Dietary lipid modulation of intestinal serotonin in Ballan wrasse- *Labrus bergylta* – In vitro analyses

Master's degree in Aquaculture biology

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Front page illustration: *L. bergylta* produced at MH Labrus in 2018. Photo from Oda Kvalsvik Stenberg

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Abstract

Salmon lice infections by *Lepeophtheirus salmonis* represent one of the biggest challenges for the salmon industry. Intensive use of chemical delousing methods has led to resistance in lice against several of the chemicals, this has sparked an interest for the use of cleaner fish as an efficient delousing solution. A goal for several salmon farmers is that farmed cleaner fish should replace wild catches, this has led to commercial farming of Ballan wrasse. In order to optimize the cultivation, knowledge about B. wrasse digestive physiology (normal functioning and regulation) and its nutritional requirements are equally important. Serotonin (5-HT) has been proposed to be an important molecule at regulating gut motility involved in digestion of nutrients as well as appetite regulation. Serotonin function in B. wrasse is unknown, elucidating its role might contribute to a better understanding of the gastrointestinal functionality that might help to overcome farming limitations of this cultured specie.

There were two separate aims of this study. A transcriptomic part; revealing the expression of genes in the intestine regulated by lipid ingestion, emphasizing genes associated to lipid and 5-HT metabolism. Here, the aim was to observe the dietary lipid modulation of the gene expression over time (post prandial incubation time from 10m to 3 hours). In addition to this, my aim was to investigate the amount of 5-HT in the gut of Ballan wrasse from the same intestines to detect potential correlation to gene expressions involved in 5-HT metabolism. These investigations were done, *in vitro* on *ex vivo* intestines of Ballan wrasse, using a lipid diet (hydrolysed fish oil).

Results showed significant response of only one gene involved in 5-HT metabolism (TPH2) and 3 genes involved in lipid metabolism after feeding (ELOVL1, PLIN2 and PPARG). Interestingly enough, 5-HT was observed to be present in the gut of Ballan wrasse with a tendency to increase 3 hours after feeding. Also, by analysing genome and transcriptomic data, I propose the presence of enterocromaffin cells (EC cells) in the gut of B. wrasse. EC cells theoretically, produce the majority of intestinal 5-HT synthesized through the enzyme TPH1. Unlike TPH1, its isoform TPH2 (exclusive to enteric neurons, another 5-HT producing cell) was observed to be significantly upregulated by feeding, suggesting an active role in nutrient sensing.

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1. Introduction

Atlantic salmon (*Salmo salar)* is a highly valued anadromous fish species that is mainly exploited in commercial and recreational fisheries (Forseth et al. 2017). The Norwegian aquaculture industry has undergone a tremendous development over the last decades with a total income growth of more than 300 % for the last 10 years. The annual production of salmon accounted for 1.3 million tons in 2016 (Directorate of Fisheries 2016) and it is expected to rise as the world population continuously grows which will have a direct impact on the need for protein-rich, nutritious food. Although aquaculture have the potential to contribute to this increasing food demand, it still faces many challenges such as how to handle spill water, escapees, water and waste recirculation, viral and bacterial diseases and the urgent need for sustainable ecto-parasite treatments (Christiansen and Jakobsen 2017).

1.1. The sea lice problem in salmon aquaculture

Sea lice are ecto-parasites of many species of fish and are a current serious threat to Atlantic salmon (*Salmon salar*) populations in Norway. The sea lice problem affects wild salmon populations to the extent that they may be critically endangered or lost, and farmed salmon resulting in severe clinical pathology (Bjordal 1990; Roth 1993; Torrissen et al. 2013). If the lice problem is not properly addressed, there is a large likelihood of even further reductions and losses in the future (Christiansen and Jakobsen 2017).

The specie that infest Atlantic salmon and has been found to be most problematic in European salmon farms is *Lepeophtheirus salmonis (Imsland et al. 2014)*. Sea lice grazes on the skin and mucosal tissue of salmon, resulting in skin erosion (open wounds) and sub epidermal haemorrhage. If sea lice are not removed, they might cause osmotic stress and ease secondary infections risking salmon welfare, which might ultimately result in death (Skiftesvik et al. 2013; Imsland et al. 2014). Sea lice reproduce year-round and therefore, a successful lice control strategy would be focused on the control of juvenile and pre-adult stages, preventing the appearance of gravid females (Burridge et al. 2010).

Medicinal treatments have historically been used to prevent the occurrence of high sea lice abundance. This has resulted in drug resistant parasites occurring on farmed and possibly wild salmon which have been already reported in Chile, Scotland, Canada, and Norway (Aaen et al. 2015).

1.1.1. Alternatives to the use of chemicals for sea lice removal: Cleaner fish

Methods other than chemical, have been designed and used in-situ by farmers. First farmers started to hang onions out in the cages when they first identified lice (Bjordal 1990). New and modern methods such as light-traps, shading cages, sound or electrical stimuli to repeal lice have been tested out as sea lice expanded but none presented promising solutions to the problem (Costello 1993). A more recent mechanical method that consists of immersing infected salmon in fresh water baths was believed as of great potential treatment for lice. However, Stone et al. (2002) concluded that short baths in fresh water (3h) did not significantly affected the survival of lice. On the other hand, Powell et al. (2015) reviewed a positive reduction in attached stages of lice in salmon after freshwater baths (likely due to mechanical action during the pumping of fish from the cages to the fresh water sites). Regardless its efficiency, this treatment represents a hypo-osmotic challenge for salmon that may result in significant osmotic stress for the fish with short-term acute physiological effects (Powell et al. 2015) and therefore, it cannot be considered as a good alternative.

Biological controls have a brighter and promising future against sea lice. Many authors claim that the utilization of cleaner fish is at present the most developed and environmentally sustainable alternative method for lice control, it is less expensive compared to medicinal treatments, and can be managed in ways causing no apparent stress to salmon (Groner et al. 2013; Skiftesvik et al. 2013; Imsland et al. 2014). Several different wrasse species has been used to aid in the control of sea lice.

1.2.**Labrus bergylta (Ballan wrasse)**

1.2.1. Ballan wrasse as cleaner fish

Up to date, Ballan wrasse (*Labrus bergylta*) is the biggest and most robust of the available wrasse species and has the greatest potential for large-scale biological delousing (Gagnat 2012). However, Ballan wrasse is temperature sensitive making it unfit for low temperatures (Imsland et al. 2014).

The use of cleaner fish in the production of Atlantic salmon and Rainbow trout in Norway surpassed 26 million fish in 2015 (Directorate of Fisheries 2016) being mainly caught from their natural habitat by the fisheries industry and provided to the farms (Skiftesvik et al. 2014). This growing demand of wrasses (Figure 1) increase the pressure on wild populations and represents a new challenge for the industry. In order to cope with this dramatic increase of the fishing pressure and be able to meet the demand from salmon farms, intensive culture of Ballan wrasse needs to be quickly developed.

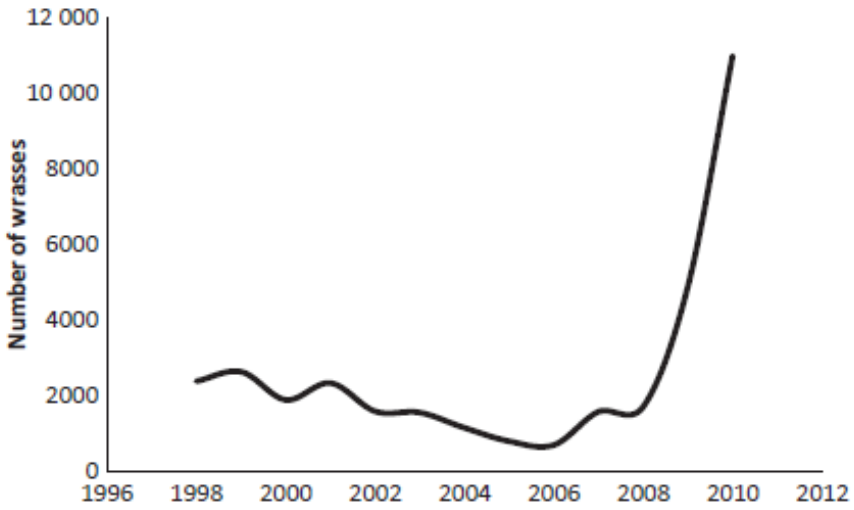

Figure 1: Catches of wrasses in Norway for use as cleaner fish in salmon cages. From (Torrissen et al. 2013).

Nonetheless, as the cultivation of this specie is new, anatomical, physiological and functional knowledge is needed for optimal rearing.

1.2.2. Wild VS. Cultivated stock

As mentioned above, Ballan wrasse populations are being over exploited for salmon delousing and this creates the necessity to cultivate them. Apart from the obvious decrease of the current pressure on wild wrasse, farmed Ballan wrasse can alleviate concerns about the risk of wrasse to salmon disease transmission as reviewed by Groner et al. (2013). Another advantage of farming wrasse might be the possibility to implement breeding programs. Breeding programs for faster growth in aquatic species have showed to have improved feed conversion and higher survival (Gjedrem et al. 2012), implying that the use of selectively bred wrasse individuals might lead to improve the biological efficiency of its production.

Scientists have wondered however whether farmed Ballan wrasse fed by dry feeds and grown in captivity was as efficient as wild individuals at removing sea lice. With the purpose of clear up that issue, Skiftesvik et al. (2013) reported an extremely efficient delousing behaviour of cultivated individuals that proved to be as efficient as wild individuals despite not having previous contact with sea lice.

Despite the promising role of farmed Ballan wrasse as cleaner fish in salmon aquaculture, there is still much to be elucidated to enhance a successful co-inhabitation, for instance, optimal ratio wrasse/salmon per cage and temperature optimum for Ballan wrasse. Also, the optimal size of Ballan wrasse for large salmon (>2kg) has not been reported as such and further aggressive behaviour

between salmon a Ballan wrasse is yet to be explored (Skiftesvik et al. 2013). One of the biggest challenges that is considered critical for production of viable offspring is the apparently lack of appetite and feed intake in the juvenile phase of farmed individuals (Hamre et al. 2013).

1.2.3. General intestinal physiology

The mechanisms by which gastric fish species digest and absorb nutrients have been widely studied. However, unlike most farmed fish (except cyprinids), Ballan wrasse lack stomach and pyloric caeca besides having a rather short gut.

The intestinal tract of Ballan wrasse (Figure 2) comprises about 1.5-2% of the total body weight and its length represents only 2/3 of the total body length (Hamre et al. 2013). The gut is short and lies in a loop (Z-shaped), the liver with the gall bladder and a prominent bile duct is connected to the proximal gut. The pancreas is commonly referred as pancreatic tissue and appears to be mixed with adipose tissue and distributed along the gut.

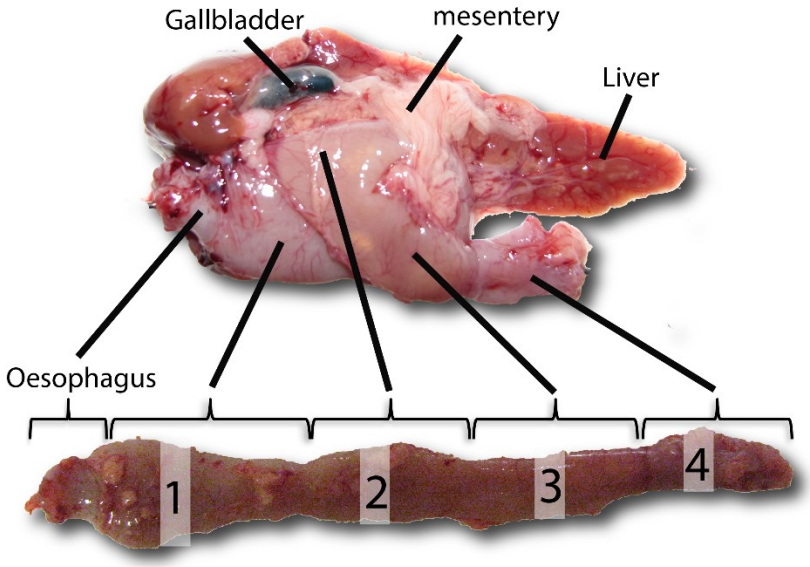

Figure 2. Figure showing the digestive organs of the Ballan wrasse out-lighting the gut. Numbers from 1 to 4 refer to the different segments of the gut being 1 the anterior part and 4 the hindgut. From (Lie et al. 2018).

