvenile gut tract and where each region was cut. Abbreviations: E, esophagus; C, cardiac stomach (cut anteriorly to the liver attachment); F, fundic stomach (cut posteriorly to the liver attachment); P, pyloric stomach; PC, pyloric caeca; AI, anterior intestine; MI, middle intestine; PI, posterior intestine; R, rectum.

Once the entire gut tract was dissected and placed in the three solutions the RNAlater samples were refrigerated overnight at 4°C before being moved to a -80°C freezer for storage in preparation of RNA extraction. The samples in NBF and bouin's were kept at room temperature for no longer than 24hrs before the tissue sample was removed from the fixative and transferred into 70% ethanol for longer term storage.

2.3.4 Histological processing and analysis

Once all samples were collected and stored from all the flounder, one juvenile and one adult samples stored in bouin's and NBF were sent to the University of Otago histology lab for histological preparation. Initially, all nine segments from each fish in both fixatives (18 samples) were sent to determine which fixative produced the clearest histology slides. After reviewing the initial slides, bouin's was the fixative which produced the clearest slides and thus for further analysis only the bouin's samples were processed (Figure 2.6). Also, from these initial two fish the nine regions were narrowed down to just five for the experimental fish processing, this included the esophagus, fundic stomach, anterior, middle and posterior intestine regions. This was due to resource constraints but also because it wasn't necessary to analyse all nine regions to see how a diet impacted the gut tract. Therefore, all nine gut regions were analysed for the six wild caught flounder and just five regions were analysed for the 14 experimental fish, these were all histologically prepared at the University of Otago and sent back to Tauranga for analyses.

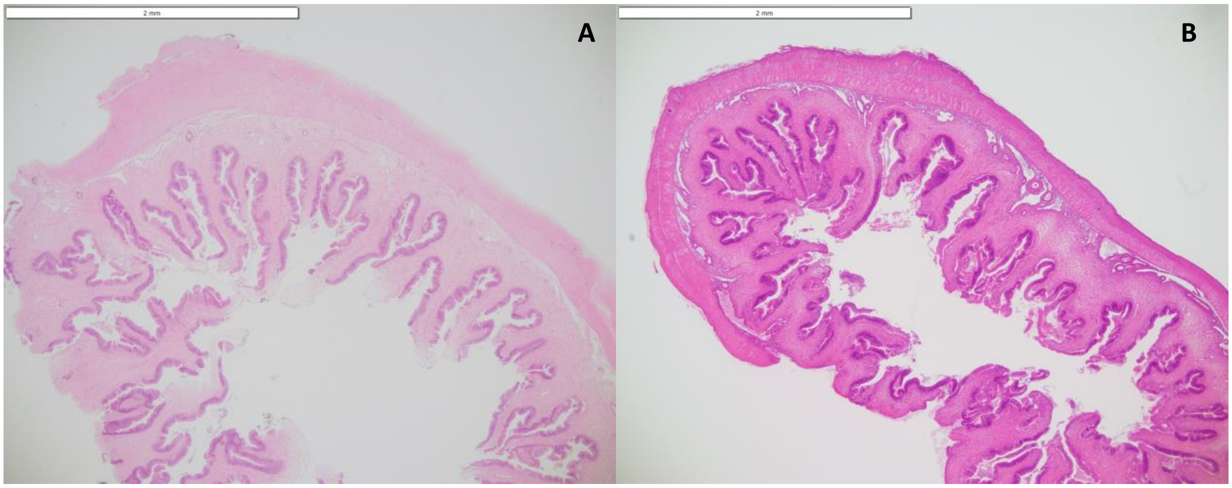


Figure 2.6. Example of an adult flounder anterior intestine that was fixed with NBF (A) and bouin's (B) and stained with hematoxylin and eosin. Scale bar, 2mm.

The histological preparation involved each sample being dehydrated and embedded in paraffin and cut in sections of 3-4µm thickness and made into two slides per sample. The first slide was stained with hematoxylin and eosin (H&E) to analyse the tissue layers and the other slide was stained with Alcian Blue and periodic acid-Schiff (AB & PAS) to clearly visualise goblet cells. These slides were analysed under an Olympus BX53 microscope with images taken at magnification on an Olympus DP27 camera through the CellSens Entry programme. These images were loaded onto ImageJ software for further analysis. Each H&E slide had four measurements taken of the mucosal fold length and muscularis externa thickness where possible (some histology slides were of poorer quality and thus taking measurements was not possible) to create averages of each sample of these measurements (Figure 2.7). Each AB & PAS slide had the number of goblet cells counted (Figure 2.8).

Figure 2.7. Example of a good quality AB & PAS-stained histology slide of a juvenile esophagus showing GC, goblet cells, ME, muscularis externa thickness and MF, mucosal fold lengths. Scale bar, 500µm

Figure 2.8. Example of a good quality AB & PAS-stained histology slide of a juvenile intestine showing clear GC, goblet cells inside the cell's epithelium. Scale bar, 200µm

2.3.5 Statistical analysis

Analysis and graphs of data were performed using R Studio version 4.3.2 with the ggplot package. Normality was assessed using the Shapiro-Wilk test, and homogeneity of variances was tested with Levene's test. To meet the assumptions of parametric testing, the data was log-transformed and tested using Two-way Analysis of Variance (ANOVA). Two-way ANOVAs were applied to the experimental diet groups for muscularis externa thickness and goblet cell counts to assess the effects of diet and gut region, as well as their interaction. The raw data, not the log-transformed data, was used for graphing. When significant differences were found ($p<0.05$), a post-hoc Tukey's test was performed. One-way ANOVAs were not conducted for the wild adult and juvenile data due to the smaller sample sizes. Bar graphs were created in Excel.

2.4 Results

Observations from the dissections of all the yellowbelly flounder individuals used in this study revealed that the species exhibits a notably uniform alimentary canal. No distinct boundaries were observed between different gut regions. The stomach was not clearly defined by changes in shape or any noticeable narrowing or widening of the tract, and no pyloric caeca appendages were present.

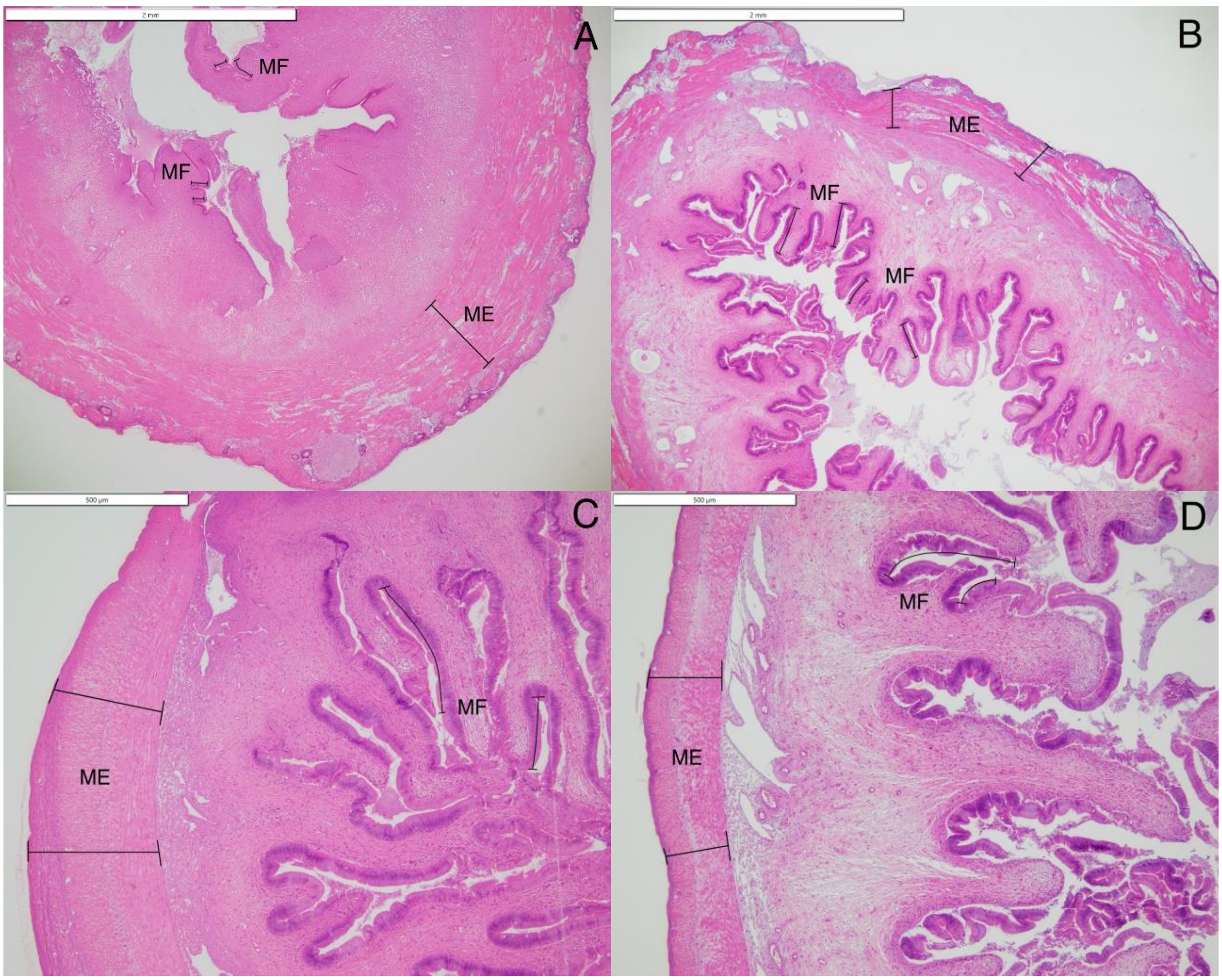
The three juvenile yellowbelly flounder used to represent the typical gut structure of wild juveniles were slightly smaller in body length (4.7 ± 0.7 cm) than the juveniles used for the experimental diets (5.7 ± 0.6 cm, 5.3 ± 0.8 cm, 6.6 ± 1.9 cm) (Table 2.1). However, the relative gut length was found to be the longest in the wild juveniles and the mussel diet groups compared to the other groups.

Table 2.1. Body length, body weight, digestive tract length and relative gut length of the wild adult and juvenile yellowbelly flounder as well as the diet-tested juveniles (only three of the four starved fish were used in these averages as one was slightly larger in size and thus was removed from the averages). Values are ±SD.

	Wild adult	Wild juvenile	Mussel diet	Pellet diet	Starved
Sample number	3	3	5	5	3
Body length (cm)	27.2 ± 1.5	4.7 ± 0.7	5.7 ± 0.6	5.3 ± 0.8	6.6 ± 1.9
Body weight (g)	353.3 ± 50.3	2.3 ± 0.7	3.5 ± 0.9	2.4 ± 0.8	2.9 ± 0.7
Digestive tract length (cm)	27.3 ± 5.0	8.0 ± 0.2	9.8 ± 0.8	7.2 ± 0.2	8.7 ± 1.1
Relative gut length (cm)	1.0 ± 0.2	1.7 ± 0.2	1.7 ± 0.3	1.3 ± 0.4	1.5 ± 0.2

2.4.1 Histomorphometric characteristics of the digestive tract

The H&E stained histology images of the stomach regions showed no clear evidence of gastric glands or pits which are typical for the stomach. Additionally, the three intestinal regions exhibited minimal variation between one another. The muscularis externa layer was well-defined throughout the gut, and the branching of the mucosal folds appeared consistent, with only slight differences observed in the lengths of the mucosal folds (Figure 2.9).



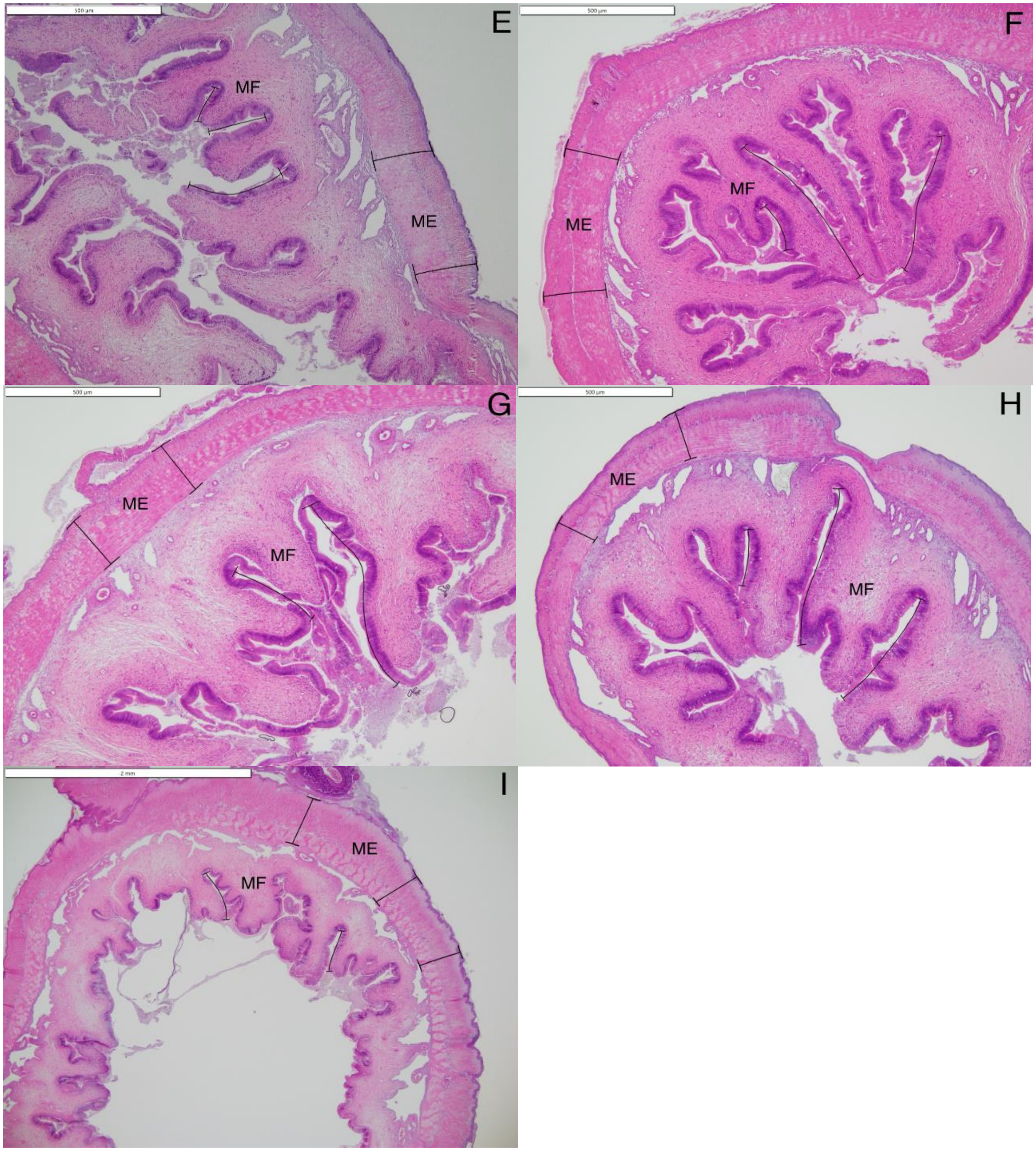


Figure 2.9. Morphological structures at each gut segment from a wild-caught adult yellowbelly flounder stained with H&E. Examples of ME, muscularis externa and MF, mucosal folds are displayed. (A) esophagus, scale bar of 2mm; (B) cardiac stomach, scale bar of 2mm; (C) fundic stomach, scale bar of 500µm; (D) pyloric stomach, scale bar of 500µm; (E) pyloric caeca, scale bar of 500 µm; (F) anterior intestine, scale bar of 500µm; (G) middle intestine, scale bar of 500µm; (H) posterior intestine scale bar of 500µm; (I) rectum scale bar of 2mm.