Lie et al. (2018) reported the higher nutrient digestion and uptake in the anterior part of the gut compared to the posterior by observing a declining expression gradient, anterior to posterior, for most genes involved in general nutrient digestion. They also showed the similarity in the gene profile between intestinal segments 1-3, and the much larger number of differentially expressed genes between segment 1-3 and segment 4 (hindgut), suggesting that the hind-gut of Ballan wrasse differs substantially from the rest of the gut.

1.2.4. Feeding and diet approaches

Wild populations base their diet on benthic invertebrates such as molluscs, decapod crustaceans, and sea urchins (Figueiredo et al. 2005).

Studies on the digestive tract and its response to feed composition have been conducted over the last years aiming to find the optimal feed and feeding regime. A first approach was done regarding the nutritional requirements of juvenile Ballan wrasse where wild populations of Ballan wrasses were also analysed and their nutritional status was assumed to be of good health, setting the bases to make comparisons. Hamre et al. (2013) showed that the optimal composition of diets for juvenile individuals was 65% protein, 12% lipid, and 16% carbohydrate. Also of note is that not only the amount of nutrients is important but also the quality. This specie is highly sensitive to diet quality and seems to have specific nutritional requirements. Variations in the quality and composition of protein and lipid sources have large effects on growth (Øystein Sæle, personal communication).

A second approach was published by Lie et al. (2018) in an attempt to determine the genetic basis for the digestive system function of Ballan wrasse. They reported the loss of all known genes related to the stomach function and claimed substantial changes in the appetite control. As stomach is mainly involved in protein digestion, these authors suggested that the lack of genes related to stomach digestion might require formulated diets with higher levels of digestible protein than those for gastric species.

The last approach was done by (Le et al. in prepp) in regards of the evacuation rates in the gut of Ballan wrasse. They observed that after 4 and up to 8 hours 90% of the digesta was transferred from the first to the second segment of the gut, dismissing that the first segment has a pseudogaster function (storage function). They also claimed that differences in dietary water levels (dietary moisture level) had no or limited effect on digestibility and gut evacuation rate.

Despite interesting studies on Ballan wrasse have recently been published , further research on the feeding physiology of this fish is crucial to determinate the optimal feed formula and optimal feeding practices aiming towards a successful cultivation of Ballan wrasse (Dunaevskaya 2012).

1.3. Lipid and fatty acids metabolism in Ballan wrasse

The digestive process involves a series of events starting with ingestion of food, followed by the secretion of enzymatic and fluid secretions, digestion via mechanical and enzymatic processes, absorption, motility (including evacuation) and final regulation of the different processes (Rønnestad et al. 2013). Genetic studies on the physiology of Ballan wrasse have shown that the anterior part of the gut plays a major role in nutrient digestion and absorption (Lie et al. 2018; Le et al. in prepp).

1.3.1. Digestion of lipids

Lipids together with proteins have a major role as sources of metabolic energy for growth, reproduction, movement and migration in fish (Langhans et al. 2010; Tocher 2010) which makes these two ingredients of great value for fish feeds. It is well known that fat is a strong stimulus for the release of hormones contributing to digestion and gut motility. Among the diverse group of lipids, triacylglycerol (TAG) and phospholipids (PL) might be the ones that have driven much of the attention in lipid digestion research. TAG is used as energy storage while PLs are vital for energy, membrane structures, posttranscriptional regulation of proteins and as a messenger molecules (Rønnestad et al. 2013). Furthermore, Izquierdo et al. (2000) high-lighted the importance of adding PL to micro-diets for an advantageous larval growth and survival in several fish species. In accordance with this, European sea bass (*Dicentrarchus labrax*) larvae fed with high levels of dietary PL displayed better maturation of the digestive tract, better survival, better growth, and fewer malformations than larvae fed on low PL diets (Cahu et al. 2003). Fish larvae need dietary PL to be able to metabolise ingested neutral lipids and therefore, phospholipids are the most crucial lipid class for the correct development of young fish larvae

In contrast to mammals, digestion of lipids in teleost has been shown to take place exclusively in the gut as teleost lack gastric lipase. Pre-intestinal lipid digestion has not been reported (Rønnestad et al. 2013). Digestion of lipids relies on bile salts from the bile which emulsify large fat droplets to finer ones called micelles in a process called emulsion. In the case of mammals, triacylglycerol (TAG) is hydrolysed by neutral lipases to free fatty acids (FFA) and monoacyglycerol (MAG) which are absorbed by the apical membrane of the enterocytes. Tocher (2010); Rønnestad et al. (2013) reviewed the impolitic activity in the gut of several fishes; In Cod and salmon the complete hydrolysis of TAG along the gut results in free fatty acids (FFA) and glycerol while the hydrolysis of TAG to FFA and glycerol only happened in the hindgut of turbot and not in the anterior part. Based on this, it is admitted that the main products of the digestion of all major lipid classes in fish are free fatty acids, which are further absorbed by the apical membrane of the enterocytes. In addition, there will be glycerol from the digestion of triacylglycerol, one lyso-PL molecule from the digestion of PL, and long chain alcohols from the hydrolysis of cholesteryl and wax esters (Tocher 2010).

1.3.2. Lipases in fish and mammals: differences

Triacylglycerol (TAG) is a major lipid class in the diet of marine fish. In mammalian gut, TAG hydrolysis is regulated by two main lipases, the pancreatic lipase-colipase system (EC 3.1.1.3) and the less specific bile salt-activated lipase (EC 3.1.1.1) (Tocher 2010).

Several types of lipases have been recognized in the digestive tract of juvenile and adult fish. Among them, non-specific bile salt activated lipase (BAL) which catalyses the hydrolysis of carboxyl ester bonds, have been suggested to play an important role in the digestion of neutral lipids as well as phospholipids in teleost fish (Izquierdo et al. 2000). In line with this, Sæle et al. (2010) reported the presence of non-functional pancreatic lipase-protein (PRLP), the lack of colipase, and the important role of BAL as the major neutral lipase in cod. Besides, although BAL may hydrolyse PL, phospholipase A2 (PLA2) is the most important enzyme for PL hydrolysis .

1.3.3. Lipid sensing

Several mechanisms are involved in dietary fat sensing and energy regulation along the digestive tract. It is currently accepted that the sensing of luminal content, lipids in this case, relies on two receptors: G-protein coupled receptors (GPCR) and solute carriers (SLC). GPRCs lie on the apical membrane of enterocytes and are the binding place for ligands (nutrients) flowing on the lumen. This binding activates intracellular G proteins which trigger a cascade-like downstream pathway eventually resulting in the absorption of micelles in exchange with small solutes across the membrane by solute carriers (SLCs) (Rønnestad et al. 2014). Sensing involves the transmission of signals from the gut to vagal nerves and this is believed to provide a very accurate measure of the energy available from ingested fat (Langhans et al. 2010). Although the function of the sensing mechanisms in fish have not been fully investigated, it is known that nutrient sensing has an important role in peristalsis and appetite regulation (Murthy 2006).

1.3.4. Absorption and transport of lipids

The gastrointestinal mucosa can be considered a complex chemosensory system where enteroendocrine cells and gut hormones work together through specific signalling cascades to enhance absorption of nutrients (Rønnestad et al. 2014).

Absorption of lipids seems to be a rather complex process that requires the presence of lipases and it starts when micelles (emulsified lipid with bile salts and phospholipids) enter the enterocyte by the apical membrane. In order for this to happen, lipids in the micelles need to be broken down into small particles. BAL has a crucial role at breaking lipids into free fatty acids in fish (Sæle et al. 2010) that can then be absorbed/ transported into the enterocyte on the apical side. It is believed that short FAs enter the cell by diffusion (or “flip-flop” pathway) whereas complex FAs are actively transported by the CD36 system. FAs in the enterocytes are further re-esterified to lipids in two pathways: the monoglyceride pathway which produces TAG, and the a-glycerophosphate pathway that produces both TAG and PL. The latter has been observed to be the major pathway for lipid re-esterification in teleost (Rønnestad et al. 2013; Rønnestad et al. 2014).

Once absorbed FAs are re-esterified to lipids in the ER of enterocytes, they bind to proteins forming lipoprotein particles which can vary in size and protein-triglyceride ratio. These lipoproteins are directly discharged into the circulatory system as VLDL or chylomicrons and will deposit lipids to cells in the body (Izquierdo et al. 2000). The characteristics of the lipoprotein particles depends on the major fatty acid being absorbed (Ockner et al. 1972).

1.4. Serotonin

1.4.1. Importance of serotonin

Serotonin (5-hidroxytryptamine, 5-HT) is a widely distributed molecule in the animal kingdom that can act both as an hormone and/or neurotransmitter (Kim and Camilleri 2000). Serotonin serves multiple functions in vertebrates such as endocrine responses, heart development, mediator of behaviour, social interactions, stress and feeding regulation (Winberg and Thörnqvist 2016; Backström and Winberg 2017). Serotonin synthesized within the central nervous system is called brain serotonin and represents only 5% of the total 5-HT while peripheral 5-HT accounts for 95% of the total body serotonin and it is mainly produced within the gut (gut serotonin) (Linan-Rico et al. 2016).

Mechanisms of brain serotonin synthesis in mammals have recently attracted much attention as it seems to regulate mood, sleep, sex, appetite, depression, and neuropsychiatric illness within others (Kim and Camilleri 2000; Gershon and Tack 2007). However, gut 5-HT also deserves great focus as it mediates many gastrointestinal functions via paracrine and endocrine pathways including motility, peristalsis , mixing movements (fed state), secretion, vasodilation, and perception of pain or nausea (Gershon 2004). In the same way that altering brain 5-HT can lead to mental instability, alteration of the gut 5-HT signalling pathway can lead to gastrointestinal disorders, for instance the inflammatory Bowel Diseases (IBD) in humans (Linan-Rico et al. 2016). Due to its important implications in humans, serotonin might be of relevance for gut motility regulation in fish as Ballan wrasse.

1.4.2. Gut as a complex intrinsic nervous system

Among the organs in the body, the bowel stands out as different as it is equipped with its own intrinsic nervous system which can and does operate independently of the brain mediating its own behaviour (Gershon 2004). This is called the enteric nervous system (ENS) and it is responsible of peristaltic reflexes (waves of contraction and relaxation) of the gut. ENS is unique both in the complexity of its organization and its resemblance to the brain. ENS is composed by efferent, afferent, and inter-neurons, acting as an integrating centre in the absence of CNS input (CNS is physically separated from serotonergic neurons of the ENS, platelets, and EC cells) (Gershon and Tack 2007). Some authors refer to the ENS as the second brain as its structural organization and neural diversity is rather complex, resembling to the brain more than peripheral nerves (Gershon and Tack 2007). Although serotonergic

neurons from the CNS do not have any input in the 5-HT system of the gut, there is communication between the gut (ENS) and the brain (CNS) through the parasympathetic and sympathetic nervous systems (Gershon and Tack 2007). In other words, there is a bidirectional communication network between the brain and the gut with serotonin functioning as a key signalling molecule in both ENS and the CNS (O'Mahony et al. 2015). However, the exact 5-HT functioning pathway has not been completely elucidated (Kim and Camilleri 2000). Experiments on in-vitro guts are characterized by the absence of CNS input and serotonin is then proposed as one of the main molecules regulating the enteric system and leading to peristalsis and motility regulation.

1.4.3. Signalling and metabolic synthesis of serotonin in the gut

Serotonin can be produced either in the brain by serotonergic neurons or within the gut. Around 90% of the gut 5-HT is synthetized, stored, and released by a subset of enteroendocrine cells called enterochromaffin cells (EC) that are scattered throughout the enteric epithelium (Kim and Camilleri 2000; Yano et al. 2015) . The other 10% is produced by enteric neurons. Irrespective of the location in the gut-brain axis, the synthetic cascade of serotonin is similar in both the gut and the brain (O'Mahony et al. 2015).

Afferent nerves of the ENS do not reach into the gut lumen where they could respond to changes in pH, the presence of nutrients or any other luminal stimuli. Instead, EC cells function as sensory transducers to monitor conditions prevailing in the lumen (Mawe and Hoffman 2013). These cells store prodigious amounts of serotonin in secretion granules laying on the base of their microvillus. At least five important enzymes are needed to complete the serotonin metabolic pathway (Figure 3). The first step in the synthesis of 5-HT is catalysed by the rate limiting enzyme tryptophan hydroxylase-1 (TPH1) that converts tryptophan into 5-Hydroxytryptophan (5-HTP). TPH-2 has the same function as TPH1 but it has uniquely been found in neurons from both the brain and the gut (Gershon and Tack 2007). 5-HTP is then converted to 5-HT by action of the enzyme L-amino acid decarboxylase (L-AADC) commonly known as DDC.