2.4.2 Muscularis externa thickness

Both the wild adult (WA) and juvenile (WJ) muscularis externa thickness exhibited similar patterns throughout the digestive tract, with the thickest measurements observed in the esophageal region (789.62 ± 93.26 µm in WA and 97.53 ± 11.5 µm in WJ) and the rectum (434.62 µm in WA and 80.32 ± 8.14 µm in WJ, Figures 2.10 and 2.11). A decrease in thickness was noted throughout the stomach (mean of 270 µm in WA and a mean of 54 µm in WJ) and intestine (mean of 171 µm in WA and a mean of 49 µm in WJ) regions. However, muscularis externa thickness could not be assessed in the fundic stomach and anterior intestine regions of the WJ because the histology slides lacked clear cross-sections suitable for measurement.

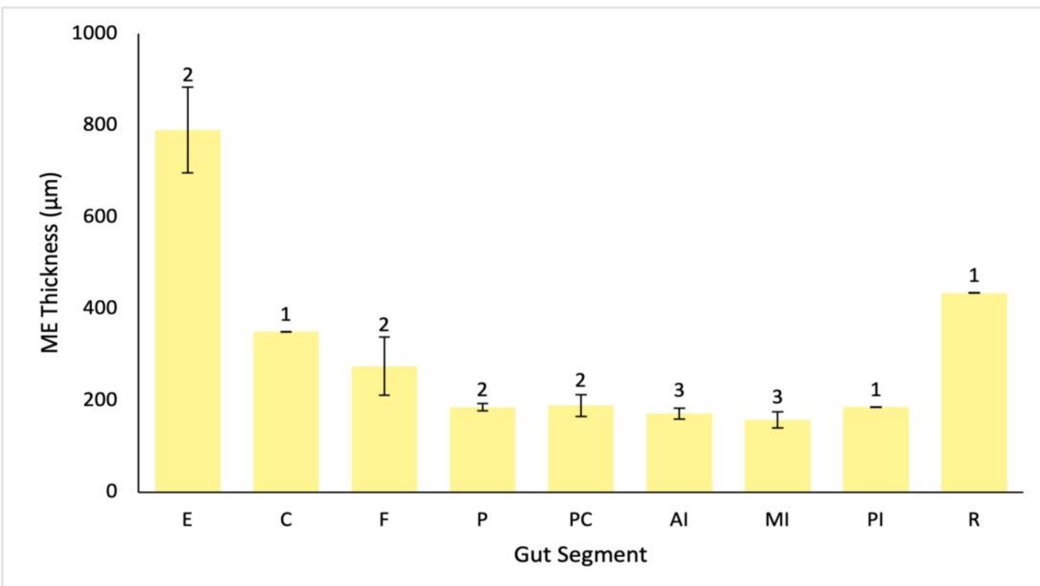


Figure 2.10. Mean thickness (µm) of the muscularis externa (ME) per gut segment from the wild adult flounder. Sample sizes for each mean are displayed above the error bars. Error bars ±SE.

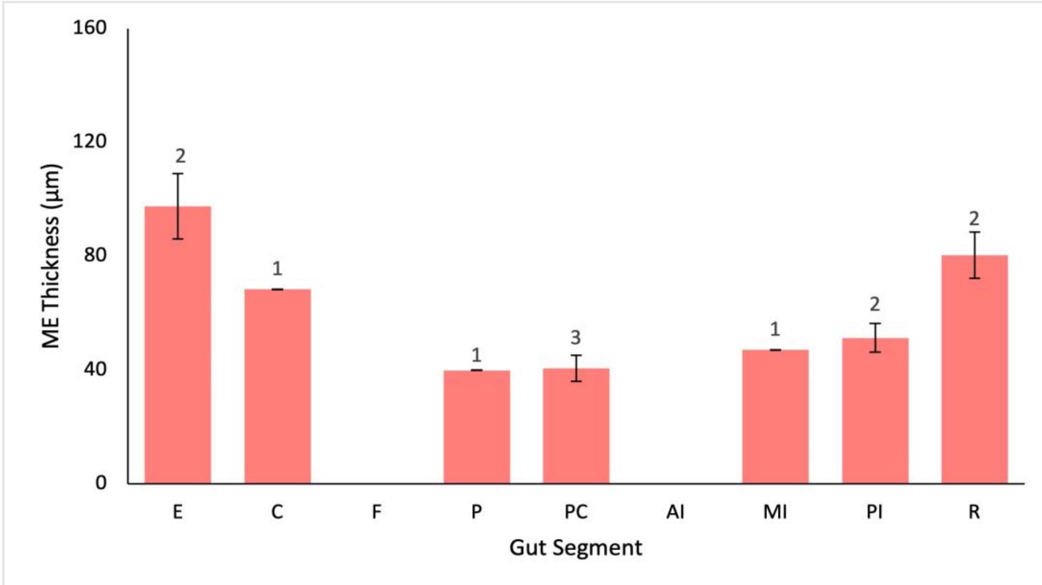


Figure 2.11. Mean thickness (µm) of the muscularis externa (ME) per gut segment from the wild juvenile flounder. Sample sizes for each mean are displayed above the error bars. The fundic stomach (F) and anterior intestine (AI) lacked suitable histology cross-sections for measurements and thus ME thickness could not be obtained. Error bars ±SE.

The mussel (MD) and starved (S) diet groups followed the same pattern, with the thickest muscularis externa region being the esophagus (144.72 ± 14.87 µm in MD and 121.62 ± 16.49 µm in S), with thickness decreasing through the rest of the gut before increasing at the posterior intestine (44.42 ± 7.25 µm in MD and 63.68 ± 13.02 µm in S) (Figure 2.12). The pellet diet group however, had an increase in thickness at the anterior intestine (73.01 ± 9.34 µm) and then decreased again through the middle and posterior intestine (49.66 ± 3.11 µm and 44.61 ± 5.88 µm).

There was a significant difference in muscularis externa thickness between gut regions ($F = 21.529$, $Df = 4$, $p =< 0.001$) however, no significant difference was found between diets, and no interaction was observed between diet and gut region. Further analysis demonstrated significant differences in the externa thickness between the esophagus and all other gut regions. This pattern was similar to the observations made regarding wild adult and juvenile flounder, although it is worth noting that the rectum was not analysed for externa thickness in the experimental fish.

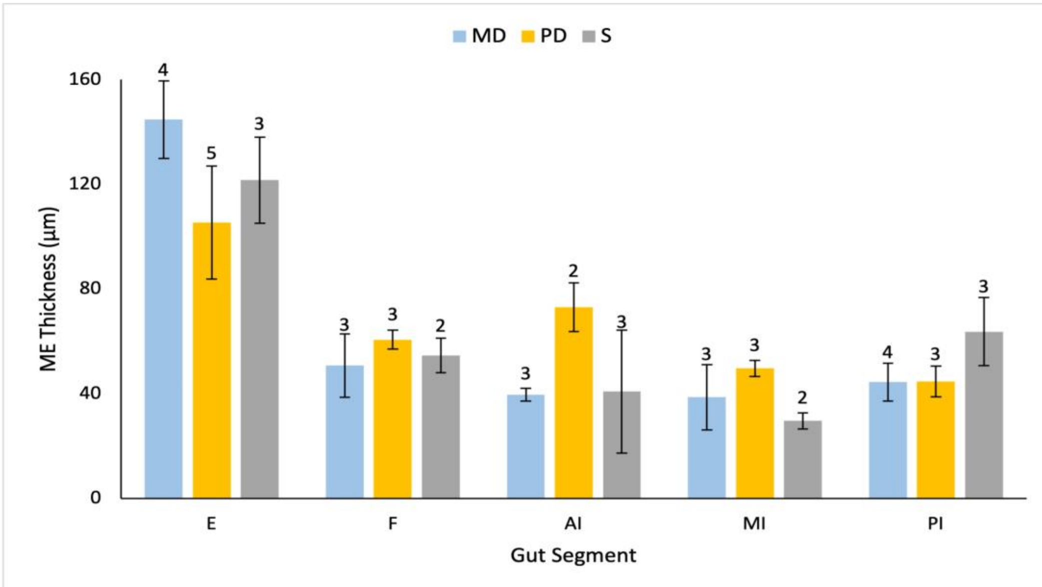


Figure 2.12. Mean thickness (µm) of the muscularis externa (ME) per gut segment from the experimental diet groups, mussel diet (MD), pellet diet (PD) and starved (S). Sample sizes for each mean are displayed above the error bars. Error bars ±SE.

2.4.3 Mucosal fold length

Mucosal fold length was only able to be measured from the wild adult histology images. The shortest mucosal folds were in the esophagus (294.81 µm) which then increased throughout the stomach regions (mean of 640.76 µm). Shorter folds were measured throughout the remaining intestine and rectum (mean of 440.68 µm) except the posterior intestine where length increased (752.63 ± 260.01 µm) (Figure 2.13).

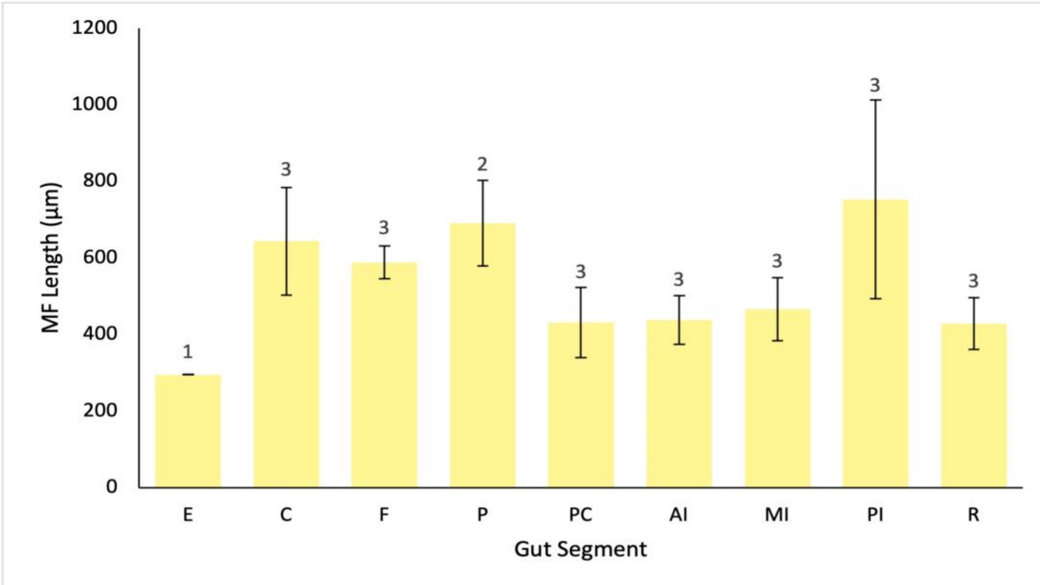


Figure 2.13. Mean length (µm) of the mucosal fold (MF) per gut segment from the wild adult flounder. Sample sizes for each mean are displayed above the error bars. Error bars ±SE.

2.4.4 Goblet cell distribution

The number of goblet cells (GC) throughout the gut tract in the wild adult and juveniles showed variation in patterns between the two. The adults displayed lower numbers of GC from the esophagus to the middle intestine (mean of 291 GC), which then increased at the posterior intestine and rectum (948 ± 219 and 1315 ± 323) (Figure 2.14). The wild juveniles however, had an increase in GC at the fundic and posterior stomach (80 and 88 ± 2.85) and variable numbers throughout the intestine (mean of 66 GC) before increasing at the rectum (137 ± 36) (Figure 2.15).

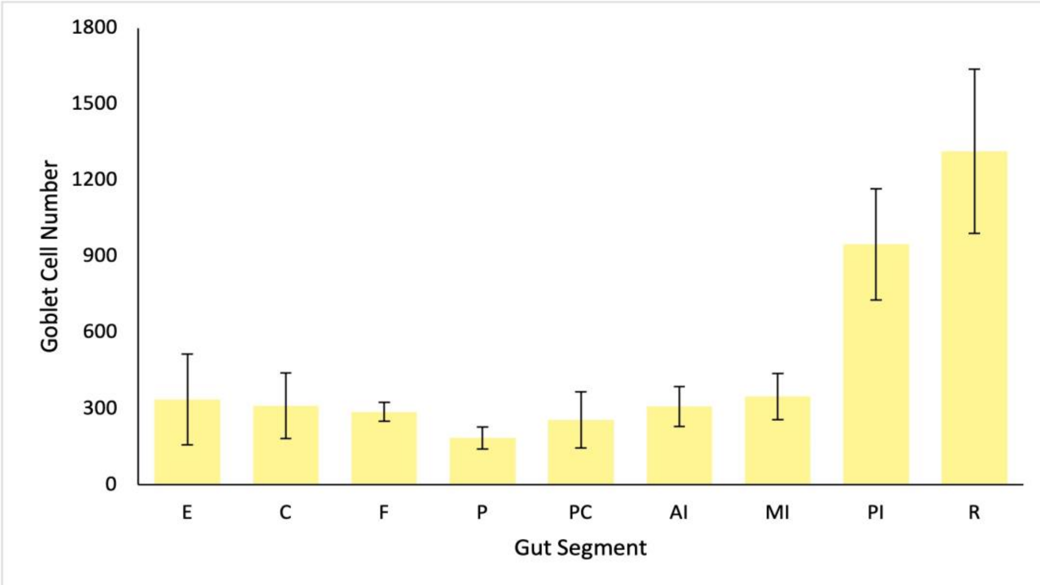


Figure 2.14. Mean number of goblet cells per gut segment from three wild adult flounder (n= 3 per segment). Error bars ±SE

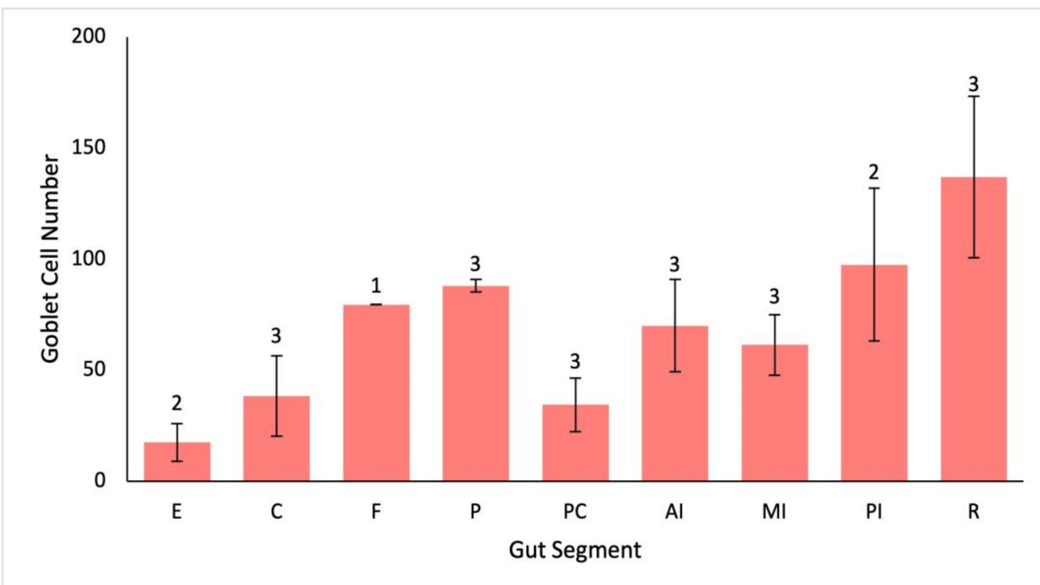


Figure 2.15. Mean number of goblet cells per gut segment from the wild juvenile flounder. Sample sizes for each mean are displayed above the error bars. Error bars ±SE.

All three diet groups displayed a similar distribution pattern of GC similar to what was seen in the wild adults. However, the diet groups had a much higher number of GC at the esophagus region

(773 ± 429 for the MD, 520 ± 304 for the PD, 639 ± 221 for the S group) in comparison to the remaining gut regions (Figure 2.16). The starved group in particular had a bigger increase in GC at the middle and posterior intestine (190 ± 14 and 503) compared to the mussel and pellet diet groups (66 and 172). There was, however, no significant difference in the number of GC between the gut regions or between the diets despite there appearing to be a difference between the gut regions.

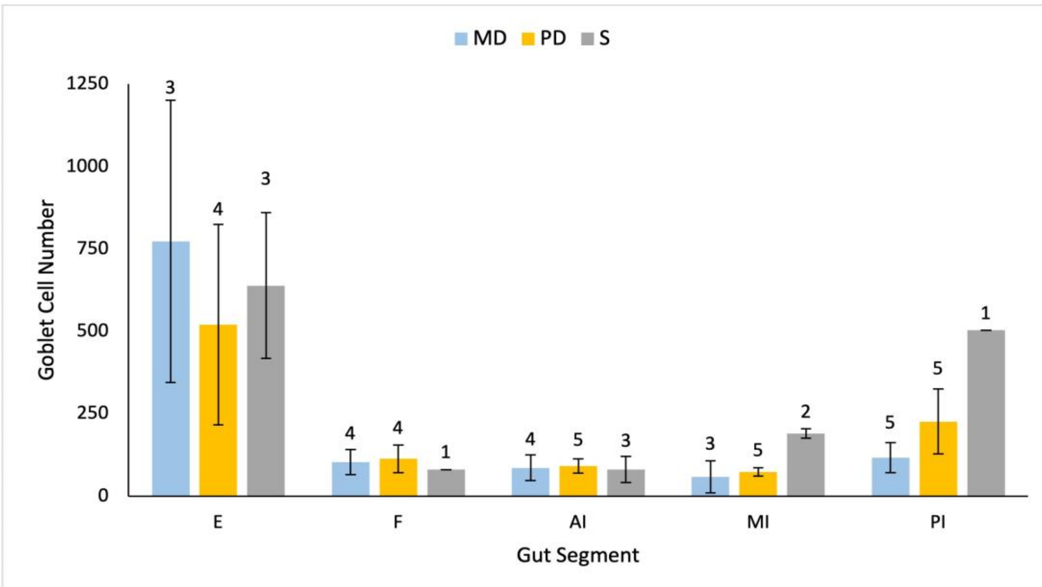


Figure 2.16. Mean number of goblet cells per gut segment from the experimental diet fish, mussel diet (MD), pellet diet (PD) and starved (S). Sample sizes for each mean are displayed above the error bars. Error bars ±SE.

2.5 Discussion

This study aimed to characterise the histomorphometric structure of the yellowbelly flounder alimentary tract in wild individuals and in juveniles fed either a 'natural' or a formulated pellet diet. The findings highlight that, while certain structural features are consistent with other cultured flatfish, the yellowbelly flounder also displays notable differences in gut morphology. Key observations include the absence of visible pyloric caeca, a lack of clear stomach differentiation, thus raising questions about the presence of a stomach, and no significant effect of diet on muscularis externa thickness or goblet cell number.

The muscularis externa (ME), consisting of the inner circular and outer longitudinal muscle layers, was thickest in the esophageal region across all wild adults, juveniles, and diet-treated juveniles. This result aligns with expectations, as the ME facilitates peristaltic movement of food through the gut and is typically more developed in areas requiring greater mechanical strength (Kasozi et al., 2024; Nasruddin et al., 2014). Similar trends have been reported in species such as marbled flounder and tigerfish (*Hydrocynus forskahlii*), where the ME is particularly well developed in the esophagus, stomach, and certain intestinal regions (Cho et al., 2023; Kasozi et al., 2024). In both the wild adult and juvenile groups, the rectum exhibited the second greatest ME thickness, while the remaining gut regions displayed relatively uniform ME structure, indicating a lesser need for reinforcement in those areas. Although the rectum was not assessed in the diet-treated groups, it would be expected to show similarly increased thickness. Within the experimental groups, ME thickness followed the same overall trend, with significantly greater thickness only in the esophagus. It can be concluded that the ME was not impacted by either three of the diet treatments. However, interestingly, the pellet-fed group showed a localised increase in ME thickness in the anterior intestine, unlike the other diet groups. This could reflect an adaptive response to promote motility in response to the pellet diet. An interesting finding has been noted in marbled flounder, where a thick ME in the pyloric stomach (603 µm) and well-developed gastric glands and thinner ME (198µm) in the cardiac and fundic stomach support the breakdown of a small, hard food diet (Cho et al., 2023). In contrast, yellowbelly flounder in this study showed no obvious gastric glands and lacked clear differentiation in the ME across much of the alimentary tract, apart from the esophagus. Although the ME was thinner in these regions, it remained well developed. This may indicate that the gut tract is suitable to consuming large volumes of soft food which is consistent with the species' natural benthic habitat (Cho et al., 2023; Lakshmi, 2010). Overall, the consistently thick ME in the esophagus highlights its role in forcefully contracting to move ingested material, while the lack of significant differences between other gut regions suggests a relatively uniform structure, typical of stomachless species (Gabaudan, 1986). The lack of variation between the diet groups and when compared to the wild caught individuals also indicates that diet treatments had minimal impact on the ME layer.

Mucosal folds (MF) increase the surface area of the alimentary canal, facilitating nutrient absorption and allowing the gut to expand during food intake (Back et al., 2020). Interestingly, in the wild adult yellowbelly flounder, the esophagus and rectum exhibited shorter MF compared to the rest of the tract. The stomach regions and posterior intestine showed the longest folds, suggesting these areas may play a larger role in food storage and processing, while the rectum may have a reduced role in waste expulsion than typically expected. Given the species' natural diet comprising of soft sediment along with crabs and worms, extensive folding in the esophagus and rectum may not be necessary. Instead, longer MF along the mid to posterior intestine may reflect the regions where digestion and nutrient absorption are prioritised. Variation in MF length along the gut has been observed in many fish species, often following a similar pattern to muscularis externa thickness. The longest folds are typically found in regions involved in food intake, digestion, or waste expulsion such as the esophagus, stomach, and rectum (Noguera et al., 2015). For example, MF are longest in the esophagus of banded tilapia (*Tilapia sparrmanii*), the fundic stomach of marbled flounder, and the intestine of Northern pike (Cho et al., 2023; Bosi et al., 2020; Okuthe & Bhomela, 2020). The variation in folds between species highlights how different fish have evolved gut structures suited to their specific feeding habits, with longer folds helping to accommodate different meal sizes and potentially creating space to separate ingested material into smaller portions for digestion (Mokhtar et al., 2017).

Goblet cells (GC) play a key role in both the ingestion of food and the egestion of waste by secreting mucus that protects the epithelial lining from physical and chemical damage, while also facilitating the smooth passage of ingested material (Back et al., 2020; Cho et al., 2023; Hur et al., 2016). In this study, high numbers of GC were observed in the pyloric intestine and rectum of both wild adult and juvenile flounder, consistent with their protective and lubricating roles. Surprisingly, the esophagus in these groups showed relatively low GC numbers, despite being commonly reported as GC dense in other species such as the marbled flounder (Cho et al., 2023). In contrast, all diet-treated juveniles displayed high numbers of GC in the esophagus, suggesting that dietary conditions can influence mucus production in this region. An increase in GC following dietary changes may enhance digestion or, in the case of starvation, serve as a protective mechanism against epithelial damage caused by poor nutritional status (Zhao et al., 2024). In the yellowbelly flounder GC were observed throughout all the 'stomach' regions, suggesting a lack of a true stomach and that mucus secretion occurs throughout the gut because GC are typically absent in the stomach. This is due to the presence of gastric glands that produce digestive enzymes like pepsin (Cho et al., 2023; Gabaudan, 1986; Le, et al., 2019b). Numbers of GC often increase progressively from the anterior to the posterior intestine to aid in food movement along the tract (Hur et al., 2016; Murray et al., 1996; Okuthe & Bhomela, 2020; Purushothaman et al., 2016; Ray & Ringø, 2014). This trend was observed in the wild flounder and in the mussel and pellet diet groups, indicating that these diets did not significantly disrupt typical GC distribution, aside from the increase seen in the esophagus. However, the starved group showed elevated GC numbers in the middle and posterior intestines, which may reflect a protective adaptation to nutritional stress, as similarly reported in rainbow trout (*Oncorhynchus mykiss*) (Berillis & Mente, 2017). Overall, these findings suggest that while dietary composition and feeding status can influence GC distribution in specific gut regions, the overall pattern of GC abundance along the alimentary tract in yellowbelly flounder largely reflects functional demands and potential stomach absence.

The relative gut length (RGL) was measured for all yellowbelly flounder used in this study. Results showed that the wild adult group had the shortest RGL, suggesting a dietary profile more consistent with carnivory. In contrast, the wild juveniles and mussel diet groups displayed the longest RGLs, aligning more closely with an omnivorous feeding strategy, similar to the pellet-fed and starved groups (Dasgupta, 2004). It was initially expected that the wild adults would have a longer RGL, despite their carnivorous diet of mud crabs, to reflect adaptations to ingesting indigestible materials such as mud and silt often consumed alongside prey. Longer intestines have been associated with such dietary components due to the need for extended processing time (German et al., 2010; Link et al., 2002; Plante et al., 1990). Additionally, species that lack a true stomach often exhibit a longer intestinal tract to compensate for the absence of gastric digestion (Le et al., 2019b).

Overall, dissection of the alimentary canal revealed no visible pyloric caeca appendages, and histological analysis suggested the possible absence of a stomach, as there was minimal tissue differentiation and an unexpected presence of goblet cells throughout the entire canal. The relative gut length indicated that wild-caught adults were more closely aligned with a carnivorous diet, whereas juveniles in this study exhibited traits more consistent with an omnivorous diet, highlighting a possible ontogenetic shift in feeding behaviour from omnivory to carnivory. The diet treatments trialled showed minimal impact on the alimentary canal, based on histological results for the muscularis externa, mucosal fold height, and goblet cell counts after up to 10 weeks of feeding. This suggests that juvenile yellowbelly flounder may be adapted to handle a broader range of feed types, including both animal and plant-based ingredients, during early development. However, further investigation is required to confirm these findings, such as examining the presence of gastric glands and pits in potential stomach regions. This was challenging in the current study due to the small sample size and difficulties in interpreting some histological images.

Chapter 3. Characterisation of Gut Cholecystokinin Expression in *Rhombosolea leporina*

3.1 Introduction

The alimentary canal is lined with gastrointestinal hormones located in endocrine cells throughout the mucosal layer (Rao & Wang, 2010; Thomas et al., 2003). Some of the main peptide hormones involved in digestion, absorption, and the regulation of secretion and pancreatic functions include gastrin, secretin, and cholecystokinin (Buddington & Krogdahl, 2004; Rao & Wang, 2010). Cholecystokinin was selected as the physiological indicator of digestion in this study, as it is commonly used in diet-related research to evaluate digestive function and response.

3.1.1 Cholecystokinin hormone

Cholecystokinin (CCK) was first described by Ivy and Oldberg in 1928 and is now recognized as a key hormone in fish, present in both the brain and gut epithelium (Cho et al., 2023; Jo et al., 2021). It plays an important role in digestion by stimulating pancreatic secretion, promoting gallbladder contraction, facilitating gastric emptying, and signalling satiation to the brain to regulate food intake (Jo et al., 2021; Liddle, 1997; Micale et al., 2014; Rønnestad et al., 2007). It is mostly concentrated in the intestine, particularly in the anterior region (Hur et al., 2013; Jo et al., 2021). The hormone is released upon food ingestion and binds to CCK receptors in the gut epithelium, triggering a signal to the brain via the vagus nerve to modulate feeding behaviour (Li et al., 2023; Wang et al., 2010).

The presence and role of CCK have been investigated in various teleost species at different developmental stages. For instance, studies on larval European sea bass (*Dicentrarchus labrax*), turbot, olive flounder, and Atlantic halibut have reported that CCK receptor cells are predominantly located in the anterior intestine in species with coiled gut tracts, whereas species with straight guts exhibit a more dispersed distribution (Micale et al., 2014; Rønnestad et al., 2007). In a 2021 study on olive flounder, Khoa et al. examined *cck* gene expression during various juvenile stages and found that expression peaked following metamorphosis. Similarly, Cho et al. (2023) reported no *cck* expression in the esophagus or stomach of marbled flounder, but expression was detected at varying levels from the pyloric caeca to the anus. Like goblet cell distribution, *cck* expression patterns can vary both between and within species.

Determining the presence and function of genes in teleosts can be challenging due to whole genome duplication (WGD). This duplication is estimated to have occurred within vertebrates approximately 545 million years ago, with a subsequent event 320–350 million years ago affecting most teleost species (Alsop & Vijayan, 2009; Jaillon et al., 2004). The significance of WGD lies in its potential to create new gene functions. The duplication of genes, resulting in paralogs, can offer adaptive advantages by expanding genetic diversity and functional capacity (Glasauer & Neuhauss, 2014; Magadum et al., 2013). These duplicated genes may retain their original roles, become non-functional, or evolve new functions (Glasauer & Neuhauss, 2014; Muncaster et al., 2023).

Whole genome duplication events in teleost fish have contributed to the diversification of the *cck* gene. The CCK receptors, originally characterised in rodent models, were first classified as CCK-A (alimentary) and CCK-B (brain) but are now more commonly referred to as CCK1 and CCK2 (Dufresne et al., 2006). In Atlantic salmon, genome duplication has led to the presence of both CCK1 and CCK2 receptors, as well as two paralogs of the CCK2 receptor, termed *cck2-R1* and *cck2-R2*. Predominantly expressed in the pancreas was *cck1* whereas *cck2-R1* was higher in the midgut, and *cck2-R2* showed the strongest expression in the brain (Rathore et al., 2013). Thus, two of the three receptor genes were mainly associated with the digestive tract. A similar pattern was observed in olive flounder, where both *cck1* and *cck2* were expressed in the brain, but intestinal expression was dominated by *cck1*, with low levels of *cck2* and minimal expression of both in the stomach (Kurokawa et al., 2003). In addition to its role in the digestive tract, *cckb* expression in the hypothalamus is closely involved in regulating gonadotropin secretion, particularly follicle-stimulating hormone (FSH), thereby influencing reproductive processes (Cohen et al., 2023;

Cohen et al., 2024; Uehara et al., 2024). These findings emphasize the importance of considering gene duplication when studying *cck* expression. Both paralogs (*ccka* and *cckb*) should be investigated to determine whether they differ in expression and function across tissues.

3.1.2 Dietary impacts on cholecystokinin expression

Due to its role in digestion, *cck* expression is closely influenced by diet and is known to decline during periods of starvation (Babaei et al., 2017). For instance, in grass carp (*Ctenopharyngodon idella*), an agastric fish, *cck* expression decreased in both the brain and intestine during fasting but increased again once feeding resumed (Feng et al., 2012). This decline aligns with *cck's* role in signalling satiety, as reduced expression during starvation is expected. However, the response of *cck* expression to fasting is species-specific. In pacu (*Piaractus mesopotamicus*), for example, *cck* expression declines only in the gut and not in the brain, unlike in grass carp (Volkoff et al., 2017). In contrast, Atlantic salmon show no decline in intestinal *cck* expression during starvation but instead, expression levels remain stable in the gut and increase in the brain (Murashita et al., 2009; Volkoff et al., 2017).

Dietary protein is a key factor stimulating *cck* expression. In gilthead sea bream (*Sparus aurata*), a high-protein, low-carbohydrate diet led to significantly elevated *cck* levels compared to fish on a low-protein, high-carbohydrate diet (Babaei et al., 2017; Santos et al., 2020). Similarly, in yellowtail (*Seriola quinqueradiata*), high concentrations of fishmeal enhanced *cck* expression, indicating that both the quality and quantity of dietary protein influence *cck* secretion (Furutani et al., 2012). In red tilapia (*Oreochromis* sp.), protein was also shown to have a stronger impact on satiety than either carbohydrates or lipids (Santos et al., 2020).

These findings highlight the significant role of diet in modulating cck expression, reinforcing the importance of understanding how different dietary components affect digestive hormone expression. This knowledge is crucial for formulating feeds that not only meet the nutritional needs of aquaculture species but also promote efficient digestion and optimal feeding behaviour, which are key factors for enhancing growth and welfare in aquaculture.