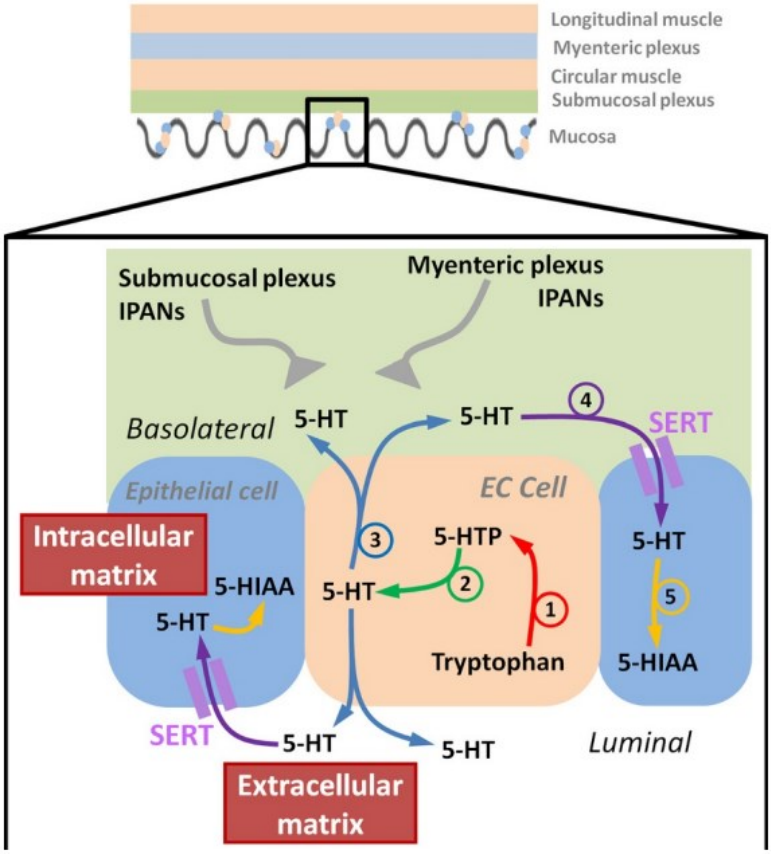

Figure 3: The metabolic pathway of serotonin in the gut. EC cells (orange) are enterochromaffin cells and neighbouring cells (blue) are epithelial cells both laying on the innermost layer called mucosa. This is followed by the submucosal plexus containing neurons from the ENS. Steps 1–5 are the processes involved in 5-HT synthesis and neurotransmission; Step 1 where 5-Hydroxytryptophan (5-HTP) is synthesized by the rate limiting enzyme tryptophan hydroxylase-1 (TPH1). 5-HTP is then converted to 5-HT by the enzyme L-amino acid decarboxylase (DDC) (step 2). 5-HT is then released into the submucosal plexus (step 3) where it can bind to corresponding 5-HT receptors (SLC6A4) on intrinsic primary afferent neurons (IPANs). Remaining 5-HT is then transported into epithelial cells via SERT transporters (step 4) and metabolized to 5-hydroxyindole acetic acid (5-HIAA) by the action if the enzymes MAO A and MAO B (step 5). The latter represents the final step in neurotransmission as 5-HT is inactivated. From (Parmar et al. 2012)

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Gut mucosa might be altered by mechanical and/or chemical stimuli such as nutrients, toxins, pressure, and/or acid (Mawe and Hoffman 2013). In response to this stimulation, intracellular levels of ca^{2+} in EC cells increases which evokes the release of 5-HT into the extracellular matrix either on the basolateral side or into the gut lumen (Bertrand and Bertrand 2010). Luminal 5-HT might stimulate cells lying in the crypts of the mucosa layer while basolateral 5-HT enters the inner walls of the GI tract (submucosal plexus) where it can either be taken up by platelets entering the circulation or bind to specific receptors in intrinsic primary afferent neurons (IPANs) (Mawe and Hoffman 2013). This activation triggers in turn a cascade of interneurons and motor neurons within the enteric circuitry causing changes in the regulation of GI motility (Parmar et al. 2012). Once IPANs get activated by 5-HT, they release Ach

and/or GCRP which will stimulate other neurons and the signal is transmitted to numerous other cells causing peristaltic activity and secretory reflexes. SLC6A4 is an important membrane 5-HT carrier that enable this communication between synaptic and presynaptic spaces (Gershon and Tack 2007). Despite the very few studies on the cellular and molecular mechanisms of 5-HT release from EC cells, a simple outwards exocytosis of 5-HT from the storage granules in multiple directions has been proposed as the likely mechanism (Linan-Rico et al. 2016).

The majority of released 5-HT that have not been absorbed by platelets nor conjugated to neural receptors is quickly cleared into neighbouring epithelial cells that surround EC cells (Parmar et al. 2012). This is done via the 5-HT selective reuptake transporter (SERT). This transporter is expressed by all epithelial cells of the intestinal mucosa and seem to have a crucial role on both strength and duration of the excitatory signals caused by 5-HT. Once 5-HT enters the epithelial cell, it is metabolized into 5-hydroxyindole acetic acid (5-HIAA) as a result of the activity of monoamine oxidases (MAO). While 5-HIAA by-product from liver and kidney is excreted in the urine, the gut has its own inactivating mechanism (Kim and Camilleri 2000).

1.4.4. Serotonin in fish: Novel research in Ballan wrasse

As mentioned before, serotonin plays a critical role in several gastrointestinal functions in vertebrates including fish. Similar to mammals, the enteric system of teleost is characterized by a well-developed myenteric plexus containing projections of serotonergic neurons with a wide variation on morphology and density (Velarde et al. 2010). Despite the little knowledge of the serotonergic system functions and its general organization in fish, it has been considered similar to that in mammals as it is highly conserved across the vertebrate subphylum (Winberg and Thörnqvist 2016). However, differences on the location of 5HT body cells in the brain of teleost in respect to that in mammals, and differences in the type of active 5-HT receptors in the gut of teleost have been characterised over the last years (Velarde et al. 2010; Winberg and Thörnqvist 2016; Backström and Winberg 2017). This proves that there are some anatomical and functional differences in the serotonergic system of teleost compared to that in mammals and further research might elucidate whether this system has remained as conserved as it has been thought within the vertebrate phylum.

In short, serotonin regulate the contractile activity of the gut showing a dual action in both mammals and teleost acting as a stimulant and /or relaxing factor of gut motility within different species (Velarde et al. 2010). Above all, the exact role of 5-HT in mediating these effects is still very limited and not fully understood. Therefore, there is a clear necessity to broaden the knowledge of the serotonergic system and how it is regulated in fish. The serotonergic system of Ballan wrasse have not been investigated before.

1.5. Aim of the master's thesis

This thesis is part of the NRC project: "Intestinal function and health Ballan wrasse", Grant No.: 244170. First of all, this master's thesis aims at revealing some of the physiological characteristics of the digestive tract of Ballan wrasse by in vitro analyses of guts. More in detail, the main focus was to investigate the serotonergic system of the gut of Ballan wrasse by tracing the expression of the main genes implicated in serotonin metabolism after a lipid meal. In order to do this, serotonin in the gut was quantified to observe its presence and to what extent, and to observe any possible correlation with gene expression. The effect of post prandial time (from feeding to up to three hours) on both the genetic expression and the amount of serotonin present in the gut was analysed.

A second but not less relevant aim of this thesis was to observe the modulation of the expression of those genes involved in lipid metabolism. For that purpose, fish oil was used as feed in the experiments which was also expected to stimulate the mechanisms involved in serotonin metabolism. In a broad perspective, the relevance of this study tries to contribute to broaden the little knowledge of the digestive system of this a-gastric species, Ballan wrasse, with the potential of improving the farming efforts of the aquaculture industry.

2. Materials and Methods

2.1. Experimental overview

As mentioned previously, this master thesis has two distinguishable parts; Serotonin measurements with enzyme-linked immunosorbent assay (ELISA) and gene quantifications by terms of RT-qPCR. A pre-trial was important to optimize the set-up of the eventual experiment. All the experiments described thereafter were performed in vitro guts of Ballan wrasses.

2.2. Fish used for ex vivo experiments

Ballan wrasses (20-30 g body weight) were provided by Marine Harvest (Øygarden, outside Bergen, the day before dissections and placed in cold room temperature (14 °C) with dimmed light. In order to reduce stress, fish was then sorted out in different buckets (4-5 fish/bucket) containing stripes of plastic bags (fake kelp) to imitate their natural environment. Overnight fasting was required to ensure empty guts for the trials.

2.2.1. Diet preparation

Hydrolysed fish oil (containing omega-3-fatty acids, Vitamin D, A and E) was used as feed for the final experiment (see result chapter). In order to hydrolyse fish oil a lipase from Pseudomonas cepacia (PS Lipase <30 U/mg, Sigma-Aldrich) was used by mixing 30 mg of the latter with 500µL of PBS (pH=8) in a small glass container together with 3 mL of fish oil. To ensure an optimal activation of the enzyme, it was crucial to maintain a pH of 8 under 35-40 °C for 3 hours. Once fish oil was hydrolysed (change in consistence), the mix was incubated at 80 °C for 1 hour to ensure deactivation of the enzyme. After deactivation, 250µL of tween 20 were added to the hydrolysate and it was stored at -20 °C until further use.

2.2.2. Dissections

Fish were anaesthetised by placing them in a bath with MS222 (30 mg/ml) before being killed with a blow in the head. Weight and standard length of each individual were recorded. After that, dissection of the abdominal part was performed with extreme care to not do any incision in the intestinal wall. Guts were then removed from the body and placed in a Petri dish filled with Ringer's solution. Gills were also retrieved together with the gut to facilitate the input of fish oil. Ringer's solution (pH=7) was made for the purpose of creating an isotonic solution to keep the tissue active during the trial. It is a solution of several salts composition (table A.1 in the Appendix). Previous to the insertion of "feed" guts were emptied by flushing with a gentle squirt of Ringer's solution to wash out food remains. Food was then added by injecting 0, 1% µl of the total body weight into the oesophagus with a pipette. In

order to prevent food from escaping anteriorly, guts were closed by tying a thread around the oesophagus right below the gills. They were then moved into glass assay tubes (Figure 4) filled with 24 ml of Ringer's solution and constant gas flow (95% oxygen + 5% C02).

After incubation guts were retrieved from the tubes accordingly to the given times. Only the first segment was used for gene analyses and serotonin quantification. The first segment (40% of the total gut length) was cut off and opened by incision. A rather small part of this tissue (around 50 mg) was taken by a transversely cut in order to avoid variation and washed by flushing it gently on the Ringer's solution. The remaining tissue from the first segment was weighted and placed in a different Eppendorf for further serotonin extraction. Samples were quickly frozen by placing them in dry ice mixed with methanol to avoid degradation of genetic material. Tissues were kept at -80°C until further use.

2.2.3. Pre-trial

The aim was to identify genes associated with lipid and serotonin metabolic pathways in the gut of Ballan wrasse that could be regulated by lipid ingestion. Ex vivo guts were prepared as described above. Three different treatments were done with 8 n in each group: intact fish oil (TG), hydrolysed fish oil (free fatty acids) (hTG) and empty (control) (c). Guts were then put in tubes with Ringer's solution and incubated for one hour. After that, Guts were sampled and RNA extracted exclusively from the first segment. RNA from the 24 samples was then transferred to a 96- well plate, sealed and sent to be sequenced by The Norwegian Sequencing Centre (NSC), Oslo, Norway (www.sequencing.uio.no). Transcriptomic data was analysed according by Kai Lie (KaiKristoffer.Lie@hi.no) at HI.

2.2.4. Final experiment

To investigate the genetic expression and serotonin profile of the gut as a function of post prandial time, a timeline experiment was set as showed in figure 4. This experiment consisted on 7 gut from 7 different fishes each of one was given a specific post prandial incubation time (10, 30, 60, 90, 120, 150, and 180 min). This experiment was repeated a total of three times (experimental triplicates) under equal conditions.

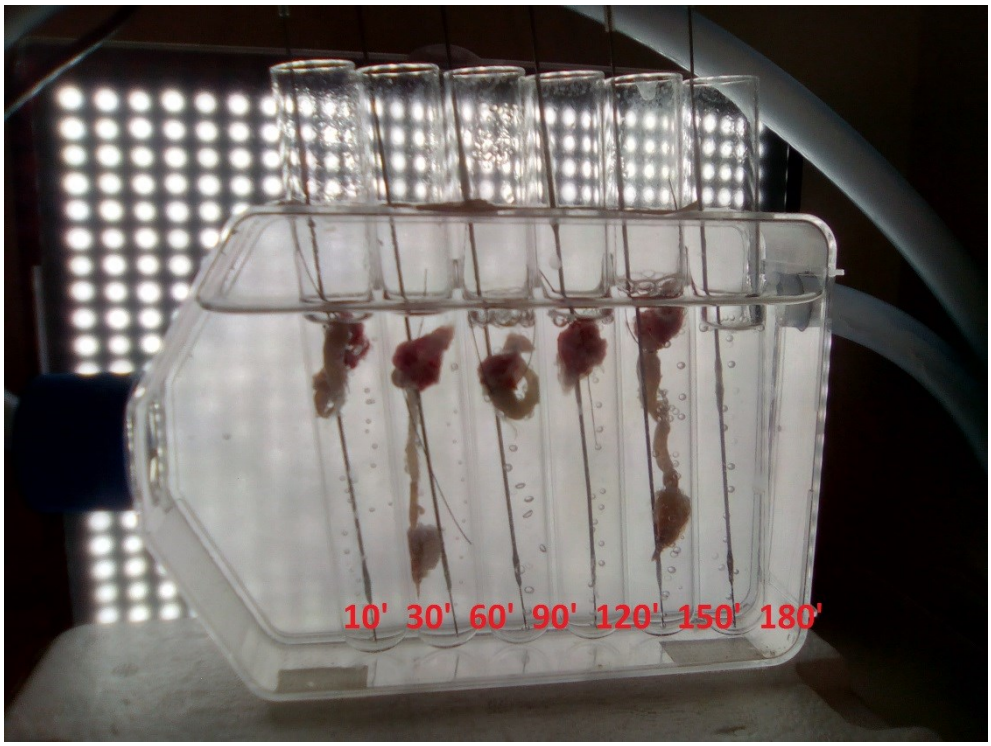

Figure 4. In vitro experimental set-up. 7 guts were placed in tubes filled with Ringer's solution and supplied with air during incubation. Numbers indicate the post prandial incubation time (10' to 180') for the different guts. Photo by Hoang Le (HI)

The term experimental triplicate is used as the experiment was run three times. However, it is crucial to be aware of the potential causes of variations between trials that might (or not) have an important effect on the results interpretation. In this case, the experimental procedure was kept identical for the three trials, the only difference was the month and the day-time when they were undergone. The first one was performed in September 2017 while the others two in the same day in January 2018. The first and the second were performed at the same time in the morning but in different dates (20/09/2017 and 30/01/2018). The third experimental replicate was done later in the afternoon (30/01/2018) so fish was kept longer in the room than the fish used for the other two experiments.

2.3. Genetic analyses by real-time RT-qPCR

Gene expression was determined by means of RT-qPCR. Gene expression analyses were performed equally to all the samples from the three replicates.

2.3.1. RNA extraction and purification:

It was important to work under sterile conditions to avoid contamination and keep the samples on dry ice while handling to minimize DNA degradation. The aim of the procedure was to obtain pure RNA by magnetic-particle technology (EZ1 RNA Universal Tissue Kit). For that purpose, 50 mg of frozen gut tissue (stored at -80 °C) was placed in 2 ml micro-centrifuge tubes. In order to get an efficient lysis of

the tissue and to inhibit RNases activity, 750 µL of QIAzol Reagent was added together with 4 stainless steel beads into each tube. The tubes were further placed in the TissueLyser for 60 seconds at 6000 rpm (6000-3x10). After the tissue was completely disrupted and homogenized the tubes were left on the benchtop at room temperature (rt) for 5 minutes. 150 µL of chloroform was added to each tube in order to separate RNA and DNA in different phases. For this, the tubes were centrifuged at 12,000xg for 15 min at 4 °C (this low temperature is crucial for optimal phase separation and removal of DNA). After centrifugation, the colourless upper phase of the tube (containing RNA) was carefully transferred to 2 ml tubes supplied by the kit. Reckless (rushing) pipetting could lead to take some of the lower, red organic phase which would contaminate the RNA samples. The EZ1 instrument (Bio-Robot EZ1) was used and the RNA Universal tissue protocol with integrated DNase digestion was set up as default program. In order to ensure fully DNA removal from the samples, 10 µL of DNase were added into the liquid in the fifth well of each cartridge supplied by the kid. It was of great importance to track the samples ID throughout the whole procedure to avoid data mix-up. Up to 6 samples were processed in a single run using the Bio-Robot EZ1. The final products were pure RNA and further quantification and purity analyses were taken. RNA must be stored at -70°C.

2.3.2. RNA quantification and purity:

It was important to check the amount and the purity of the extracted RNA to ensure optimal conditions before moving on. Concentration of RNA can be determined by measuring the absorbance at 260nm (A_{260}) in a Nano-spectrophotometer. RNA eluate was first spin and placed in a suitable magnet to minimize magnetic particle carryover during the A_{260} reading. 1, 8 µL of the eluate was enough volume to measure the concentration and purity of each RNA sample.

Purity relies on $A_{260/280}$ and $A_{260/230}$ ratio.

- **$A_{260/280}$ ratio**: This value indicates the purity of the sample and should range between 1, 8-2, 2. If lower values are obtained, the sample could contain some leftovers of DNA, proteins or phenol.
- **$A_{260/230}$ ratio:** A lower value than 1, 8 could imply the presence of salt and/or phenol leftovers.

2.3.3. RNA quality/Integrity:

Checking the integrity of the RNA beforehand was required to minimize potential failures. By using an Agilent 2100 bio-analyser instrument (Agilent technologies, Waldbronn, Germany), RNA integrity can be obtained in terms of RIN number which range from 1 to 10. An optimal RIN number can be that close to 10 but it is expected to be lower with RNA extracted from tissues, especially in this case where guts were dissected out of the body and repeatedly handled by flushing during dissection. Besides the

RIN number, the Agilent bio-analyser instrument also provides an electro-photogram (graph) showing the ribosomal RNAs 28S and 18S which should appear as sharp bands or peaks. If the ribosomal peaks of a specific sample are not sharp, but rather appear as a smear towards smaller sized RNAs, it is likely that the sample suffered major degradation either before or during RNA purification.

It was required to know the RNA concentration of the samples in advance as it had to range between 25-500 ng/µL in order to be detected by the instrument. Previous dilution in ddH_2O was performed for those samples exceeding the required concentration. All the samples were then transferred to micro-centrifuge tubes and placed in a heating block for denaturation.

Gel-Dye Mix together with RNA marker and a ladder (Pre-denatured for 3 minutes at 70 °C) were also needed. The chip was placed in the priming station provided by the kit and 9 µL of Gel-Dye Mix were pipetted in the well-marked **G**. A plunger was then used in order to spread the gel along the micro-channels by making sure its initial position was 1 mL and pressing down until it was held by the clip. After 30 seconds, the clip was released and the plunger pulled back to 1 mL position. 9 µL of the gel-dye mix were pipetted in each of the two wells marked **G** and the remaining gel was discarded. 5 µL of RNA marker were pipetted in each of the 12 sample wells and in the one marked as the ladder. Subsequently, 1 µL of the ladder was pipetted into the ladder-well. Each of the RNA samples (previously denatured by heat) were loaded into the Nano-chip (Agilent RNA 6000 Nano kit). Each RNA chip consists on a set of interconnected micro-channels that is used for separation of nucleic acid fragments by electrophoresis (based on their size as they are driven through the gel). Once all these loading steps were completed, the chip was vortexed for one minute at 2400 rpm so that all the reagents mixed properly. The chip was then ready to be placed in the bio-analyser. Cleaning up the electrodes of the bio-analyser with ddH_2O water was required before the chip was placed in and the program run.

2.3.4. Primer design:

Once relevant genes (8 genes associated with lipid metabolism and 6 genes associated with serotonin metabolism) were selected based on the pre-trial, primers were designed for the mRNA sequences of PLPP3, PLD1, PLIN2, CD36, SLC27A4, ELOVL1, APOA-IV, PPARG, DDC, TPH1, TPH2, SLC6A4, MAO A, and MAO B. RPL37 and ubiquitin (Ubi) were chosen as reference genes based on (Sæle et al. 2009). Gene specific primers (table 1) were designed using NCBI data bases and its primer design tool (NCBI, Maryland, USA).

Table 1. Primer sequences used for RT-qPCR. The coefficient variance (CV) is a measure of the stability of the reference genes. CV for RPL37 and Ubi was 0,082 and 0,079 respectively and considered as suitable reference genes (CV <0, 25).

Gene	*Forward sequence*	*Reverse sequence*
PLPP3	GAGACACCATTAGCGATGCG	CTGACCCCACAACCGAACAG
PLIN2	CAGGAGTATGGTCACGAGGC	TGTAGACGTGTGTGGCAGAG
CD36	ACGGAGGGATAAAACGCACA	TATGCTGTGGTTCCAGGCTC
SLC27A4	TGCTCGTCGGCTCTTATTCC	TTGTAGCCGATAAGCTCGCC
DDC	CACAAACTCACCACGTGCC	CACATCTGGGTAGACTGGTCG
TPH1	GAGGGACCACGTAGAGGAAGAT	CCTTCACTAGTCCTCCCACTTC
TPH2	TGAGGCATGCTTTGTCCGAT	AACGGACGCTTGATCGTCTT
SLC6A4	GTGTCCTGGATTAGGGGCAA	AAATCACTCATGCCTGGGCT
PLD1	GCCATCGAGAAGAGCGAACA	TACACGCGGTACCTTTTACCC
ELOVL1	GAGGAAGCTGAGCAGAGAACT	ACTGCGTCACCCGTTTATCC
APOA-IV	TAGCTTGGAGCCATGAGGGT	TGCATCAATCAGCCCATCCAT
MAO A	CAGCTCATCTGCTCCGGAAA	TTGGCTGCCGGTATTTCCAT
MAO B	CCAACTCAGAACCGCATCCT	AGGGAAGGAGCCTTTGAACG
PPARG	GCTGCAACAAATGCAAAGCCC	GAATCCCACAGGCCAGGCTA
RPL37	CCTCCGTGTTTCACTGGGCAGAC	ACCCAGACGTGCTGCAGTGG
Ubi	GGCCAGCTGTCTGAGAGAAG	GTCAAGGCCAAGATCCAAGA

2.3.5. Reverse Transcription (RT) reaction- from RNA to cDNA:

In order to run the RT-qPCR reaction, RNA samples were converted into complementary DNA by means of the enzyme reverse transcriptase. TaqMan reverse transcription reagents were used (Applied Biosystems, Foster City, CA, USA).

A RNA pool containing 2 µl of each of the RNA samples was prepared in order to create the standard curve. This standard curve was made with six serial dilutions using a dilution factor of 0, 5 resulting in concentrations ranging from 3,125 to 100 ng/µl per well. RNA samples were then diluted to a concentration of 50 ng/µl. Two negative controls were also prepared; the non-amplification control (nac) which did not have any enzyme, and the non-template control (ntc) with ddH_2O instead of RNA.

Table A.2 in appendix shows volumes and reagents required for the reaction. The reaction mix comprising the non-enzymatic reagents was made, vortexed and put on ice. The nac control was then prepared by mixing 38, 33 µl of the reaction mix with 1, 67 µl ddH_2O at RT temperature. Posteriorly, the multiscribe enzyme Transcriptase was added to the RT reaction mix and 40 µl of the latter was

pipetted in each of the 96 well RT plate except for the nac well. Both samples and standards were prepared in triplicates. Posterior loading of the plate was done by adding 10 µl of the RNA samples into the wells always keeping track of what is loaded and where (RNA was added to the nac control but only H_2O in the ntc control). The final volume of each well of the plate was 50 µl.

The plate was then covered by a 96-well plate cover which was previously cleaned with soap, ddH_2O, and 70 % EtOH (crucial step to avoid contamination). The plate was centrifuged at 50x g for 1 minute and ready for reverse transcription which was performed by GeneAmp PCR 9700 (Applied Biosystems, Foster City, UA, USA). Table A.3 in the appendix shows the instrument set up for the reaction. The run was 90 minutes long. When finished, the cDNA plate was sealed with a tape pad and stored at -20°C until further used for real-time quantitative PCR.