3.2 Aim for Chapter 3

This chapter aims to quantitatively assess the expression of *cck1* (*ccka*) and *cck2* (*cckb*) in the alimentary canals of wild adult and juvenile yellowbelly flounder, as well as in juveniles fed experimental diets for a minimum of six weeks. The insights gained will contribute to the growing understanding of how dietary variation influences the expression of this critical gut hormone.

3.3 Methods

Quantitative gene expression of *cck* was measured on the same six fish used for histology (three wild adult and juvenile yellowbelly flounder which were dissected immediately after capture). Of the 14 diet-treated experimental fish used for histology 11 were used for *cck* gene expression (five mussel diet (MD), five pellet diet (PD) and one starved fish (S) as samples from the remaining starved fish were not stored in RNAlater). Following dissection, the fresh gut tissue was immediately fixed in RNAlater (Thermofisher Scientific) and were refrigerated overnight at 4°C before being moved to a -80°C freezer for storage in preparation for RNA extraction

3.3.1 Quantitative gene expression process and analysis

To analyse gene expression, RNA was extracted from all nine gut segments of wild adult (WA) and wild juvenile (WJ) yellowbelly flounder. For the experimental fish, the number of gut segments was reduced from nine to six, this included the esophagus, cardiac stomach, fundic stomach, anterior intestine, middle intestine, and posterior intestine. This reduction helped minimise the number of samples requiring extraction, conserving both time and resources. In total, 54 gut samples were extracted from the wild fish and 66 from the experimental diet groups (MD, PD, and S, Table 3.1). All RNA extractions, complementary DNA (cDNA) synthesis, and quantitative PCR (qPCR) procedures were conducted at the University of Waikato Tauranga laboratory.

Table 3.1. Number of replicates per gut segment and treatment (Wild Adults, Wild Juveniles, Mussel Diet, Pellet Diet and Starved).

Fish type	E	C	F	P	PC	AI	MI	PI	R	Total
WA	3	3	3	3	3	3	3	3	3	27
WJ	3	3	3	3	3	3	3	3	3	27
MD	5	5	5	0	0	5	5	5	0	30
PD	5	5	5	0	0	5	5	5	0	30
S	1	1	1	0	0	1	1	1	0	6

3.3.2 Primer design

Ribosomal protein L18 (*rpl18*) is a protein-coding gene commonly used as a reference gene in qPCR studies and was selected for this study (Hu et al., 2018; Xie et al., 2021). Another reference gene, TATA-binding protein (*tbp*), was also trialled; however, due to low amplification success, only *rpl18* was used in the final analyses. The expression of the *cck* hormone throughout the gut tract was the target of interest. Due to gene duplication events, two target genes, *ccka* and *cckb*, were examined.

As no published yellowbelly flounder sequences were available, two primer sets for each *cck* gene and *rpl18* gene were designed based on existing olive flounder (*Paralichthys olivaceus*) mRNA sequences available through the National Centre for Biotechnology Information (NCBI) database. Each primer set was tested at different annealing temperatures, and the most suitable set for each gene was selected based on qPCR cycling and melt curve results. Key information for each primer is provided in Table 3.2, and the full olive flounder gene sequences used for primer design are available in **Appendix B**.

Table 3.2. Primer sequences for reference (*rpl18*) and target (*ccka* & *cckb*) genes designed for the yellowbelly flounder. The average efficiency of each primer ± standard deviation was calculated.

Gene	Primer sequence (5'-3')	Annealing temp (°C)	Tm (°C)	Amplicon size (bp)	GC (%)	Efficiency (Mean%± SD)
rpl18	FW: GCTCCTGGTCAAGTTCGTACCG	64	63.2	140	57	0.94±0.03
(set 1)	RV: GACATCTTCATCTTACGGATCAGGC	64	61.6		48	
ccka	FW: AGCAACTACAACCAACTACAGGAGG	60	62.7	187	48	0.91±0.02
(set 1)	RV: AAGTCCATCCAGCCGACATAATCC	60	63.7		50	
cckb	FW: CTCGGCGAGACTCATCTCCAC	60	63.1	96	60	0.93±0.03
(set 2)	RV: CCTGTCCGCTATCCGATGG	60	63.8		63	

3.3.3 RNA extraction

RNA was extracted from RNAlater fixed tissue using the Direct-zol RNA Miniprep kit (Zymo Research Corporation). All gut samples were removed from -80°C storage in batches and allowed to defrost on ice before following the manufacturer's protocol for RNA extraction. Samples were transferred into a 2ml screw cap RNase/DNase free tube filled with 800µl TRIzol Reagent and 10-15 2.3mm diameter zirconia/silica beads. These were homogenized on a hard setting of 6800rpm for three, 20 second bursts with 30 second intervals in between, this setting was repeated 2-3 times. Some of the tougher adult samples like the esophagus needed more homogenising as tissue was still visible after the first one or two rounds. Once the TRIzol appeared clear with no remaining tissue the supernatant was pipetted out into a new 2ml RNase/DNase free tube filled with 800µl 100% ethanol.

These were briefly vortexed to fully combine before being transferred into a Zymo-Spin column and centrifuged at 13500rpm for one minute. The flow through was discarded and the column was transferred into a fresh collection tube. A DNase I treatment was then applied by adding

400µl RNA wash buffer to the column and centrifuging. Once complete, 75µl DNA digestion buffer and 5µl DNase I treatment was pipetted directly onto the filter in each column and left at room temperature (20-30°C) for 15 minutes.

After the 15 minutes, 400µl of Direct-zol PreWash was added to each column and centrifuged once more with the flowthrough being discarded. This was repeated to ensure the complete removal of the DNase treatment. Following this, 700µl RNA Wash Buffer was added to each column and centrifuged for two minutes as opposed to one. The column was carefully transferred into a new RNase/DNase free 1.5ml tube and 50µl DNase/RNase free water was added to elute the RNA held within the column filter by centrifuging for one minute. The samples RNA concentration and purity was ready to be tested on the Denovix DS-11 spectrophotometer before being used for cDNA synthesis with the excess RNA being stored in a –20°C freezer to reduce degradation.

3.3.4 RNA quantity and purity

To assess the concentration and purity of the RNA extracted a DeNovix DS-11 spectrophotometer machine was used where it was first blanked with 1µl of PCR-Grade water, wiped clean with a Kimwipe (Kimtech Science wipe) before 1µL of an RNA sample was loaded and red before being wiped clear and the next sample tested. The DeNovix measured the amount of light absorbed by a sample at a specific wavelength. A reading taken at 260 nm compared to 280 nm assessed the purity of nucleic acids and should be recorded at ~2.0 otherwise, it could indicate protein contamination (DeNovix Inc, 2023). Whereas the ratio of the 260 nm and 230 nm should also read ~2.0 but otherwise indicates the presence of unwanted organic compounds like TRIzol (DeNovix Inc, 2023). For each sample the A260, A260/230, A260/280 and concentration (ng/µL) were recorded (**Appendix C**)**,** and the concentration was used to calculate the amount of RNA needed during cDNA synthesis.

3.3.5 Complementary DNA synthesis

Complementary DNA (cDNA) was made from the newly extracted RNA and the Quantabio qScript XLT cDNA SuperMix kit was used which was kept in –20°C freezer until needed. Each cDNA sample synthesis must be a total volume of 20µl of which 4µl of that is the SuperMix and the remaining 16µl is a combination of RNA and PCR-Grade water (depending on the RNA volume being used). The volume of RNA used for each cDNA synthesis varied due to the concentration of RNA (previously determined from the DeNovix (**Appendix C**)**.** The amount of RNA to be targeted in each cDNA sample can be anywhere from 1-10µg, in this instance 2µg of RNA was targeted due to many of the RNA concentrations being relatively low. This meant that either all or most of the available 16µl was filled with RNA with little to no water added. The following equation was used to calculate the volume of RNA needed based off the DeNovix concentration reading (ng/µl).

$$RNA\,Volume\,(\mu l) = \frac{2}{\left(\frac{RNA\,conc\,(ng/\mu l)}{1000}\right)}$$

If the volume equated to more than the available 16µl then just 16µl of RNA was used. It is important to note that because of this not all of the cDNA was standardised perfectly due to not being able to include the correct amount of RNA. Similarly with the RNA concentrations being overall relatively low thus were only representative of a very small population.

Once all the RNA volumes were calculated cDNA could be synthesised using 200µL RNase/DNase free PCR tubes with the appropriate volumes of RNA, PCR-Grade water and SuperMix added to give a final volume of 20µl (**Appendix D**)**.** These tubes were then placed into a SimpliAmp Thermal Cycler and incubated at 25°C for 5 minutes, 42°C for 60 minutes, 85°C for 5 minutes before being held at 4°C. The cDNA was then transferred into a 1.5ml DNase/RNase free tube and stored at -20°C before being used for gene expression.

3.3.6 Relative quantitative PCR (qPCR)

A 48-well Magnetic Induction Cycler (MIC) qPCR machine was used to test the amplification of the cDNA samples by quantifying the expression levels of the reference gene *rpl18* and the target genes *ccka* and *cckb*. The concentrated cDNA was diluted with PCR-Grade water to either a 1/50 or 1/25 dilution, this was dependent on a successful amplification curve and Cq value. Often a

1/25 dilution was needed when testing the target genes as the template concentration was much lower compared to the reference gene. However, a more concentrated dilution increased the sensitivity of the assay to pipetting errors, making any discrepancies in sample volumes more noticeable and potentially impacting the consistency of the results thus extra care was required. The qPCR protocol used Quantabio Perfecta SYBR Green FastMix as the reagent, with each reaction containing 10 µL. Reactions were prepared in 200 µL DNase/RNase-free tubes containing the component ratios listed in Table 3.3, making a final volume of 20 µL per tube before being loaded into the MIC. Each sample was tested in duplicates, and every MIC run included no-template controls (NTCs) where PCR-grade water replaced the cDNA to check for potential contamination (Figure 3.1).

Table 3.3. Ratio of components for each qPCR reaction with the appropriate primer used (*rpl18*, *ccka* or *cckb*)

qPCR Component	Volume per reaction (µl)
Forward primer	1
Reverse primer	1
cDNA (1/50 or 1/25 dilution)	8
SYBR Green FastMix	10

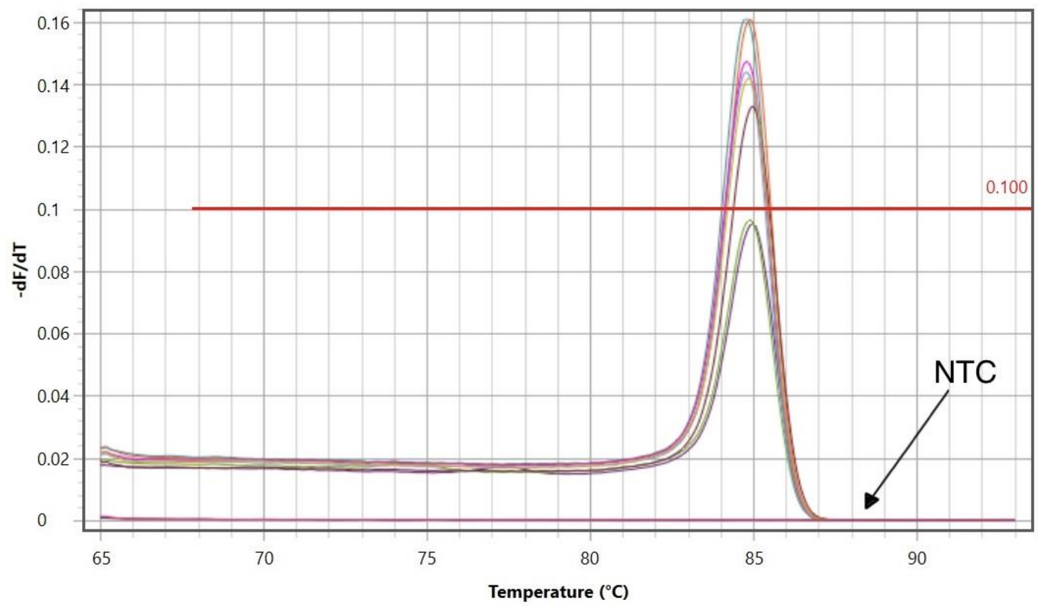


Figure 3.1. Melt curve using CCKA primer set 1, amplifying a single product in wild-caught adult yellowbelly samples with no amplification seen in the No Template Controls (NTC).

In addition to the NTCs, a positive control sample was included in each run as an inter-plate calibrator to verify consistency in the quantification cycle (Cq) values across runs. Each primer assay began with an initial hold at 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds and the appropriate annealing temperature for 30 seconds. A melt curve was generated at the end of the run, ranging from 65°C to 95°C, to confirm primer specificity and detect potential contaminants. Any samples showing divergent peaks were identified as potentially contaminated and were rerun to ensure reliable results (Figure 3.2).

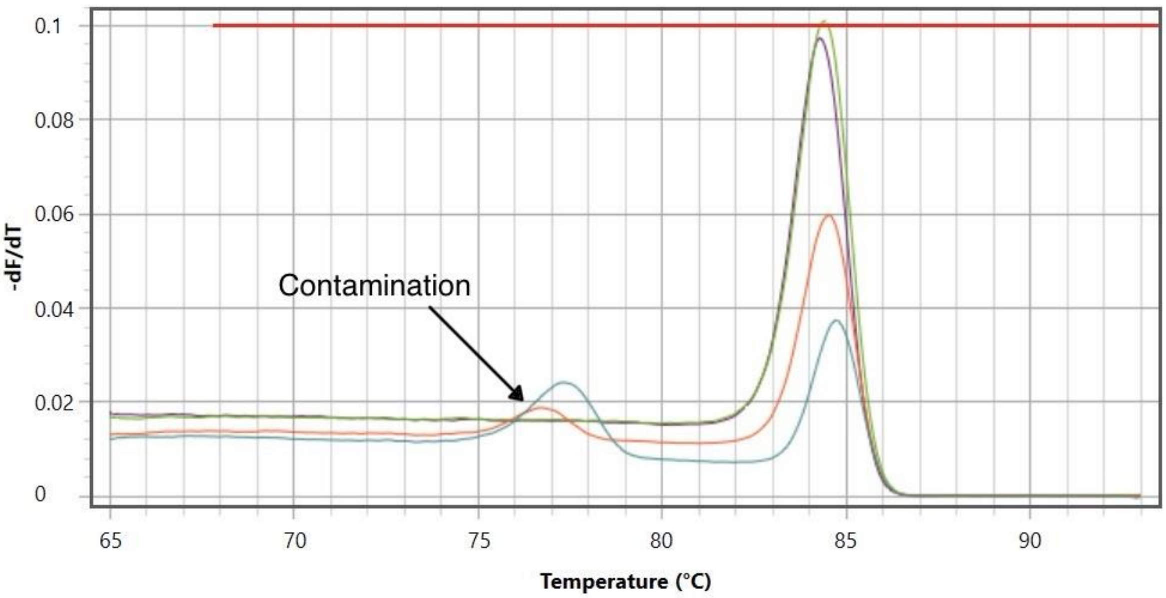


Figure 3.2. Melt curve using RPL18 primer set 1, amplifying multiple products in wild-caught juvenile yellowbelly samples with amplification seen in two of the samples therefore indicting contamination.

Cq values from each run that had a difference greater than 0.5 were also repeated (Institute for Research in Immunology and Cancer, n.d.). The efficiency of each sample was recorded, and most samples ran at >90% with only a few samples slightly less, if they were much less, they were also repeated to get a better efficiency result. Samples with a Cq value greater than 30 were excluded from the analysis, indicating that the gene concentration was very low. These samples were re-tested at a 1/25 dilution (if not already tried). If no improvement was observed, they were excluded from further analysis, resulting in a reduced sample size for some data sets.