2.3.6. Real Time quantitative PCR (RT-qPCR):

Both reverse and forward primers were diluted with TE buffer to a concentration of 0,05nmol/µl before being used. They were then vortexed for 15 seconds and stored at -20°C to avoid degradation of the primer DNA.

Gene expression was quantified by means of qPCR by measuring cDNA amplification and fluorescence. The cDNA plates were used as a template for this reaction which was measured by a Light cycler 480 (Roche Applied Sciences, Basel, Switzerland). A reaction mix (SYBR Green) was prepared for each primer and the volume of such depended on the number of samples of the cDNA plate (Table A.4 in the appendix). SYBR Green master mix is a dye that becomes fluorescent when conjugated with cDNA. The quantity of cDNA is expressed as the cycle threshold value (ct) which stands for the number of heating/cooling cycles that it takes before the increase in fluorescence is linear. The already prepared cDNA plates were thawed on ice, centrifuged at 1000 x g for one minute and shaken for 5 minutes at 1500rpm.

By using a pipetting robot programmed to transfer 8µl of the reaction mix (containing the primer) and 2µl of cDNA from a single well to each well on a 384-well qPCR plate. When the plate was filled, it was covered with an optical adhesive cover by flatting the surface with an applicator being careful not to touch the film with the hands. Centrifugation of the plate at 1500 x g for 2 minutes was required. The plate was then placed into the Light cycler 480 Real Time PCR System for approximately 90 min. The set-up program for the qPCR was as followed; Pre-incubation for 5 minutes at 95°C to achieve denaturation and activation of the enzyme Taq DNA polymerase. This was followed by amplification which consisted in 45 cycles of three steps: Denaturation of DNA for 10 seconds at 95°C, annealing or primer binding to DNA strands for 10 seconds at 60 °C, and elongation process for 10 seconds at 72°C where double stranded DNA was synthetized. The plate was read at the end of each cycle. Following

the 45 cycles, the melting point was measured by gradually increasing the temperature of the plate from 65 °C to 97 °C in intervals of 0, 5°C and readings were obtained every second. Figure A.1 in the appendix shows a general picture of the standard protocol for SYBR green 384 well-plate.

2.3.7. Calculation of mean normalised expressions

Genetic data analysis such as reference primer efficiencies, data normalization and relative quantification were displayed using the CFX Manager Software (Bio-Rad CFX Manager 3.1).

2.4. Serotonin quantification

ELISA analyzer (Serotonin ELISA; LifeSpan BioSciences, Inc. (LSBio), Seattle) was used to measure serotonin in tissue. Serotonin was extracted from only the first segment of the guts from the triplicates at different time intervals (10, 30, 60, 90, 120, 150, and 180 min). Tissue samples were firstly minced and homogenized with buffer as following explained before measuring the concentration of serotonin.

2.4.1. Serotonin extraction: Homogenized

PBS solution 0, 02 M (pH=7) was used as buffer to perform the extraction. After dissection, all the tissue samples were adjusted to a finale concentration of 80 mg/ml regardless their initial weight. Needed volumes of buffer (PBS) were then calculated and extraction was carried out as following: tissue was rinsed with the corresponding amount of buffer together with 4 stainless steel beads into a tube. The tubes were further placed in the TissueLyser for 60 seconds at 6000 rpm (6000-3x10). Tubes were immediately placed in wet ice for cell lyses by ultrasonication, 3 rounds of 10 seconds each. Tubes were then centrifuged for 5 minutes at 5000X g and 20 °C. Further collection of the supernatant was required for assaying.

2.4.2. Serotonin quantification

A. Assay principle:

Serotonin ELISA bio-analyser kit (Serotonin ELISA; LifeSpan BioSciences, Inc. (LSBio), Seattle) was used. The assay principle is based on the competition antigen-antibody principle. A pre-coated plate with a target specific capture antibody was required. Both standards and samples together with a fixed amount of biotin- conjugated target antigen are added to the wells. The antigen from the samples compete with the biotin-conjugated antigen to bind the capture antibody. Unbound antigen is washed away. A conjugate (HRP) which binds to biotin is then added followed by a TMB substrate that reacts with HRP resulting in colour development. This colour signal is measured in terms of its optical density (OD). The OD of an unknown sample can then be compared to an OD standard curve to determine the

antigen concentration of the unknown sample which is serotonin. As a result, the higher the amount of serotonin in the sample, the lower the OD reading is.

The coated 96-well plate, standard stock, sample diluent or buffer, reagent A and reagent B, wash buffer, TMB substrate, and a stop solution were provided by the kit (ELISA bio-analyser).

B. Assay preparation:

Sample preparation: Dilution of the samples was required as the sensitivity of the method was unknown. A dilution series of 4 dilutions was performed for each sample using a dilution factor of 0, 5. The initial concentration for all the samples was 80 mg/ml as mentioned above. The resulting concentrations varied from 1:1 for the non-diluted to 1:16 (5 mg/ml) for the most diluted one (Figure 5).

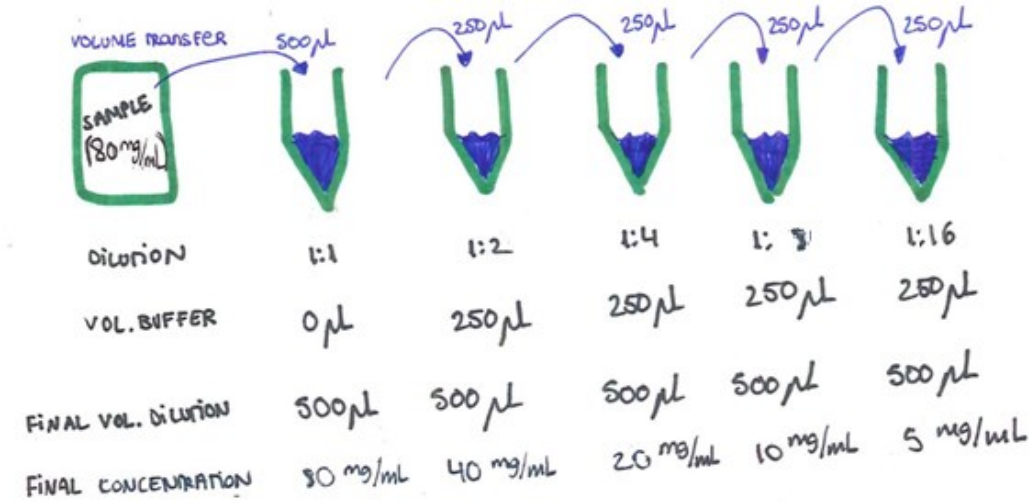

Figure 5. Dilution series preparation of samples: Dilution factor of 0, 5.

Standard dilution preparation: To calculate the amount of serotonin in the samples it was needed to have a Standard stock with a fixed serotonin concentration of 50 ng/ml which was provided by the kit. A dilution series of 7 dilutions was prepared out of the Standard stock. The dilution factor was also 0, 5 resulting in the concentrations showed in Figure 6. A control (Zero) was also prepared using only buffer.

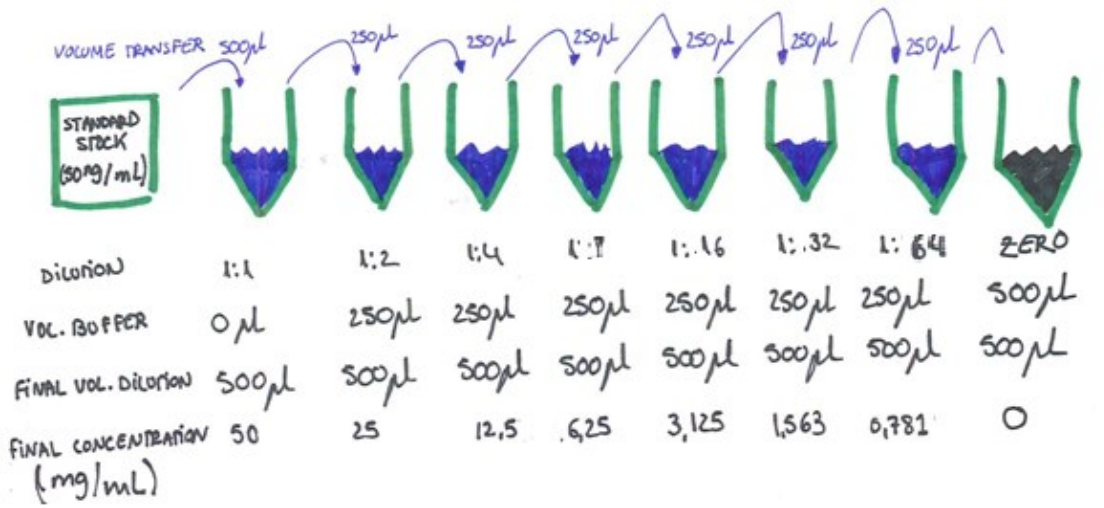

Figure 6. Dilution series preparation of standards with a dilution factor of 0, 5.

Once samples and standards dilutions were finished, the rest of the needed reagents were prepared. Detection Reagent Working Solution A and B were prepared in sufficient volumes for all the 96 wells of the plate. For that, Detection Reagent A and B were diluted to a ratio of 1:100 using Assay Diluent A and B respectively. 750 mL of Wash buffer was prepared by mixing 720 ml of deionized water to the supplied 30 ml of 25x Wash buffer concentrate and kept at 4°C. No further preparation was required for neither TMB Substrate Solution nor Stop solutions which were brought to room temperature, as all the others, prior to start. TMB required sterile precautions and was also protected from light.

2.4.3. Plate preparation for 5-HT measurement:

The plate was then filled by adding 50 µl of standards, blank, or Sample in the wells. All the samples were done in duplicates. Immediately after, 50 µl of solution A were added to each well with a multiple micropipette and the plate was incubated for 1 hour at 37°C with gentle mixing (micro-oscillator at low frequency). The liquid from each well was then aspirated and washed three times with a soaking time of two minutes between each wash by a DELFIA 1296-026 Microplate Washer. When the washing was finished, the plate was taped against absorbent paper to get rid of remaining drops. 100µl of the solution B were then added to all wells and the plate was again incubated for 45 min at 37°C. It was crucial to seal the plates while incubating to avoid possible evaporation. A second wash of the plate was required as described before but for 5 cycles. TMB solution was now added (90 µl per well) followed by gently agitation to ensure thorough mixing. It was incubated for 15-20 minutes at 37°C protected from light and periodically monitored until optimal colour development was achieved. In order to cease the enzymatic reaction, 50 µl of Stop solution were added to each well and the optical density (OD) of the plate was quickly measured by a Multilabel Plate Reader set to 450 nm.

2.4.4. Calculation of serotonin concentration:

A linear standard curve was designed for each plate (one plate per replicate) by plotting the log of the ELISA serotonin concentration on the X-axis against the mean absorbance (OD reads) from the standard dilutions on the Y-axis. A logarithmic trend-line was drawn as it best fitted the points on the graph. Microsoft Excel (Microsoft®Office®2003, Microsoft Corp., USA) was used for this purpose. The given linear equations (Y= mx + b) were then used to calculate the concentration of serotonin in our samples where Y were the OD values of each sample and X was the log of the concentration of serotonin to be calculated. M and b values were given by the standard curve.

Each sample was diluted following a series of 4 dilutions with a dilution factor of 0, 5. This was done in order to find those dilutions which OD values ranged within the OD values from the standard curve. The OD values from the standards that flattened the standard curve out were removed as were not considered trustable to measure serotonin concentration. In order to calculate the real concentration of serotonin from a sample that has been diluted, the concentration read based on the standard curve must be multiplied by the dilution factor.

In order to assess correlation, if any, between serotonin measurements in tissue and genetic expression, Pearson's correlation coefficients were used.

2.5. Statistics

Regression models were aimed to observe serotonin and gene expression modulation with time. Weighted least square regression ($1/\sigma^2$) was selected as the only suitable modelling approach considering that the variance component was not homogeneous (lack of homoscedasticity) for the various genes and neither for serotonin measurements from tissue. For this purpose, the measured data were modelled as a function of time by using the general expression of the form:

$$\gamma = m \times t + b$$

Where γ represents the gene expression/ serotonin concentration in tissue, *m* is the slope of the model (aka variation of the gene expression/serotonin concentration in tissue per time unit), t the time in minutes and *b* the intercept of the model.