3.3.7 Statistical analysis

The normalised fold expression of *ccka* and c*ckb* were calculated using the geomean of the relative normalised expression of the target genes (*ccka* or *cckb*) (Taylor et al., 2019, Figure 3.3). Relative quantity was calculated using the average efficiency for each gut segment, plus one, instead of the fixed value of two, e.g. $1.92^{\wedge \Delta Cq}$, as the latter assumes 100% efficiency.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Target	Biogroup_ sample	Mean Cq	Control group Avg Cq per Target	ΔCq per sample Per target (Col. 4-Col 3)	Relative quantity (RQ) 2^ΔCq	Norm. factor GEOMEAN [RQ (HPRT + Actin)]	Normalized expression per sample (A)	Log_2 Normalized expression (B)	Bio group expression (C)	Log_2(C) (D)	Standard deviation SD) of (B) (E)	Standard error of the mean (SEM) (F)	Confidence interval lower limit	Confidence interval upper limit
Actin	Untreated_S1	28.74	28.74	0.00	1.00									
Actin	Untreated_S2	28.85		−0.12	0.92									
Actin	Untreated_S3	28.63		0.11	1.08									
Actin	Treated_S4	28.84		−0.10	0.94									
Actin	Treated_S5	29.08		−0.34	0.79									
Actin	Treated_S6	28.76		−0.02	0.99									
HPRT	Untreated_S1	29.00	28.50	−0.50	0.71									
HPRT	Untreated_S2	28.00		0.50	1.41									
HPRT	Untreated_S3	28.50		0.00	1.00		$\frac{\text{RQ (DER5)}}{\text{Norm. factor}}$	Log_2 (A)	GEOMEAN(A)		SD(B)	$\frac{SD(B)}{\sqrt{n}}$	$(1+E)^{D-t*F}$	$(1+E)^{D+t*F}$
HPRT	Treated_S4	27.80		0.70	1.62									
HPRT	Treated_S5	29.20		−0.70	0.62									
HPRT	Treated_S6	28.50		0.00	1.00									
DER5	Untreated_S1	31.26	31.08	−0.18	0.88	0.84	1.05	0.07	1.00	0.00	0.11	0.06	0.83	1.20
DER5	Untreated_S2	31.01		0.07	1.05	1.14	0.92	−0.12						
DER5	Untreated_S3	30.97		0.11	1.08	1.04	1.04	0.06						
DER5	Treated_S4	32.65		−1.57	0.34	1.23	0.27	−1.87	0.43	−1.21	0.73	0.42	0.12	1.51
DER5	Treated_S5	32.03		−0.95	0.52	0.70	0.74	−0.43						
DER5	Treated_S6	32.40		−1.32	0.40	0.99	0.40	−1.31						

Figure 3.3. The model used for calculating the normalised fold expression (column C) of *ccka* and *cckb*. Actin and HPRT act as reference genes, which were replaced with rpl18 in this study. DER5 was originally the target gene, but in this study, *ccka* and *cckb* were substituted as the target genes (Taylor et al., 2019).

Due to there only being a single sample for each gut segment in the starved fish, it was not possible to use the normalised fold expression calculation as recommended by Taylor et al. (2019). This calculation considers variance, which is not possible with a single fish. Therefore, the Pffafl method was used to calculate relative expression ratios for the juvenile *cck* expression data. Despite the lack of statistical replication in the starved fish the effects of starvation on *cck* expression was considered biologically relevant to include for graphical comparison to that of the dietary treated juvenile fish. Where the target represents the *ccka* or *cckb* gene, the reference is the *rpl18* gene, and the control corresponds to the wild juvenile data.

$$\Delta Ct = Ct\ target - Ct\ reference$$

$$\Delta\Delta Ct = \Delta Ct\ sample - \Delta Ct\ control$$

$$RQ = 2^{-\Delta\Delta Ct}$$

Data analyses were performed using R Studio version 4.3.2 with the ggplot package. Normality was assessed using the Shapiro-Wilk test, and homogeneity of variances was tested with Levene's test. As the assumptions were violated, the data was log-transformed before conducting a Two-way ANOVA. However, log transforming the *cckb* data did not fix the violations of assumptions and so a Welch's corrected Two-way ANOVA was conducted instead to account for the non-normal distributions. This was followed by a post-hoc pairwise t-test with Benjamini & Hochberg correction as a Tukey test assumes the assumptions were met. A standard Two-way ANOVA was performed on the log-transformed *ccka* data to detect whether diet or gut region had an impact and if there was an interaction between those two. A post-hoc Tukey's test was used when significant results were found where $p < 0.05$. The starved diet group was excluded from analyses due to having a low sample size. One-way ANOVA's were not conducted on the wild adult and juvenile *cck* data due to sample sizes being smaller. Bar graphs were created in Excel.

3.4 Results

3.4.1 Wild adult yellowbelly flounder *cck* expression

In every gut region apart from the cardiac stomach, *ccka* was more highly expressed in the wild adults compared to *cckb*. While expression varied for both genes throughout the gut, the greatest difference was evident in the mid intestine where *ccka* expression was approximately seven-fold higher than that of *cckb* (1 ± 0.74 and 0.13 ± 3.17 respectively, Figure 3.4). This data was unable to be calculated for the wild juveniles due to having samples being excluded from the qPCR results and therefore not enough replicates to calculate the normalised fold expression.

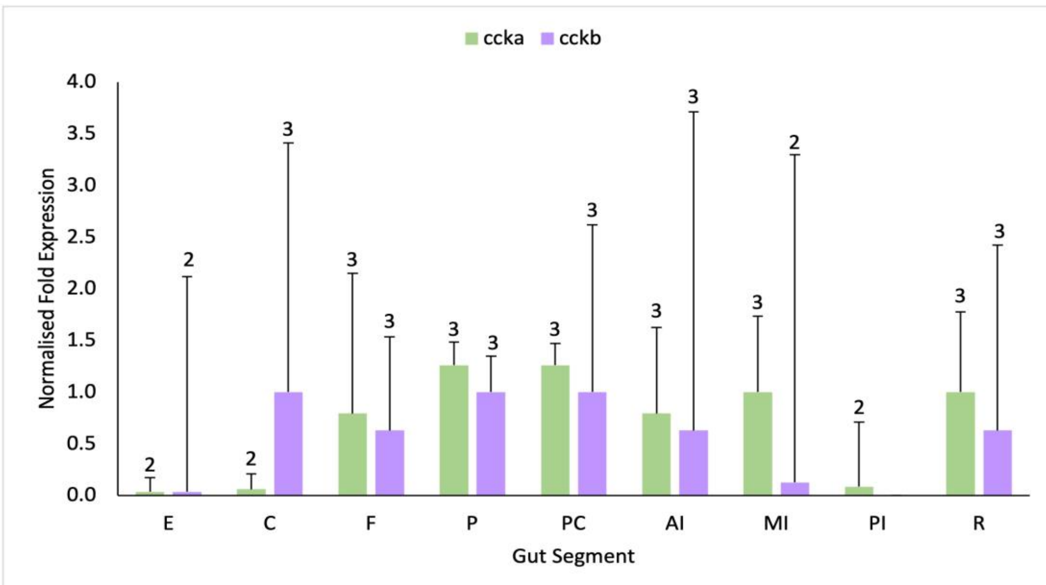


Figure 3.4. Normalised fold expression of *ccka* and *cckb* per gut segment from wild-caught adults. Sample sizes for each bar are displayed above the error bars. Error bars ±SE.

3.4.2 Diet impact on overall *cck* expression

In the mussel and pellet diet groups, *ccka* expression was greater than *cckb* in the fundic stomach, and all intestinal regions (Figures 3.5 & 3.6). Whereas *cckb* expression was greater in the esophagus for both diet groups and in the cardiac stomach region in just the mussel diet group.

Expression of *cck* was relatively uniform throughout the gut tract for both diet groups with a few exceptions.

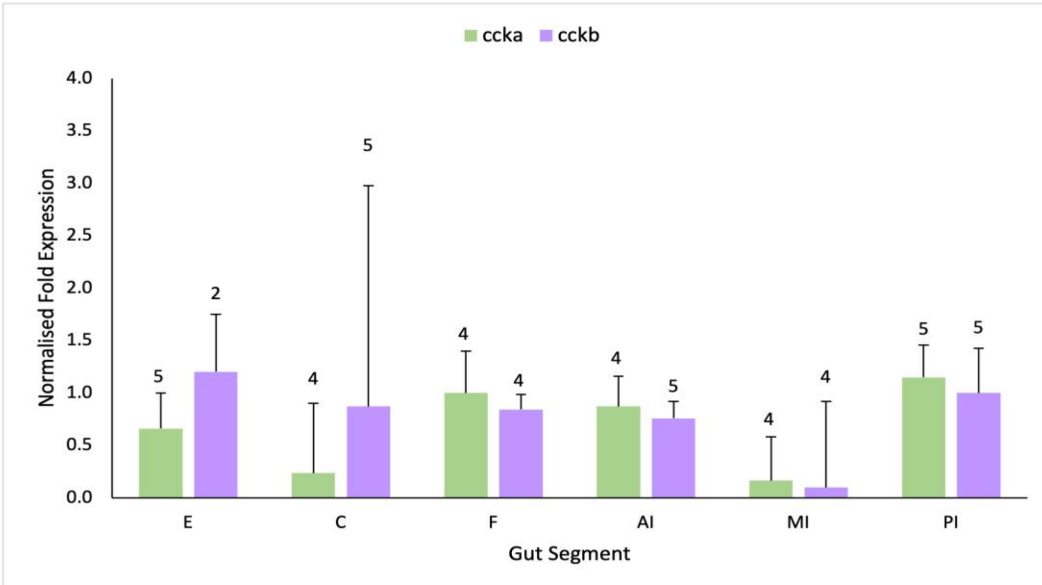


Figure 3.5. Normalised fold expression of *ccka* and *cckb* per gut segment from the mussel diet (MD) juveniles. Sample sizes for each bar are displayed above the error bars. Error bars ±SE.

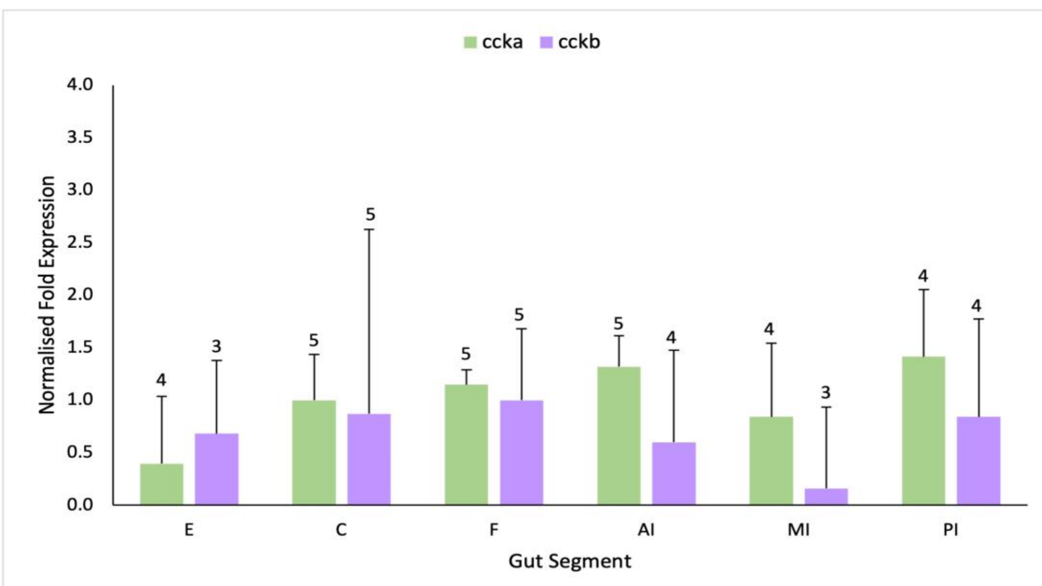


Figure 3.6. Normalised fold expression of *ccka* and *cckb* per gut segment from the pellet diet (PD) juveniles. Sample sizes for each bar are displayed above the error bars. Error bars ±SE.

3.4.3 Diet impact on *ccka* expression

Both the mussel and pellet diets exhibited similar levels of *ccka* expression throughout the gut, with two notable exceptions, the cardiac stomach and middle intestine (Figure 3.7). In these regions, the mussel diet showed lower expression levels compared to the pellet diet group (cardiac stomach: 0.24 ± 0.67 vs. 1 ± 0.44, middle intestine: 0.16 ± 0.42 vs. 0.84 ± 0.7).

There were significant differences found in *ccka* expression for both the diets ($F = 7.479$, $Df = 1$, $p < 0.01$) and gut region ($F = 3.693$, $Df = 5$, $p < 0.01$) however, there was no interaction between the two. Fish fed the pellet diet had greater *ccka* expression overall compared to the mussel diet ($p < 0.01$). Regardless of the diet the posterior intestine had the highest *ccka* expression and was significantly higher than both the mid intestine and esophagus ($p < 0.05$).

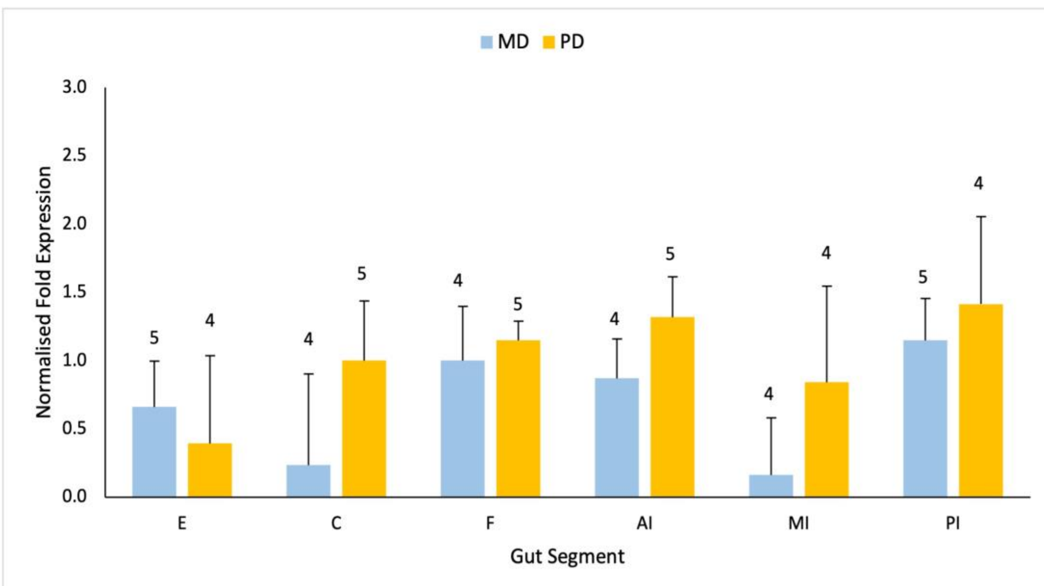


Figure 3.7. Normalised fold expression of *ccka* per gut segment from the mussel (MD) and pellet (PD) diet experimental groups. Sample sizes for each bar are displayed above the error bars. Error bars ±SE.

3.4.4 Diet impact on *cckb* expression

Similar results were found with the *cckb* expression which remained relatively consistent along the gut regions for both diet treatments (Figure 3.8). The esophagus showed the greatest variation in expression and the mussel diet had greater expression than the pellet diet (mussel diet: 1.20 ± 0.54, pellet diet 0.68 ± 0.70).

There was a significant difference in *cckb* expression in the gut regions ($F = 6.841$, $Df = 5$, $p < 0.001$) but no interaction was found between the diets and gut regions. Post hoc analysis revealed the cardiac stomach had significantly higher *cckb* expression when compared to the mid intestine ($p < 0.05$).