Statistics-Microsoft Excel (Microsoft®Office®2003, Microsoft Corp., USA) was used for analysing heteroscedasticity of the data. The acceptability of heteroscedasticity and the adequacy of the regression models were tested by comparing the lack-of-fit to pure error variances at a 95% confidence level. An Excel template developed at Havsforskning Institutet (HI) containing all the routines for

automatic calculation of the ordinary and weighted regressions and the analysis of their residuals was used. The Excel template is available upon request at HI (par@hi.no).

As a complement of the weighted least square regression ($1/\sigma^2$) models, statistical analysis were performed using R Foundation for Statistical Computing, v2.11.0 (R Development Core Team, Vienna, Austria). Gene data followed normal distributions but presented considerable heteroscedasticity. Data from serotonin measurements did not show neither normal distribution, nor homoscedasticity. For this reason, generalized linear mixed effect model (glmmPQL) with Gaussian error term was considered as adequate to measure significances throughout the feeding trial. P values of <0.05 were considered statistically significant.

3. Results

3.1. Transcriptome analyses

Transcriptome analyses (RNA-sequences) of gut samples were based on comparisons between hydrolysed fish oil diet (hTG) versus control (empty guts) and intact fish oil diet (TG) versus control. Principal component analysis (Figure 7) showed a clear separation between the hTG compared to the control (C) group.

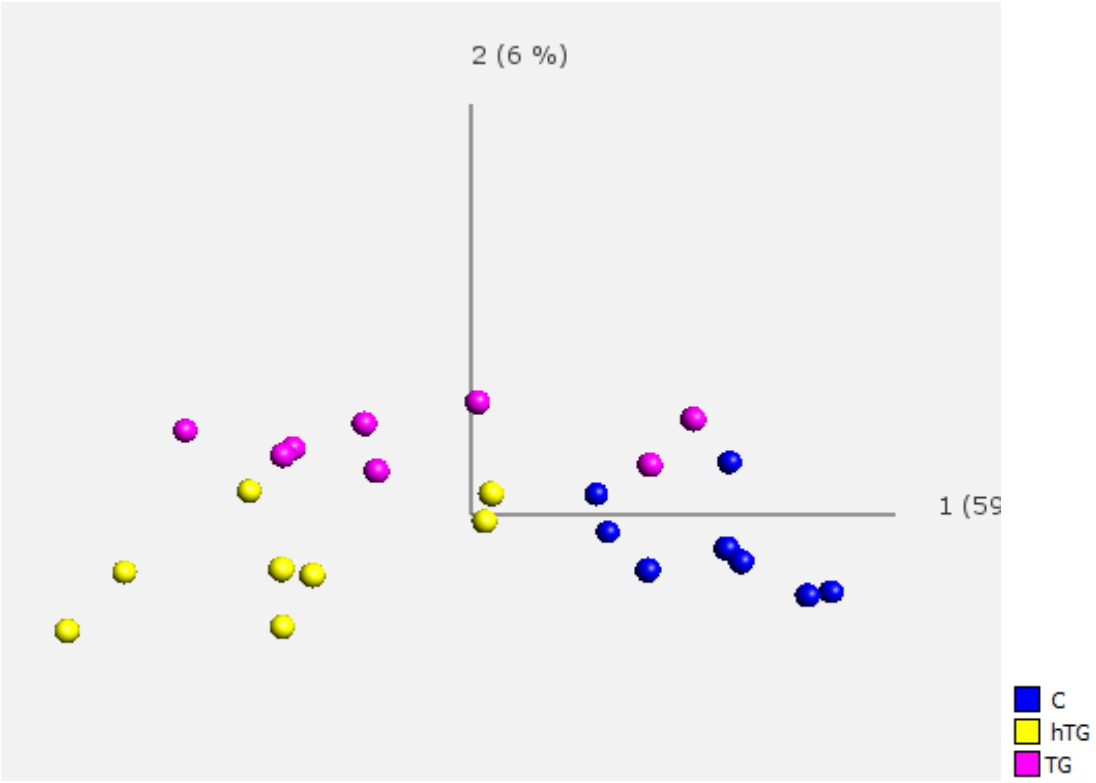

Figure 7. Principal component analysis (PCA) of RNA-sequencing data from gut gut in Ballan wrasse fed either control (C), hydrolysed (hTG), or intact (TG) fish oil diet.

For further data analyses and gene expression profiles a heat map showing differentially expressed genes between the three dietary treatments were done (Figure 8). The intact fish oil group (TG) clusters closer to the control group (c) compared to the hydrolysed fish oil group (hTG) concluding that hTG diet evokes significantly different expression of genes than TG and control. The hydrolysed fish oil treatment (hTG) was therefore chosen as the diet for further experiments.

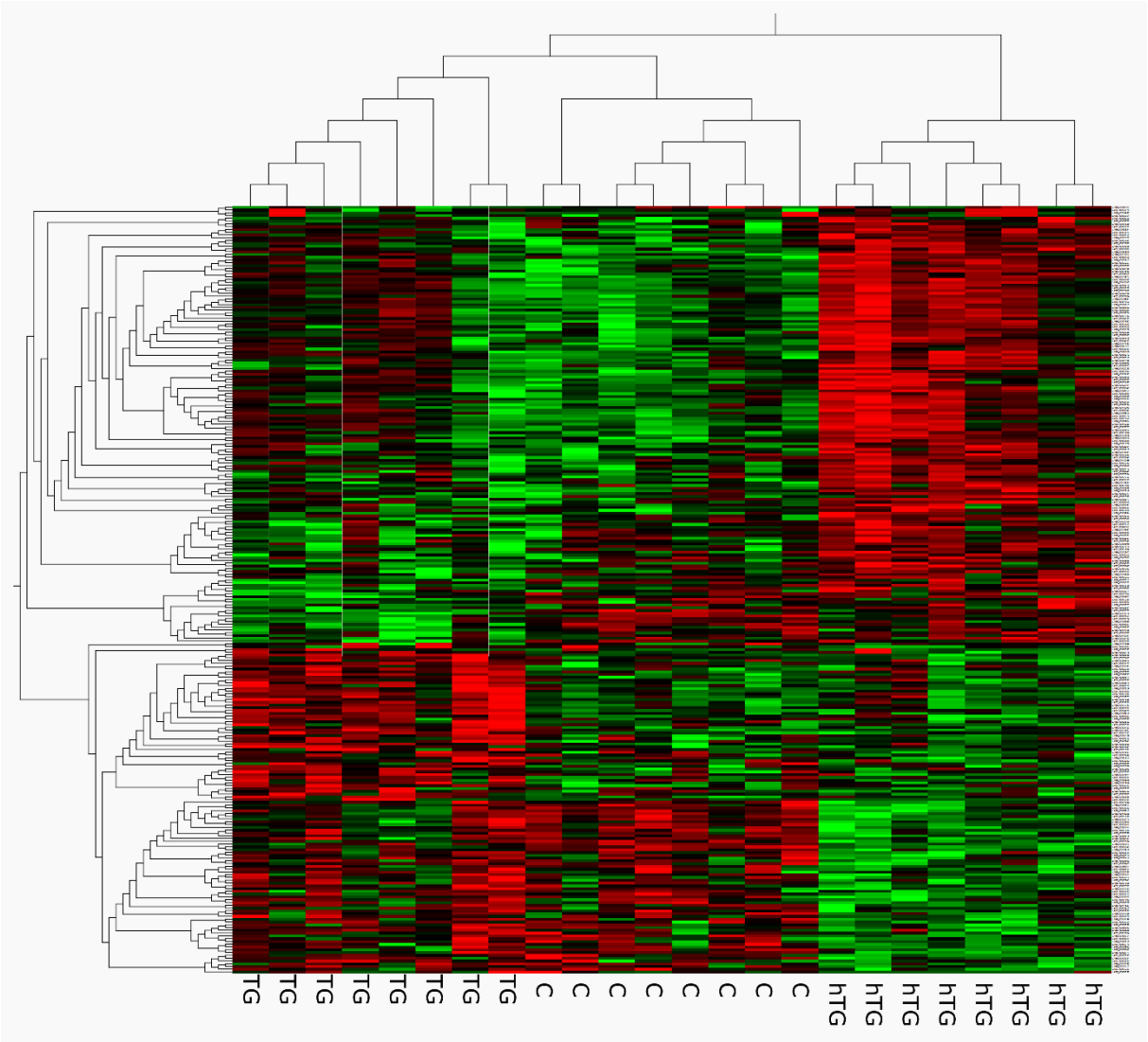

Figure 8: Hierarchical clustered heat map of the 195 differentially expressed intestinal transcripts from Ballan wrasse fed either control (C), hydrolysed (hTG), or intact (TG) fish oil diet ($p < 0.05$). The vertical distances on each branch of the heat map represent the degree of similarity between dietary treatments gene expression profiles. Expression level is colour coded: red for up-regulated, black for unchanged expression, and green for down-regulated genes.

Genes considered as relevant for serotonin and lipid metabolism were selected to be target genes in further experiments: RT-qPCR (table 2).

Table 2. List of the selected genes for further experiments (RT-qPCR). They are also classified by their function in either lipid or serotonin metabolism. Transcriptomes analysis identified differentially expressed genes (adjusted p< 0, 05) from RNA-sequencing of gut samples fed with hydrolysed fish oil compared to control diets. NA indicates lack of gene expression. P values (p < 0, 05) were significantly lower when hTG diets are used in opposite to TG diets.

Diets		***hTG vs control***		***TG vs control***		
Gene	**accession no.**	**log2FoldChange**	**p-value**	**log2FoldChange**	**p-value**	**Function**
DDC	LABE_00062252	0,394	0,043	0,281	0,230	Biosynthesis of serotonin
MAO B	LABE_00012540	-0,476	0,040	-0,299	0,295	Deamination of serotonin
MAO A	LABE_00010029	-0,433	0,045	-0,368	0,144	Deamination of serotonin
SLC6A4	LABE_00037267	0,258	0,122	0,133	0,546	Transport of serotonin
TPH2	LABE_00054456	0,962	0,001	0,683	0,044	Biosynthesis of serotonin
TPH1	LABE_00005595	0,282	0,281	0,335	0,256	Biosynthesis of serotonin
APOA4	LABE_00076033	-0,779	0,038	-0,297	0,390	Chylomicron assembly
ELOVL1	LABE_00047458	-0,203	0,142	NA	NA	Fatty acid elongation
PLD1	LABE_00056986	-0,686	0,001	-0,468	0,065	Phospholipase
PLIN2	LABE_00009890	-0,009	0,983	0,585	0,058	Lipid storage droplets
PLPP3	LABE_00030241	-0,485	0,000	-0,202	0,076	De novo synthesis of some lipids
PPARG	LABE_00007366	-0,312	0,132	-0,296	0,216	Adipocyte differentiation
SLC27A4	LABE_00024491	-0,301	0,144	-0,151	0,582	Fatty acid transport
CD36	LABE_00046348	-0,422	0,115	0,086	0,842	Regulator of fatty acid transport

3.2.Genetic expression-quantification

3.2.1. Genes involved in lipid metabolism

The mean of the expression of the genes (RNE) related to lipid transport (Figure 12) and those related to lipid metabolism (Figure 13) are shown thereupon. The expression of only three genes (ELOVL1, PLIN2, and PPARG) was significantly affected by feeding (glmmPQL p ELOVL1= 0.005, glmmPQL p PLIN2= 0.016, glmmPQL p PPARG= 0.014) where ELOVL1 and PPARG were down-regulated whereas PLIN2 was up-regulated. Not significant effect of diet was observed for the rest of the genes (glmmPQL p APOA4= 0.17, glmmPQL p CD36= 0.15, glmmPQL p PLPP3= 0.84, glmmPQL p SLC27A4= 0.67, and glmmPQL p PLD1= .60).

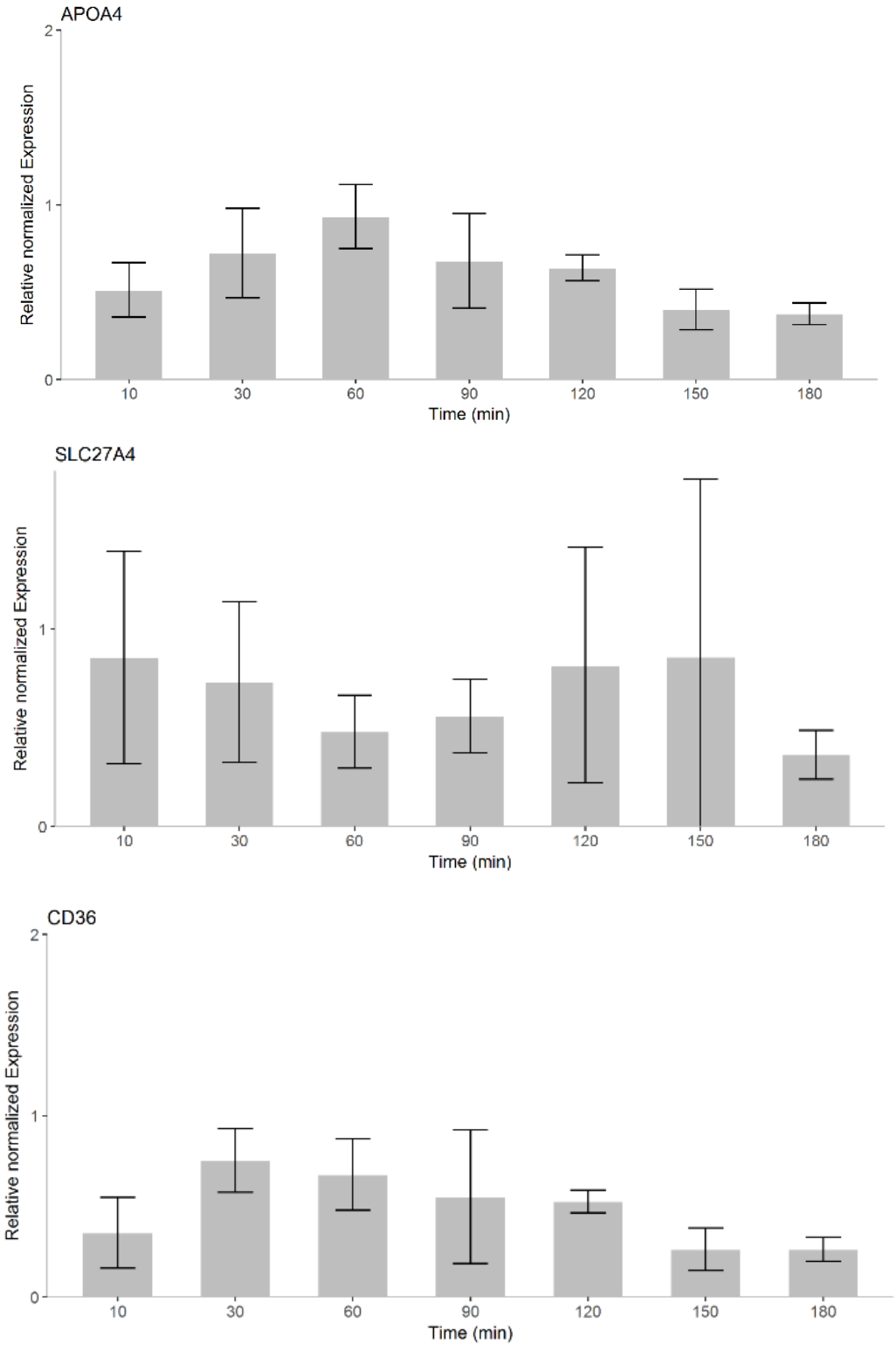

Figure 12. Average of the relative normalized expression (RNE) and the SD of genes related to lipid transport with time by means of RT-qPCR. Feeding did not modulate its expression (glmmPQL p> 0, 05).

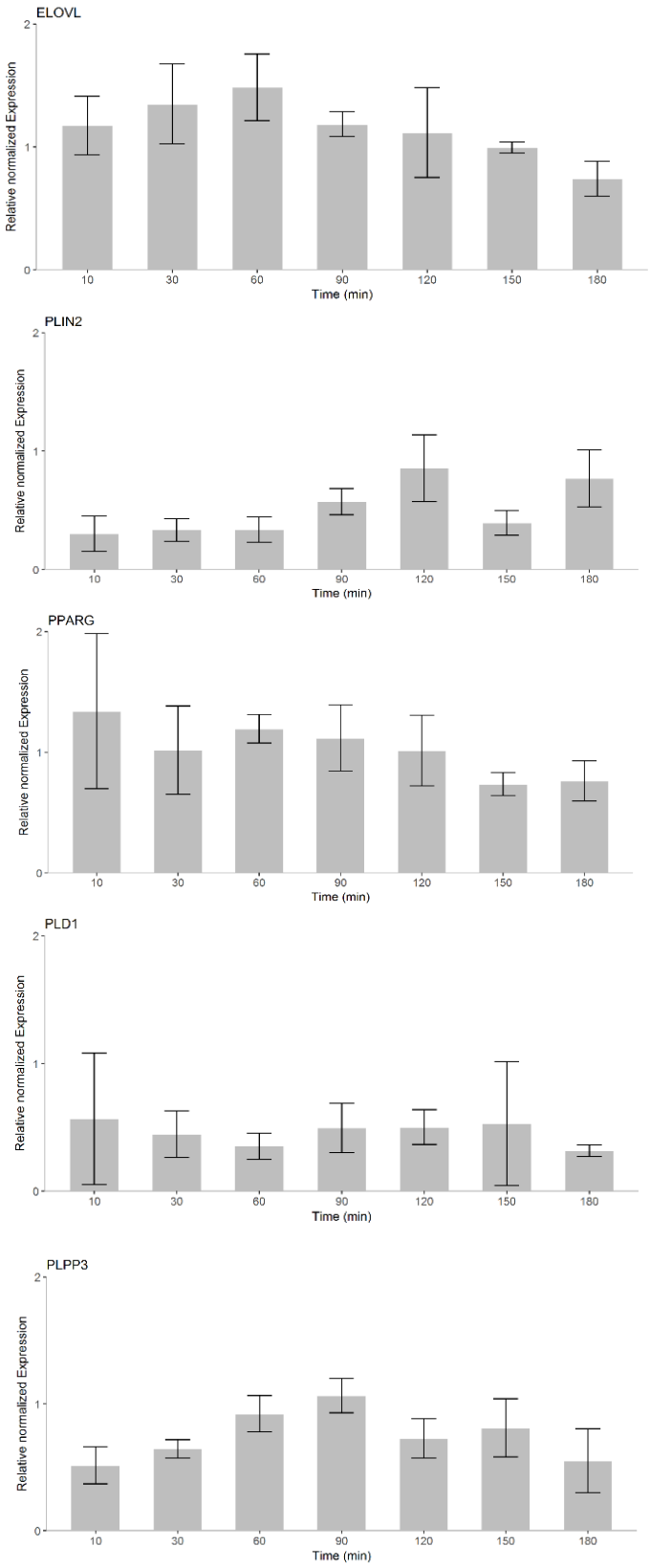

Figure 13. Average of the relative normalized expression (RNE) and the SD of genes related to lipid metabolism with time by means of RT-qPCR. Feeding did not modulate the expression of PLD1 nor PLPP3 (glmmPQL p> 0, 05) but had a significant effect on ELOVL1, PLIN2 and PPARG (glmmPQL p< 0, 05).

Weighted calibrations

Once the variances were normalized, weighted regressions of the expression of all the genes involved in lipid metabolism except for PLIN2 and PLPP3 showed a tendency to decrease after feeding (table A.6 in the Appendix). PLIN2 and PLPP3 showed a weighted regression ($1/\sigma^2$) with a positive slope (m) suggesting upregulation of the latter genes (table A.6). It is important to remark that the expression of only three genes (ELOVL1, PLIN2, and PPARG) showed to be significantly affected by feeding and the regressions for the rest of the genes (APOA4, SLC27A4, CD36, PLD1, and PLPP3) are mere trends (p values > 0, 05) (table A.6).

3.2.2. Serotonin related genes

Genes involved in serotonin synthesis

TPH1 was the highest expressed gene among all the genes involved in 5-HT metabolism. However, not significant effect of time on TPH1 expression was found (glmmPQL p=0.94). On the contrary, TPH2 expression did increase significantly with time (glmmPQL p=6*10-6) reaching the highest expression level 180 min after feeding (Figure 9). DDC expression was not modulated by feeding (glmmPQL p=0, 11) which can be observed by the flat bars in (Figure 9).

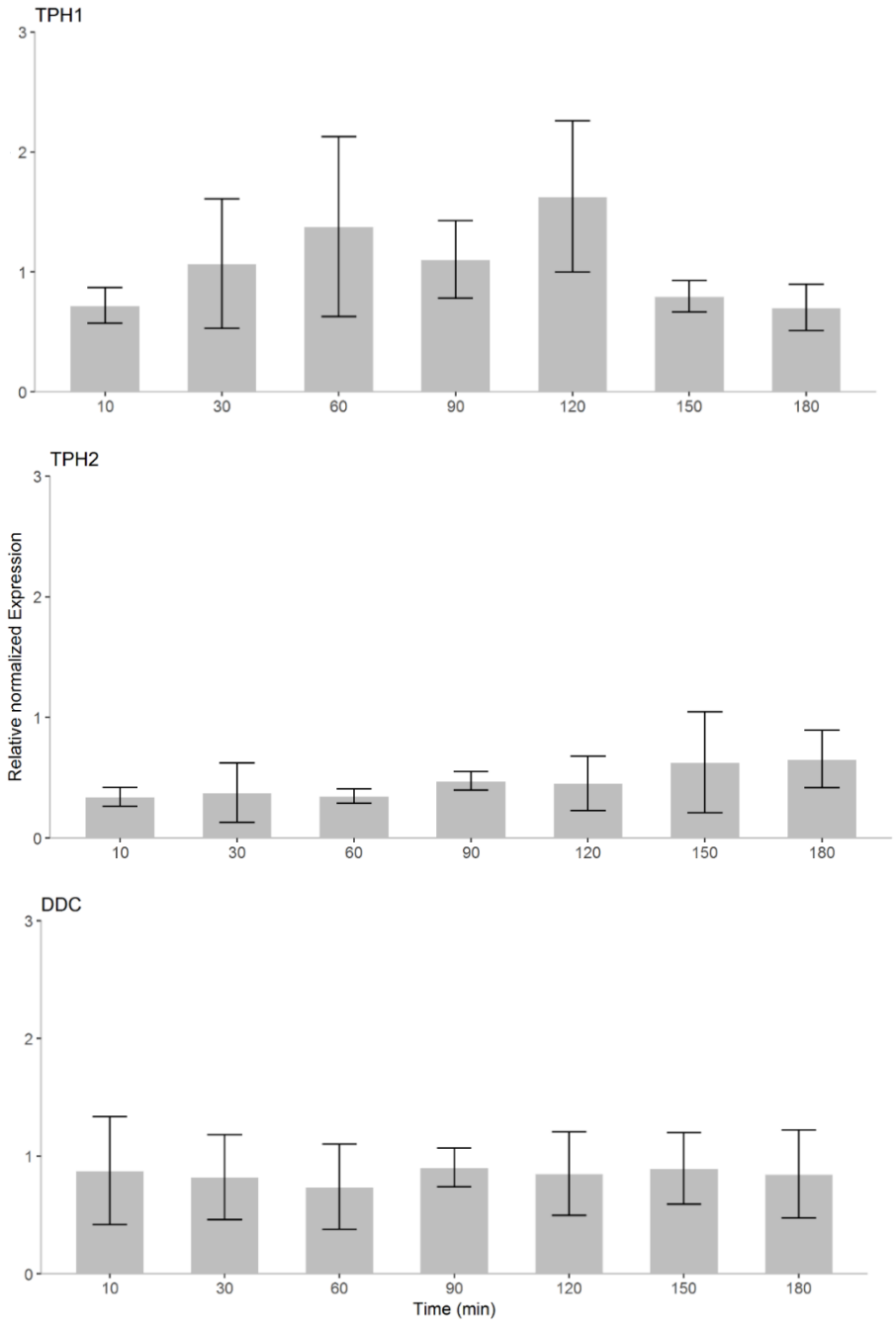

Figure 9. Average of the relative normalized expression (RNE) of TPH 1, TPH2 and DDC with time by means of RT-qPCR. The expression of both TPH 1 and DDC does not vary significantly with time (glmmPQL p>0,05) while TPH2 expression showed to be modulated by time (glmmPQL p<0,05).

Genes involved in serotonin degradation

Both MAO A and MAO B showed a similar post prandial pattern of expression (Figure 10). The mean of the expression of both genes (RNE) tended to show a peak expression 60 minutes after feeding and from there on, there is a tendency towards decreased expression. However, not significant effect of feeding on none of the genes was found (glmmPQL p MAO A= 0, 24 and glmmPQL p MAO B=0, 57).