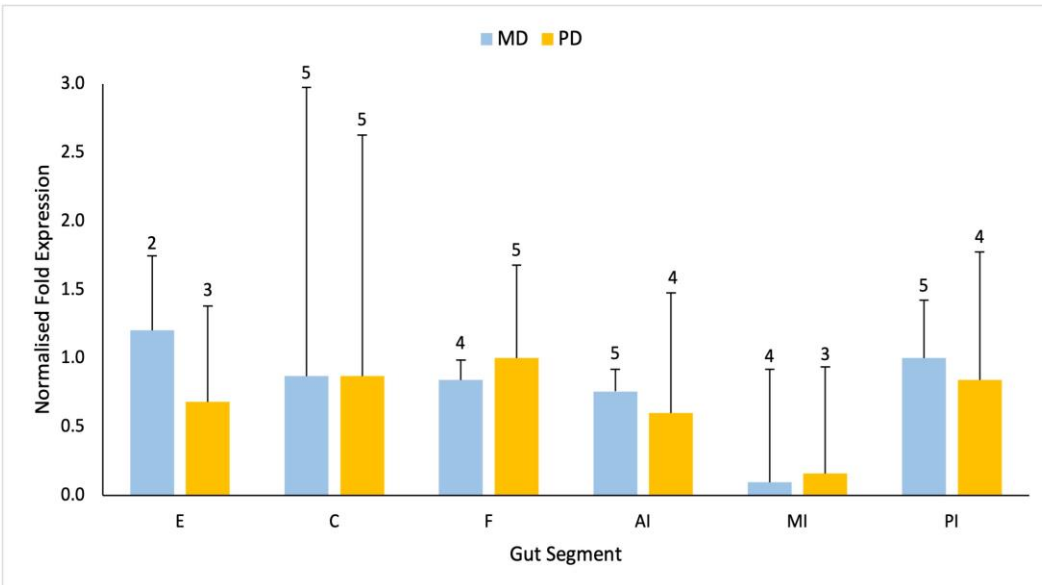


Figure 3.8. Normalised fold expression of *cckb* per gut segment from the mussel (MD) and pellet (PD) diet experimental groups. Sample sizes for each bar are displayed above the error bars. Error bars ±SE.

<u>3.4.5 Diet impact on the relative expression of cck</u>

Expression of *ccka* was upregulated in the middle intestine for the mussel and starved diet-treated groups compared to the wild juvenile group. However, it was downregulated across all three diet-treatments in the esophagus, cardiac and fundic stomach (Figure 3.9). In contrast, *cckb* expression was upregulated in the fundic stomach across all diet-treatments and was also upregulated in the middle intestine of just the starved group when compared to the wild juveniles (Figure 3.10).

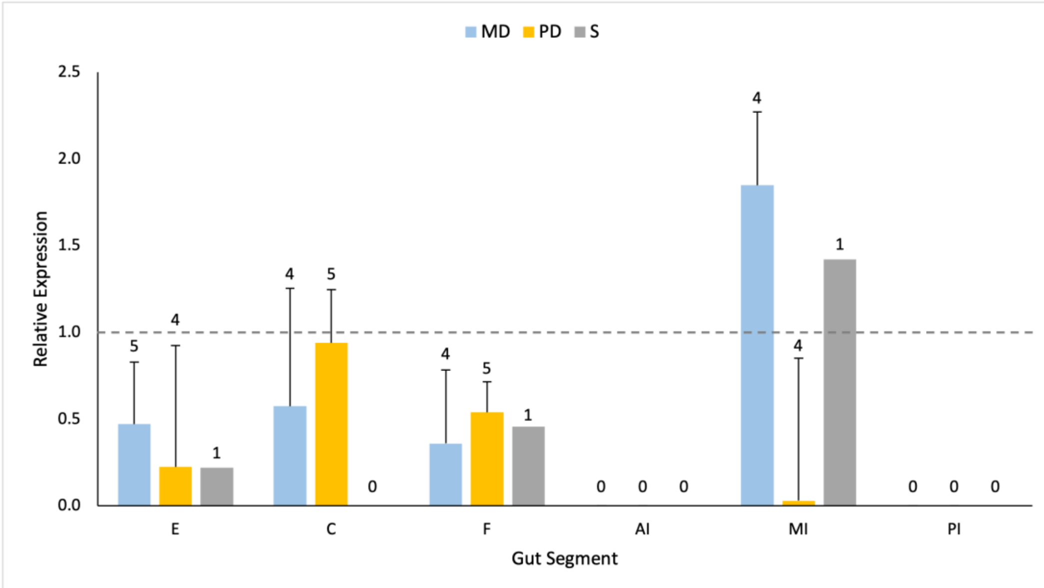


Figure 3.9. Relative expression of *ccka* per gut segment from the mussel (MD), pellet (PD) and starved (S) experimental groups. Sample sizes for each bar are displayed above the error bars. Error bars ±SE. No results were available for the anterior and posterior intestine (AI and PI) due to having no detectable *ccka* data from the wild juveniles to reference.

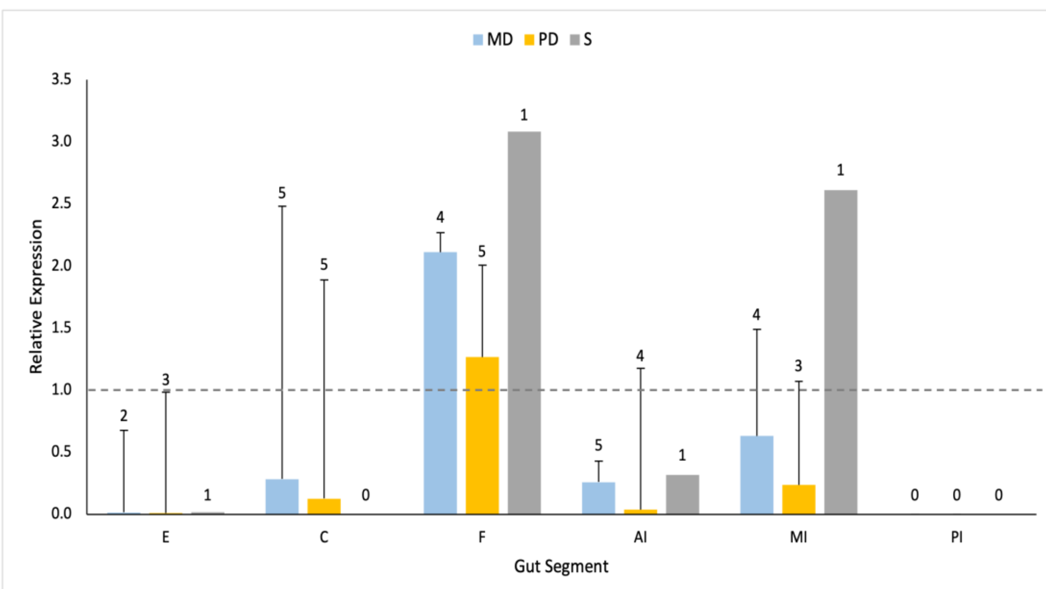


Figure 3.10. Relative expression of *cckb* per gut segment from the mussel (MD), pellet (PD) and starved (S) experimental groups. Sample sizes for each bar are displayed above the error bars. Error bars ±SE. No results were available for the posterior intestine (PI) due to having no detectable *cckb* data from the wild juveniles to reference.

3.5 Discussion

This chapter investigated the expression profiles of *cck* along the alimentary canal in wild adult and juvenile yellowbelly flounder, as well as captive juveniles fed different dietary treatments for six to ten weeks. The findings provide novel data on the gene regulation of a digestive hormone in this species. To date, limited research has explored both paralogs of CCK (*ccka* and *cckb*) expression in flatfish, particularly in terms of expression across the gut and the potential influence of diet. Results from this study indicate that both diet and gut region can influence *cck* expression in yellowbelly flounder, contributing to a better understanding of digestive gene regulation in flatfish and offering insight relevant to diet formulation in aquaculture.

The expression of *ccka* in this study followed patterns similar to those observed in olive flounder and Atlantic halibut, where *ccka* was typically the dominant paralog (Kurokawa et al., 2003; Gomes et al., 2022). In contrast, *cckb* expression was generally lower across all gut regions, except in the cardiac stomach of wild adults and mussel diet juveniles, and the esophagus of both mussel and pellet diet groups. This dominance of *ccka* over *cckb* suggests that *ccka* has retained its traditional digestive role following the teleost-specific whole genome duplication, whereas *cckb* may have undergone neofunctionalisation, adopting a distinct physiological role within the brain (Glasauer & Neuhauss, 2014; Gomes et al., 2022; Rathore et al., 2013). CCKB has been reported to be more involved in satiety, while CCKA is more directly involved in digestive contractions (Le et al., 2019a). Unexpectedly, neither *ccka* nor *cckb* showed a clear regional expression pattern along the gut in the wild adults or in the mussel and pellet-fed juveniles. Typically, *cck* expression is concentrated in the anterior intestine and diminishes towards the rectum, correlating with its role in gallbladder contraction, enzyme secretion, and stimulation of intestinal motility (Cho et al., 2023; Hur et al., 2013; Jo et al., 2021). The absence of this pattern in yellowbelly flounder supports the idea that this species may possess a more uniform, undifferentiated gut tract without clear functional regionalisation.

Further findings support the idea that yellowbelly flounder lack a stomach and distinct gut regions, as reflected in the regional expression patterns of *cck*. The low expression of both *cck* paralogs in the esophagus of the wild adults aligns with previous research showing that *cck* expression typically begins in the anterior intestine which is likely corresponding to the cardiac stomach region in this species (Hernández et al., 2018; Hur et al., 2013; Hur et al., 2016). In the mussel and pellet diet-treated groups, *ccka* expression was significantly higher in the posterior intestine compared to the middle intestine and esophagus, while *cckb* expression was significantly elevated in the cardiac stomach compared to the middle intestine. These findings are consistent with those reported in the agastric ballan wrasse, where *cckb* expression is dominant in the anterior intestine and *ccka* expression increases along the posterior gut (Lie et al., 2018; Le et al., 2019a). Assuming the yellowbelly flounder has a similarly undifferentiated gut that visually resembles a continuous intestinal tube, this expression pattern suggests functional parallels. In this context, dominance of *cckb* in the anterior gut (i.e., the cardiac stomach) and higher expression of *ccka* in the posterior intestine support the idea that these regions have distinct physiological roles. Since *ccka* is associated with promoting gut motility, its lower expression in the anterior intestine may help slow transit to allow time for digestion in the absence of a stomach. As food moves along the tract, increased *ccka* expression in the posterior regions may reflect a need to stimulate motility and facilitate waste expulsion (Le et al., 2019a).

The diets used in this study influenced *cck* expression, with differences particularly evident in the esophagus when comparing the diet-treated groups to wild adults. This variation may reflect developmental differences, as the diet-treated groups consisted of juveniles, while the wild adults had matured on a wild natural diet. Such dietary differences over time may lead to functional specialization of gut regions, including the esophagus (Rønnestad et al., 2013). Notably, *ccka* expression was significantly higher in fish fed the pellet diet than those fed the mussel diet. This may be attributed to differences in nutrient composition, specifically, protein content. Higher dietary protein levels have been shown to stimulate *cck* expression in species such as gilthead sea bream (*Sparus aurata)* and Japanese amberjack (*Seriola quinqueradiata*) (Babaei et al., 2017; Liang et al., 2024; Murashita et al., 2008). This could explain the elevated *ccka* expression in fish fed the protein-rich formulated pellet. In contrast, diet had no significant effect on *cckb* expression, which aligns with its post-duplication neofunctionalization, where *cckb* is likely predominantly associated with brain-related functions rather than digestive roles (Le et al., 2019a; Rathore et al., 2013).

Relative expression results revealed an interesting trend when comparing diet-treated fish to wild caught juveniles. Most *ccka* and *cckb* expression levels appeared downregulated in the diet-treated groups after the experimental period, in contrast to the juveniles dissected immediately after wild capture. This may suggest that the natural diet consumed by wild juveniles stimulates higher levels of *cck* to support digestion however, *cck* expression is known to increase shortly after food intake (Jeon et al., 2020). As the wild juveniles were dissected on the day of capture, it is likely they had recently fed, whereas the experimental groups were fasted for at least 12 hours prior to sampling likely resulting in lesser *ckk* expression. For instance, *cck* levels increased within 1.5–3 hours in yellowtail and 1–3 hours in Siberian sturgeon (*Acipenser haerii*) following feeding (Murashita et al., 2007; Zhang et al., 2017). Conversely, fasting has been reported to decrease *cck* expression in species like gilthead sea bream, although other studies have reported variable outcomes, including upregulation or no change (Babaei et al., 2017; Jeon et al., 2020). In this study, the starved group showed inconsistent relative expression patterns of *ccka* and *cckb* across gut regions, making it difficult to identify a consistent trend. However, given that this group consisted of a single fish and the wild juvenile comparison was also based on a small sample, these findings should be viewed as preliminary.

The unexpected expression of *cck* throughout the entire alimentary canal suggests that yellowbelly flounder may share digestive characteristics with agastric species such as the ballan wrasse. This implies that typical *cck* expression patterns observed in other teleosts may not apply to this species. Notably, *ccka* was predominantly expressed over *cckb*, although this dominance may not extend to the brain. While these findings offer valuable insights into yellowbelly flounder digestion with implications for aquaculture and feed formulation, a larger sample size would help strengthen the conclusions. This is particularly important as some wild juvenile samples had to be excluded due to low gene expression levels. It is possible that rapid post-capture dissection allowed residual gut contents to contaminate tissue samples, highlighting the need to carefully control this in future experimental designs.

Chapter 4. General Discussion

This study aimed to characterise the gut morphology of yellowbelly flounder (*Rhombosolea leporina*) across developmental stages and assess how experimental diets may influence gut structure and function. By combining histological, morphometric, and molecular analyses, this research offers new insights into the digestive anatomy and physiological responses of this species to different dietary treatments.

The yellowbelly flounder is currently commercially harvested, yet wild populations have been declining, highlighting the need for sustainable aquaculture solutions. Based on the success of other farmed flatfish such as European turbot and olive flounder, yellowbelly flounder may be well suited to land-based recirculating aquaculture systems (Daniels & Watanabe, 2010; Stieglitz et al., 2021). The findings from this study offer important implications for advancing aquaculture of this species, particularly in feed development. Overall, the results suggest a potential ontogenetic shift in feeding behaviour, where yellowbelly flounder transition from an omnivorous diet during early life stages to a more carnivorous diet as they mature. This developmental shift implies that juveniles may exhibit greater dietary flexibility, which is encouraging for aquaculture feed formulation. The observed dietary flexibility, coupled with minimal histological impacts from the diet treatments, suggests that yellowbelly flounder may tolerate a range of feed types during early life stages. This is advantageous in aquaculture, where cost-effective, sustainable, and nutritionally balanced feeds are essential (Manam, 2023). However, the gut responses also indicate sensitivity not only to protein content but to the physical consistency of feeds. The lack of a distinct stomach structure further highlights the importance of optimizing feed digestibility, texture, and nutrient composition to support efficient digestion, absorption, and growth in this potentially agastric species.

4.1 Formulating diets for an agastric species

Designing feeds for agastric species such as the supposed yellowbelly flounder requires careful consideration of both nutritional content and physical characteristics, particularly because these fish lack a stomach for initial food storage and enzymatic breakdown. As a result, digestion and nutrient absorption must occur entirely along the intestine. Although agastric and gastric fish generally have similar nutritional requirements, their strategies for acquiring and processing nutrients can differ markedly due to anatomical constraints. For example, Day et al. (2011) found that both carnivorous needlefish (*Tylosurus gavialoides* and *Strongylura krefftii*) and herbivorous halfbeaks (*Hyporhamphus regularis ardelio* and *Arrhamphus sclerolepis krefftii*) exhibited comparable levels of trypsin and lipase, key digestive enzymes involved in protein and lipid digestion. Despite their dietary differences, these species maintained similar enzyme levels to meet their physiological needs, however, the way they sourced nutrients varied. Herbivores consuming lower protein diets fed more continuously and relied more heavily on carbohydrate metabolism, while carnivores consumed less frequently but retained food for longer durations to optimize nutrient extraction (Day et al., 2011).

In the case of yellowbelly flounder, which appear to lack a true stomach and instead possess a relatively uniform gut morphology, a strategy of frequent feeding and extended intestinal retention likely compensates for the absence of gastric processing. Some agastric fish, such as the ballan wrasse, require a longer intestine to retain food for prolonged periods, facilitating the extraction of nutrients as food moves through the digestive tract (Le et al., 2019b). The feeding ecology of yellowbelly flounder, which leads to the ingestion of undigested materials like mud and silt, further supports this interpretation, suggesting that they rely on volume-based intake and prolonged intestinal digestion to meet their nutritional needs.

This highlights the importance of formulating feeds that are not only nutritionally appropriate but also physically suited for gradual and extended digestion. Feeds must be formulated to achieve an optimal feed conversion ratio, which depends not only on nutritional composition but also on the physical characteristics of the pellet, such as size, shape, and texture (Manam, 2023). The histomorphometric results from this study suggest that yellowbelly flounder may have a gut better suited to processing softer foods, which should be considered when formulating diets. Pellets that are too firm may cause digestive disturbances or hinder nutrient assimilation, while overly soft feeds risk premature nutrient leaching. However, in species where mechanical feed breakdown is limited, softer textures may facilitate digestion by reducing the workload on the gut (Cahu & Infante, 2001; Sorenson, 2012). Such considerations are essential for supporting growth, digestion, and feed efficiency in agastric species.

4.2 Feed opportunities for the yellowbelly flounder

The dietary flexibility observed in the yellowbelly juveniles also opens the door to the use of alternative ingredients, including plant and insect-based proteins. These alternatives must offer good digestibility, palatability, nutrient utilisation, availability, and affordability, while being low in anti-nutritional factors (ANFs), which can impair nutrient uptake (Glencross et al., 2007; Hodar et al., 2020; Prabu et al., 2017). The absence of adverse histological changes across experimental diets suggests a degree of gut plasticity in juvenile yellowbelly flounder that may allow them to accommodate novel feed components which is an important consideration given the environmental costs of traditional fishmeal and fish oil (Back et al., 2020; Choi et al., 2020).

In fish, nutrient absorption occurs in both the stomach and the intestine. Protein digestion begins in the stomach via the enzyme pepsin and continues in the intestine through enzymes such as trypsin (Bougatef, 2013; Fabay et al., 2022). The stomach provides an acidic environment due to hydrochloric acid secretion, which activates pepsin and contributes to the denaturation of dietary proteins (Fabay et al., 2022; Gomes et al., 2014; Koelz, 1992). This combination of acidity and enzymatic action aids in breaking down proteins and reducing the potency of protein-based ANFs such as trypsin inhibitors found in plant alternatives such as soybean (Chakraborty et al., 2019; Krogdahl et al., 2010; Norvdi et al., 2023). Because enzymes have a limited pH range in which they function, the acidic stomach phase acts as a barrier that partially degrades ANFs before they reach the intestine (Krogdahl et al., 2010; Smith, 1978). As a result, gastric species may experience less severe effects from ANFs. However, high inclusion levels can still cause problems, as seen with intestinal inflammation in Atlantic salmon fed 30% soybean meal compared to a fishmeal-based diet (Krogdahl et al., 2023). As little inclusion of 20% in the diet of olive flounder showed reduced growth, palatability issues and poor amino acid profiles (Deng et al., 2006). This highlights the need for moderation when using plant-based ingredients. Anti-nutritional factors can also be pre-treated, such as through heat extrusion, to reduce their effects (Hajra et al., 2013).

In contrast, agastric species lack this acidic phase, allowing ANFs to enter the intestine largely intact (Smith, 1978). This may lead to stronger inhibition of digestive enzymes, resulting in reduced protein digestion, slower growth, and increased disease susceptibility (Tao et al., 2025). However, a trial in Nile tilapia (*Oreochromis* niloticus) supplemented with exogenous enzymes like protease showed improvements in growth and health, suggesting this approach could help mitigate ANF impacts (Tao et al., 2025). Therefore, if yellowbelly flounder are indeed agastric, plant-based ingredients may not be well tolerated unless ANFs are treated effectively, or enzyme supplementation is trialled. Given these factors, alternative protein sources such as insect meals may offer a more suitable and sustainable option for this species.

Insect-based proteins are increasingly recognised as viable substitutes for fishmeal, particularly black soldier fly larvae (BSFL) (Mohan et al., 2022). The BSFL substitute is being actively explored as a sustainable alternative due to its favourable amino acid profile, high protein content, and low environmental footprint (Khieokhajonkhet et al., 2022; Mohan et al., 2022). One consideration with BSFL, however, is its chitin content, a structural polysaccharide that can be difficult to digest in some species (Cha et al., 2008). For example, inclusion levels above 1% in the diets of Atlantic salmon have been associated with negative growth outcomes, whereas species such as Atlantic cod (*Gadus* morhua) and Atlantic halibut tolerated inclusion levels up to 5% without adverse effects (Karlsen et al., 2015). Despite potential digestibility challenges, chitin may offer immunostimulatory and prebiotic benefits, particularly in species that naturally consume chitin-rich diets (Pascon et al., 2025; Perry et al., 2020). In the case of yellowbelly flounder, chitin may pose less of a concern during adult stages, as wild populations are known to consume chitin from their intake of crustaceans such as crabs. Indeed, Tran et al. (2022) identified that optimal chitin levels for marine teleosts such as the olive flounder may be as low as 2.2%. However, juvenile flounder, such as those examined in this study, are unlikely to encounter high levels of chitin in their natural diets and may therefore be more sensitive to its inclusion. Thus, while BSFL holds promise as a feed ingredient, its application in juvenile diets should be approached cautiously. Further trials assessing histological and molecular responses to insect meal would help determine its suitability for early life stages of this species.

4.3 Limitations and future recommendations

One of the main constraints of this study was the small sample size of juvenile yellowbelly flounder, which impacted the reliability of the results, particularly where lower-quality histology slides and low gene expression concentrations were involved. Increasing the sample size in future studies would improve the statistical power and consistency of both histological and gene expression data. Extending the duration of the experiment could also allow for more meaningful observation of dietary effects over time. Including adults in the dietary trial, alongside juveniles, would offer valuable insight into whether developmental stage influences dietary response.
Another consideration is the feeding status of wild-caught fish. Cholecystokinin (*cck*) expression in wild juveniles and adults may have been elevated due to recent feeding prior to capture, which could limit direct comparability with experimental diet groups that were fasted for at least 12 hours before dissection. Additionally, the use of wild-caught flounder posed challenges, as transitioning these fish to formulated feeds was difficult which has also been noted in the greenback flounder (Daniels & Watanabe, 2010). Utilizing hatchery-reared fish in future trials would help streamline feeding and reduce stress, hopefully improving experimental outcomes.

This study represents a step toward establishing foundational knowledge on the gut physiology of New Zealand yellowbelly flounder. However, recent research highlights an additional role for *cck* in regulating reproduction in teleost fish (Cohen et al., 2023; Cohen et al., 2024; Uehara et al., 2024). Studies in other flatfish have shown that *cckb* is more prominently expressed in the brain than *ccka* (Gomes et al., 2022; Kurokawa et al., 2003; Le et al., 2019a; Rathore et al., 2013).

Brain expression of *cckb* has been linked to the regulation of follicle-stimulating hormone (FSH), a key gonadotropin which typically stimulates the production of gonadal sex steroids to initiate gonadogenesis (Cohen et al., 2023; Cohen et al., 2024; Uehara et al., 2024). While CCKA is primarily involved in functions such as enzyme secretion, gallbladder contraction and gut motility, CCKB may play a more dominant role in the brain signalling satiety (Jeon et al., 2020; Rønnestad et al., 2007). Exploring *cckb* expression in the brain of yellowbelly flounder could not only clarify its role in reproduction but also indicate whether dietary treatments influence this paralog in the brain, as was observed for *ccka* in the gut. Controlling reproduction is an essential step toward domestication of a species, and understanding these distinct regulatory roles would provide deeper insight into how CCK supports both digestion and reproduction in this species.

While this study suggests that the yellowbelly flounder may be agastric, this conclusion warrants further investigation. The absence of visible gastric glands in histological slides does not definitively confirm the lack of a stomach. Gastric glands secrete hydrochloric acid and pepsin which are key components in protein digestion (Gabaudan, 1986; Koelz, 1992) thus testing for pepsin activity in gut regions could provide a more robust assessment of gastric function (Gomes et al., 2014). An additional limitation was the absence of growth data. Although growth rate is a critical indicator of dietary suitability, it was not measured due to the small size and fragile condition of the juveniles, and the exploratory nature of this trial. Including growth measurements in future studies would help clarify the functional outcomes of different diets. For example, one might expect the starved group to show reduced growth compared to the mussel and pellet-fed groups. Similar outcomes have been reported in other species, such as grouper (*Epinephelus fuscoguttatus*♀×*E. lanceolatus*♂) and Nile tilapia, where periods of starvation resulted in decreased digestive enzyme secretion and slower weight gain (Liu et al., 2020; Sakyi et al., 2020).

Finally, future research should explore the use of alternative diets, such as those that replace fishmeal, with more sustainable protein sources to examine their effects on both gut morphology and growth performance. This information will be crucial for formulating feeds that not only support optimal growth and feed conversion ratios but also align with sustainability goals in aquaculture.

4.4 Conclusion

This study represents the first in-depth investigation into the alimentary canal of the yellowbelly flounder, examining gut morphology and dietary effects through histological analysis of tissue structure, goblet cell distribution, and expression of the digestive hormone cholecystokinin. The findings suggest the possible absence of a stomach in this flatfish, as well as a notable adaptability of juveniles to different diets however, further research is required to confirm these patterns. Minimal variation observed between the three dietary treatments indicates a potential resilience to alternative feeds, highlighting the species' suitability for sustainable diet development aimed at achieving low feed conversion ratios. Collectively, these insights expand the knowledge base for yellowbelly flounder cultivation in New Zealand, supporting efforts to replenish wild stocks and meet growing commercial demand.

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Appendices

Appendix A

Recipe for the gel-pellet diet

- 1 tsp powdered gelatin
- 1 Tbsp crushed fishmeal pellet
- 1 ½ Tbsp boiling water

1. Pellets were crushed into a fine crumb consistency using a mortar and pestle
2. Gelatin was mixed through the crushed pellets and poured into a small dish
3. Boiling water was added to the powder mixture and stirred thoroughly for ~30 seconds to dissolve the gelatin
4. Mixture was left to cool at room temperature before being moved to the fridge to set
5. Once set the gel-pellet feed could be removed from the dish and sliced and diced into small mouth sized cubes when needed to feed to the juvenile flounder. When not needed the feed was stored in the fridge for 3-5 days before a new batch was freshly made.

Appendix B

I. mRNA sequence of the *rpl18* gene of olive flounder used for primer design (sourced from the NCBI database). The forward and reverse (reverse compliment) sequences are highlighted

```
>XM_020093129.2 PREDICTED: Paralichthys olivaceus ribosomal protein
L18 (rpl18), mRNA
TCCTCACAGATGTTATTTGACCGTCACCCGCAGGAAGCGCGTGCTCACCAGCCAATCAGATTGCCGAGAT
TTCACAGCATCGTTTATCGCGAGACCTCGGCTCTTCCTTTTCCCTCTTCGCTCTGAGTCCAAGATGGGAG
TCGACATCAGGCACAACAAGGACCGTAAGGTGCACAGGAAAGAGCCAAAGAGCCAGGATATCTATCTGAG
GCTCCTGGTCAAGCTGTACAGGTTCCTGGCCCGTCGCTCTAATGCTTCCTTCAACAAGGTGGTCCTGAGG
AGGCTCTTCATGAGCAGGACCAACAGGCCTCCCATCTCCATCTCCCGCCTGATTCGTAAGATGAAGATGC
CTGGCCGTGAGAACAGAACCGCTGTTGTTGTGGGAACAGTCACTGATGATGTCAGGATTCAGGATATCCC
CAAGCTTAAGATCTGTGCTCTGAGGGTTACAGATGGTGCCCGCCGCAGAATCCTGAAGGCAGGCGGTCAG
GTGATGACCTTTGACCAGCTGGCTTTGGCTTCTCCCAAAGGACAGGGCACAGTGCTGCTGTCAGGACCCC
GCAAAGGCAGAGAGGTGTACAGGCACTTTGGAAAAGCCCCTGGAACCCCTCACAGCCACACCAAGCCCTA
CATCCGCTCCAAGGGCAGGAAGTTCGAGAGAGCTCGTGGTCGCAGAGCCAGCCGTGGCTACAAGAACTAG
TGTCTTTTTGTTTTTCATGCAATCTGATACAAATAAAAGATTTGGCTGTATAAAATGA
```

II. mRNA sequence of the *ccka* gene of olive flounder used for primer design (sourced from the NCBI database). The forward and reverse (reverse compliment) sequences are highlighted

```
>XM_020091065.2 PREDICTED: Paralichthys olivaceus cholecystokinin a
(ccka), mRNA
TCTCTGTCCCTCAGTCACGGCCTCAACACTGGACGTGCAAAGCTCAGCATCCTCAGCCACACCAACAGCC
GCAGTCATGAATGTAGGTATCTGTGTGTGTGTTCTCCTGGCTGCCCTGTCCAGCAGTTCCCTGGGTCTGC
CCTCACAGTCCATGCCACAGAGAGCTGAGGGTGAGGCTCTCATGTCAGACAGCCTGCCCCCCCCCTCATC
CAACCACACGCGTCAGGCCCGCTCAGCTCCAGCGCCACCCTCAGGGC

AGCTCGCCAACTACAACCAACAC
AAGGAGAACACAGACCCTCGAAACAGCCTGAACCAGCTTCTGGCCAGACTCATCTCCAGGAAAGGCTCTC
CCCACCAGACCAGATCCTCCCTCACCAGCAGAGCCAGTGGTCTTGCCCCGGGCCACAGGATAAAGGACAG
AGATTATCTCGGCTGGATGGACTT

TGGGAGACGCAGTGCAGAGGAGTATGAATACTCCTCCTAAAGGCAT
GGCCCCCTTCTACTGAAAAAACCTGAGTTACAAAAAAACAACAACCCTTAACCGCCAATAAAAGTTCGCA
GAATTAATGAGCCCTTTTCACACTGCATTTACTGTAACCCAGGGCTGAGGTGACTGGATCTGAGTGAACT
ATATAATGATGTGGCTTAACACAAAAAATTGCTTTCCTGGGATTTCCTGGTTAAAATGAAAAATATCATC
AAACCAGAATATCACCAGACATTGACGCTATAAAACAAGGTCAAACTCAGCTTTAACTTGTGTTTAAAGC
TCAACAGACATTTACGCTTAAGTCTTAATACTTAATTCTTATATTATAGTAATAATATTTTAAATCAAGA
ATAATATACTTGCAATTGACCCCTAACATTTGGATGGTATGAGAAATGATTCTCAAGCAAGAAATTTGCT
TTAAAATTGAATTTCAGCCTTATTTCTGTGAGAGTGAATGTGTTAAGGCTCAAGTTTATGCCACTTAAAT
AAGTACATGTGTTAGAGGCCTTTTATTGGACCTAAATGAGCAACCAAATTTAAGCAGAAGTGCAGATTTA
TGTCTGTATCAGCTCAGATCACTTCAATTTTACACCAGATCAGATTTCGATAAAACATCTCACTATGTCA
TGTGCATCTGAAGAGAACAGAGAGCTGTGGTATTAAGAATCAACAGGTTCAAGTGTCAGTGTGGAGTCAG
```

TAACAGTTTCTATGGACTGGAAATAAAACAGCACCTGAAGGTAACCCTGGGCTGAAAATGTGTAGTCTGA
AAAGCACAGAATGTTTTGAAAAGCACAACGTGTCTATATGAGACGCTTGGTAGTCTCCCTATCATTGCAT
AATGGACTTTAGTGGCACACACATTGAAAATCAGGACTACCAGTACTGTAGATATGAGTTGAGGAGAGTC
TTTCTTTCTTTTGGAGGATCATCACATACGTATGTATATATGCATTCTTACCGGTTCGGTCTGTATGTTT
CTACAAACTTTCAGATTCAGCACAGTTCAAAATAAAGCTCCACTCTCTGACCACA

III. mRNA sequence of the *cckb* gene of olive flounder used for primer design (sourced from the NCBI database). The forward and reverse (reverse compliment) sequences are highlighted

>XM_020090543.2 PREDICTED: Paralichthys olivaceus cholecystokinin b (cckb), mRNA
ACACACACACACAGTGTGCTGAGCGGGGAGTATAAAAGGGGCATCCTTCCCACTCAGCAGAGAAGTACTC
TCCTCAGTCTCCCACTCTCCTCCAACACGCTGAACCTCTTTTCTCACGATGACTGCAGGGCTGTGTGTGT
GTGTCCTTTTGGCAGTCCTGTGTACAAGCTGTTTGGGGCACCCCATCTCCTCTCAGCACCTAGATGAGGG
CCAGCGCTCCATCTCCACTCCCTCTGAAGCCCTCCTTGAGGCGGACACCCACAGCTTGGGAGAGCCCCAC
CTCCGACAAAGCCGCTCTGCCCCCCAGCTGAAATCTCTTCCTGTGGCTGAAGAGGATGGAGACTCCCGGG
CCAACCTCAGCGAGCTG
CTGGCAAGACTCATCTCGTCCAGGAAAGGTTCTGTGCGCAGAAACTCCACGGC
GTACAGCAAAGGACTGAGCCCCAACCCATCGGATAGCAGACAGG
GACTACTTGGGCTGGATGGATTTCGGG
CGCCGCAGCGCAGAGGAGTACGAGTACTCCTCGTAAAAAGGGCAAACAAGAAAAAGAGGAAAAAACCCCA
TGCAACCTGGCCCCTCGGTGTCTCACAATAAGAGTCTATTTATGATGTATTTGTTGTACATTTGTTTGTA
AAACTGTAATAATGCAATATACATATATGCCAAATTTTGCAGAAAAGCTCCCACTGTCCAAGGTTTGTTT
GTTTCTGGTTTTCTCTGGTTTCTTTATCATTTTTTGGGTGGAAAGGATCCAGGAGCTGTCAGGGTCCGTT
GAGACTAACTGGACTGAATAAAGACTGAAAATATCA

Appendix C

Additional Table C. DeNovix readings of the purity and concentration of each samples RNA

Fish type	Sample	A260	A260/230	A260/280	Concentration (ng/µL)
WA20	462	0.19	1.34	1.74	7.61
WA20	465	0.55	0.89	1.71	21.83
WA20	468	0.88	1.76	1.91	35.14
WA20	471	0.77	1.80	1.95	30.98
WA20	474	3.59	2.15	1.97	143.46
WA20	477	1.07	2.09	1.82	42.66
WA20	480	0.97	1.30	1.78	38.60
WA20	483	0.73	1.56	1.85	29.26
WA20	486	0.65	1.66	1.77	26.12
WA20	462B	0.39	1.34	1.91	15.68
WA20	468B	0.41	1.90	1.95	16.58
WA20	471B	0.72	2.05	2.01	28.96
WA21	489	2.57	2.11	1.97	102.81
WA21	492	3.17	2.17	1.94	126.76
WA21	495	3.19	2.10	1.96	127.53
WA21	498	1.29	1.93	1.87	51.62
WA21	501	4.14	2.10	1.96	165.68
WA21	504	1.19	2.07	1.92	47.66
WA21	507	1.82	2.00	1.95	72.95
WA21	510	2.18	1.86	1.95	87.36
WA21	513	4.19	2.14	1.97	167.79
WA21	489B	1.16	1.97	1.96	46.23

WA21	489C	0.07	0.45	3.52	2.86
WA21	498B	0.37	1.53	1.78	14.63
WA21	498C	1.08	1.23	1.99	43.28
WA22	516	1.77	1.74	1.94	70.76
WA22	519	1.71	2.12	2.19	68.52
WA22	522	1.47	1.90	2.02	58.65
WA22	525	2.36	2.09	2.05	94.44
WA22	528	2.10	2.10	2.12	83.82
WA22	531	1.80	1.98	2.16	71.98
WA22	534	2.33	1.33	2.13	93.33
WA22	537	1.95	2.05	2.25	78.14
WA22	540	2.63	2.07	2.12	105.00
WJ17	381	2.54	1.96	2.02	101.67
WJ17	384	1.77	1.99	2.20	70.61
WJ17	387	3.54	2.06	2.04	141.46
WJ17	390	2.92	1.98	2.05	116.68
WJ17	393	2.89	2.14	2.11	115.59
WJ17	396	4.18	2.08	2.07	167.28
WJ17	399	2.16	1.95	2.04	86.25
WJ17	402	1.84	1.82	2.13	73.53
WJ17	405	2.21	1.79	2.04	88.22
WJ18	408	1.03	1.44	2.18	41.05
WJ18	411	1.89	1.95	2.01	75.54
WJ18	414	1.65	1.78	2.10	66.07
WJ18	417	2.56	1.99	2.16	102.59
WJ18	420	2.10	1.98	2.09	84.04

WJ18	423	1.48	1.74	2.16	59.20
WJ18	426	2.58	0.98	1.74	103.21
WJ18	429	0.35	1.29	2.20	14.10
WJ18	432	0.91	1.61	2.16	36.57
WJ19	435	1.01	2.01	1.96	40.24
WJ19	438	0.87	1.67	2.06	34.75
WJ19	444	1.54	1.92	1.98	61.73
WJ19	447	1.88	1.92	2.03	75.04
WJ19	450	1.17	1.04	1.88	46.95
WJ19	453	1.43	1.12	1.92	57.34
WJ19	456	0.52	0.58	1.55	20.93
WJ19	459	0.76	2.04	2.09	30.41
MD11	219	0.84	1.73	1.98	33.57
MD11	222	0.99	1.95	1.91	39.97
MD11	225	4.53	2.18	2.00	181.31
MD11	234	2.34	2.04	1.98	93.42
MD11	237	0.76	2.03	2.06	30.50
MD11	240	1.40	1.97	2.01	55.86
MD12	246	1.75	1.17	1.96	69.92
MD12	249	2.42	2.00	1.92	96.86
MD12	252	4.01	2.18	1.98	160.47
MD12	261	3.17	2.21	1.96	126.84
MD12	264	1.57	1.99	1.95	62.97
MD12	267	2.26	1.93	1.93	90.60
MD13	273	1.92	2.18	1.97	76.88
MD13	276	2.75	1.88	1.92	110.19

MD13	279	2.44	2.15	1.99	97.56
MD13	288	1.83	1.99	1.94	73.16
MD13	291	1.26	1.48	1.87	50.55
MD13	294	1.53	0.89	1.76	61.16
MD14	300	2.73	1.31	1.84	109.08
MD14	303	2.58	1.82	1.94	103.38
MD14	306	3.63	2.00	2.02	145.16
MD14	315	2.49	2.13	1.98	99.42
MD14	318	2.35	1.86	1.94	94.13
MD14	321	1.26	1.38	1.89	50.58
MD15	327	1.76	1.92	1.93	70.55
MD15	330	1.82	2.08	2.03	73.68
MD15	333	1.66	2.07	2.04	66.60
MD15	336	1.76	2.07	2.06	70.23
MD15	339	1.79	2.13	2.06	71.65
MD15	342	2.43	1.94	2.08	97.39
MD15	345	1.80	2.00	2.02	72.07
MD15	348	1.40	2.01	1.97	56.01
S23	543	1.21	1.73	1.97	48.27
S23	546	3.03	2.17	2.00	121.12
S23	549	2.97	2.11	1.94	118.63
S23	558	2.42	2.06	1.96	96.79
S23	561	0.95	1.91	1.96	37.81
S23	564	1.20	1.72	1.95	48.18
PD10	192	0.52	1.15	1.86	20.70
PD10	195	0.93	2.04	1.99	37.39

PD10	198	1.40	2.02	1.99	56.11
PD10	207	1.48	1.48	1.92	59.29
PD10	210	1.46	1.54	1.91	58.33
PD10	213	0.80	0.89	1.73	32.03
PD16	354	0.49	0.77	1.64	19.71
PD16	357	1.66	1.02	1.90	66.33
PD16	360	2.66	2.17	1.99	106.33
PD16	369	1.91	1.48	1.92	76.44
PD16	372	2.03	2.04	1.98	81.30
PD16	375	0.77	1.67	1.95	30.61
PD7	111	0.42	1.71	2.04	16.80
PD7	114	1.24	1.94	1.98	49.50
PD7	117	1.97	2.11	1.98	78.80
PD7	126	1.55	1.68	1.93	61.94
PD7	129	0.97	1.34	1.91	38.87
PD7	132	0.72	1.67	2.08	28.98
PD8	138	0.45	1.09	1.79	17.89
PD8	141	1.33	0.77	1.67	53.13
PD8	144	1.16	1.62	1.93	46.40
PD8	153	0.65	1.55	1.95	25.92
PD8	156	0.56	0.96	1.90	22.37
PD8	159	0.60	1.65	2.00	23.96
PD9	165	0.40	1.63	1.93	15.98
PD9	168	1.56	1.58	1.91	62.52
PD9	171	1.32	1.91	1.91	52.70
PD9	180	0.96	1.84	1.89	38.29

PD9	183	0.83	1.78	1.98	33.35
PD9	186	0.98	0.82	1.75	39.27

Appendix D

Additional Table D. Concentration of RNA required, and the volume of reagents used for each samples cDNA synthesis

Fish type	Sample	RNA required (µL)	RNA added (µL)	Water (µL)	Supermix (µL)	Total (µL)
WA20	462	197.1	16.0	0	4	20
WA20	465	68.7	16.0	0	4	20
WA20	468	42.7	16.0	0	4	20
WA20	471	48.4	16.0	0	4	20
WA20	474	10.5	10.5	5.5	4	20
WA20	477	35.2	16.0	0	4	20
WA20	480	38.9	16.0	0	4	20
WA20	483	51.3	16.0	0	4	20
WA20	486	57.4	16.0	0	4	20
WA20	462B	127.5	16.0	0	4	20
WA20	468B	120.6	16.0	0	4	20
WA20	471B	69.1	16.0	0	4	20
WA21	489	14.6	14.6	1.4	4	20
WA21	492	11.8	11.8	4.2	4	20
WA21	495	11.8	11.8	4.2	4	20
WA21	498	29.1	16.0	0	4	20
WA21	501	9.1	9.1	6.9	4	20
WA21	504	31.5	16.0	0	4	20
WA21	507	20.6	16.0	0	4	20
WA21	510	17.2	16.0	0	4	20

WA21	513	8.9	8.9	7.1	4	20
WA21	489B	32.4	16.0	0	4	20
WA21	489C	700.5	16.0	0	4	20
WA21	498B	102.5	16.0	0	4	20
WA21	498C	46.2	16.0	0	4	20
WA22	516	28.3	16.0	0	4	20
WA22	519	29.2	16.0	0	4	20
WA22	522	34.1	16.0	0	4	20
WA22	525	21.2	16.0	0	4	20
WA22	528	23.9	16.0	0	4	20
WA22	531	27.8	16.0	0	4	20
WA22	534	21.4	16.0	0	4	20
WA22	537	25.6	16.0	0	4	20
WA22	540	19.0	16.0	0	4	20
WJ17	381	19.7	16.0	0	4	20
WJ17	384	28.3	16.0	0	4	20
WJ17	387	14.1	14.1	1.9	4	20
WJ17	390	17.1	16.0	0	4	20
WJ17	393	17.3	16.0	0	4	20
WJ17	396	12.0	12.0	4	4	20
WJ17	399	23.2	16.0	0	4	20
WJ17	402	27.2	16.0	0	4	20
WJ17	405	22.7	16.0	0	4	20
WJ18	408	48.7	16.0	0	4	20
WJ18	411	26.5	16.0	0	4	20

WJ18	414	30.3	16.0	0	4	20
WJ18	417	19.5	16.0	0	4	20
WJ18	420	23.8	16.0	0	4	20
WJ18	423	33.8	16.0	0	4	20
WJ18	426	19.4	16.0	0	4	20
WJ18	429	141.9	16.0	0	4	20
WJ18	432	54.7	16.0	0	4	20
WJ19	435	49.7	16.0	0	4	20
WJ19	438	57.6	16.0	0	4	20
WJ19	444	32.4	16.0	0	4	20
WJ19	447	26.7	16.0	0	4	20
WJ19	450	42.6	16.0	0	4	20
WJ19	453	34.9	16.0	0	4	20
WJ19	456	95.6	16.0	0	4	20
WJ19	459	65.8	16.0	0	4	20
MD11	219	59.6	16.0	0	4	20
MD11	222	50.0	16.0	0	4	20
MD11	225	11.0	11.0	5	4	20
MD11	234	21.4	16.0	0	4	20
MD11	237	65.6	16.0	0	4	20
MD11	240	35.8	16.0	0	4	20
MD12	246	28.6	16.0	0	4	20
MD12	249	20.6	16.0	0	4	20
MD12	252	12.5	12.5	3.5	4	20
MD12	261	15.8	15.8	0.2	4	20

MD12	264	31.8	16.0	0	4	20
MD12	267	22.1	16.0	0	4	20
MD13	273	26.0	16.0	0	4	20
MD13	276	18.2	16.0	0	4	20
MD13	279	20.5	16.0	0	4	20
MD13	288	27.3	16.0	0	4	20
MD13	291	39.6	16.0	0	4	20
MD13	294	32.7	16.0	0	4	20
MD14	300	18.3	16.0	0	4	20
MD14	303	19.3	16.0	0	4	20
MD14	306	13.8	13.8	2.2	4	20
MD14	315	20.1	16.0	0	4	20
MD14	318	21.2	16.0	0	4	20
MD14	321	39.5	16.0	0	4	20
MD15	327	28.3	16.0	0	4	20
MD15	330	27.1	16.0	0	4	20
MD15	333	30.0	16.0	0	4	20
MD15	336	28.5	16.0	0	4	20
MD15	339	27.9	16.0	0	4	20
MD15	342	20.5	16.0	0	4	20
MD15	345	27.8	16.0	0	4	20
MD15	348	35.71	16.0	0	4	20
S23	543	41.43	16.0	0	4	20
S23	546	16.51	16.0	0	4	20
S23	549	16.86	16.0	0	4	20

S23	558	20.66	16.0	0	4	20
S23	561	52.90	16.0	0	4	20
S23	564	41.51	16.0	0	4	20
PD10	192	96.6	16.0	0	4	20
PD10	195	53.5	16.0	0	4	20
PD10	198	35.6	16.0	0	4	20
PD10	207	33.7	16.0	0	4	20
PD10	210	34.3	16.0	0	4	20
PD10	213	62.4	16.0	0	4	20
PD16	354	101.5	16.0	0	4	20
PD16	357	30.2	16.0	0	4	20
PD16	360	18.8	16.0	0	4	20
PD16	369	26.2	16.0	0	4	20
PD16	372	24.6	16.0	0	4	20
PD16	375	65.3	16.0	0	4	20
PD7	111	119.1	16.0	0	4	20
PD7	114	40.4	16.0	0	4	20
PD7	117	25.4	16.0	0	4	20
PD7	126	32.3	16.0	0	4	20
PD7	129	51.5	16.0	0	4	20
PD7	132	69.0	16.0	0	4	20
PD8	138	111.8	16.0	0	4	20
PD8	141	37.6	16.0	0	4	20
PD8	144	43.1	16.0	0	4	20
PD8	153	77.2	16.0	0	4	20

PD8	156	89.4	16.0	0	4	20
PD8	159	83.5	16.0	0	4	20
PD9	165	125.2	16.0	0	4	20
PD9	168	32.0	16.0	0	4	20
PD9	171	37.9	16.0	0	4	20
PD9	180	52.2	16.0	0	4	20
PD9	183	60.0	16.0	0	4	20
PD9	186	50.9	16.0	0	4	20