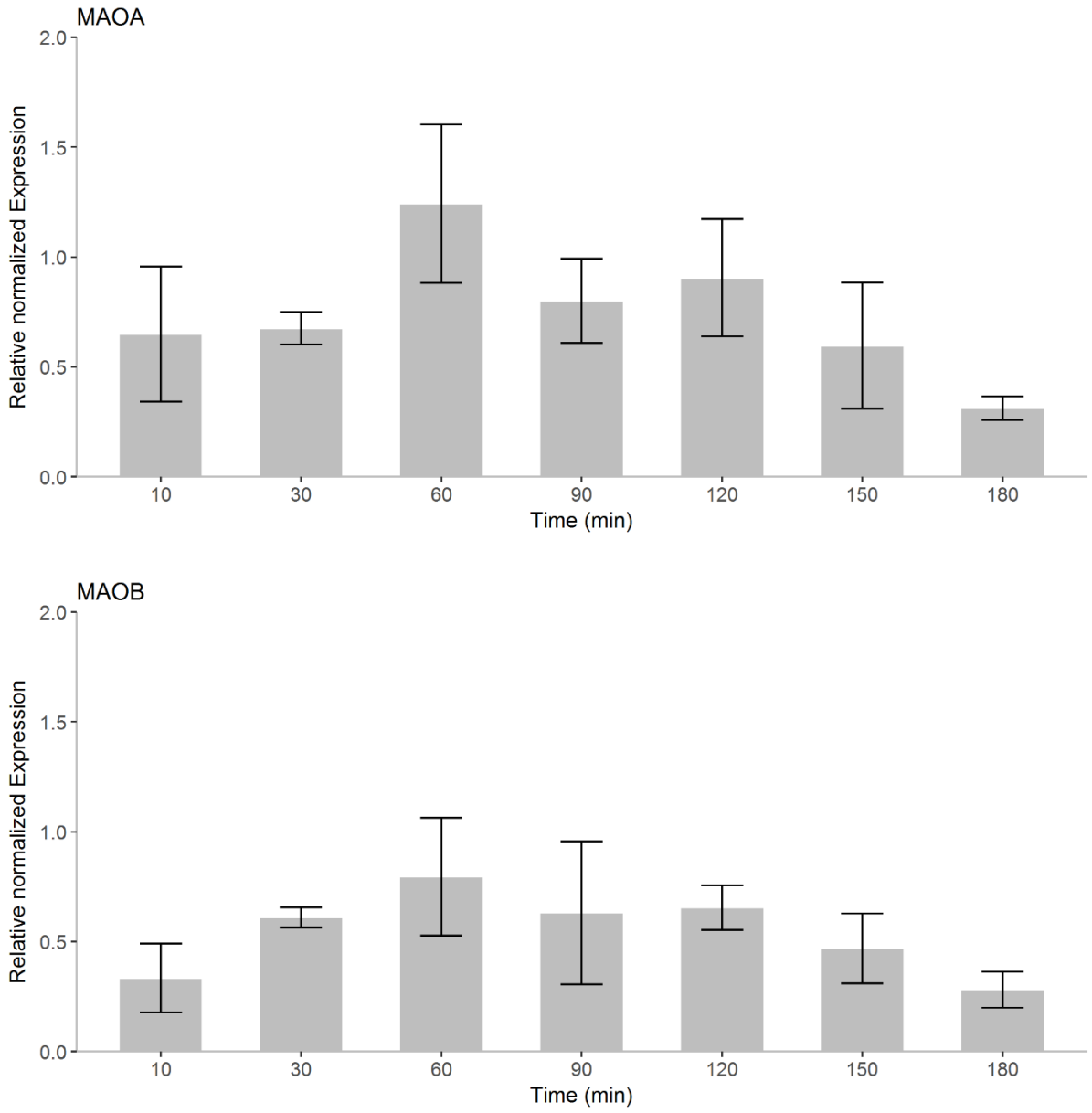

Figure 10. Average of the relative normalized expression (RNE) of MAO A and MAO B with time by means of RT-qPCR. No significant variation in gene expression with time for both genes (glmmPQL p > 0, 05).

Serotonin carrier encoding gene

SLC6A4 expression was downregulated with a p value very close to significance (glmmPQL p= 0, 053) showing its lowest value at the end of the trial (180 minutes after feeding) (Figure 11).

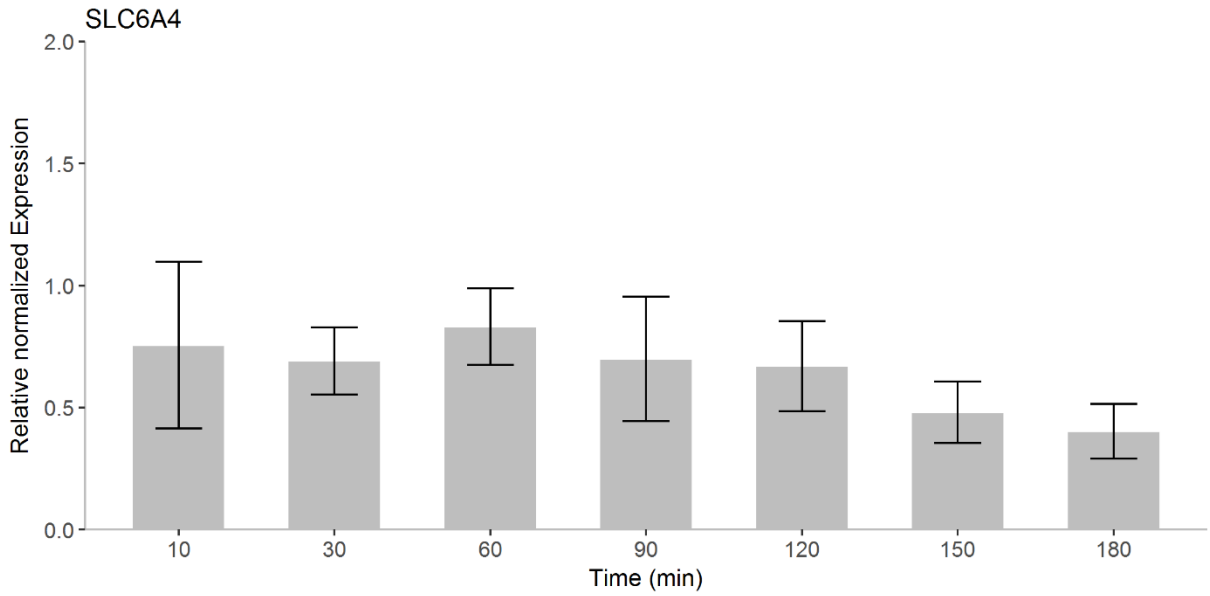

Figure 11. Average of the relative normalized expression (RNE) of SLC6A4 with time by means of RT-qPCR. Time did not modulate its expression (glmmPQL p= 0, 05)

Weighted calibrations

The violation of the assumption of data with equal variances (which precluded the implementation of ordinary least square methods) and the weighted models for all the genes involved in 5-HT metabolism with their corresponding normalized residuals are shown (table A.5 in the Appendix)

Once the data (variances) were normalized for each gene, weighted calibrations showed a linear down-regulated trend for MAO A, MAO B, and SLC6A4. A clear up-regulated trend for TPH2, and an almost inappreciable up-regulated trend for TPH1 and DDC (table A.5). Although only the expression of TPH2 was significantly affected by feeding (p = 6*10-6) as mentioned before, weighted least square regressions (table A.5) were crucial to define the tendency of the gene expression throughout time.

3.3. Serotonin in intestinal tissue

3.3.1. Optimal initial concentration of the samples

The OD values (absorbance) obtained from 1:16 diluted samples (the third dilution of the dilution series) were within the limits of the standard curves for the three replicates (Figure 14). 1:16 diluted samples were then used for calculating serotonin concentration in tissue.

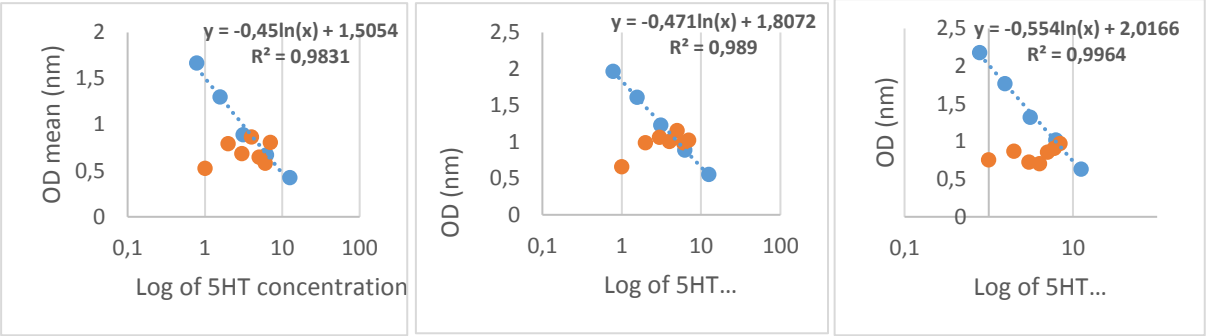

Figure 14. Standard curves of the OD values (blue spots) and their logarithmic trend line (blue line) for the three different replicates (A, B, and C correspond to replicate 1, 2 and 3). Equations together with the R-squared values are displayed for each chart. Orange dots represent the OD values from the 1:16 diluted samples which are clearly plotted within the trend-lines limits.

3.3.2. Serotonin quantification

Figure 15 shows the average of serotonin (ng/mg) found in tissue for the three experimental replicates. Serotonin levels did not show large fluctuations over the first two hours and a half after feeding where values varied between 0,55 and 0,70 ng/mg. By looking at figure 15 and table A.7 (in the Appendix), one could expect a significant effect of feed in the concentration of 5-HT as the average concentration after three hours (180 min) increased nearly twice as much as in the beginning (after 10 minutes of incubation). Nonetheless, not significant effect of feeding was found along the post prandial incubation time frame (glmmPQL p = 0,299).

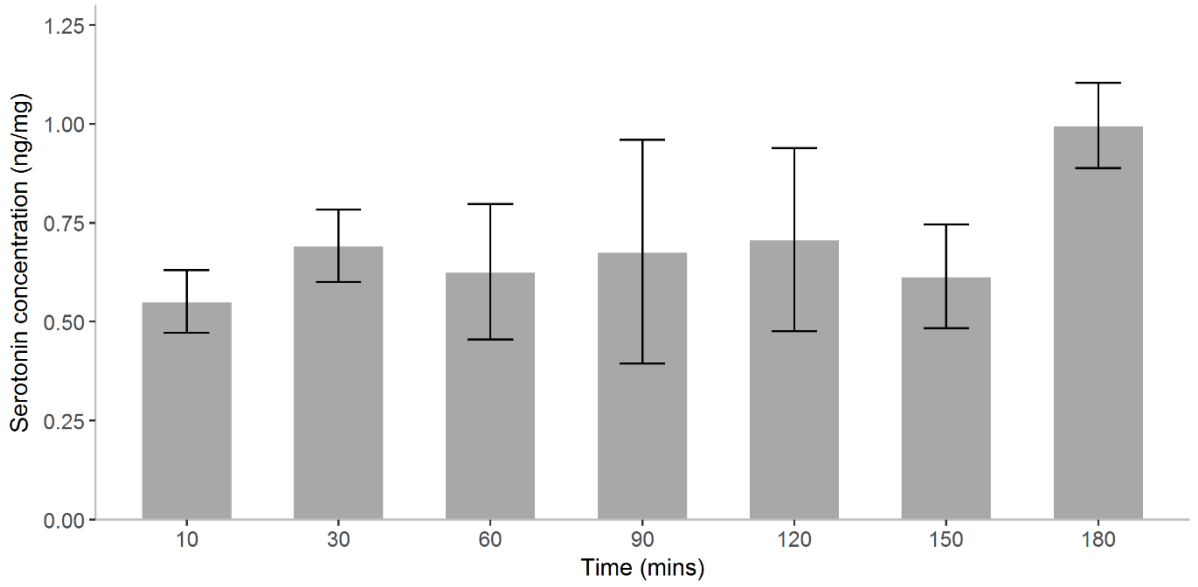

Figure 15. Average and SD of serotonin concentration (ng/mg) in tissue from the three experimental replicates. Feeding did not have a significant effect on 5-HT concentration in tissue (glmmPQL p = 0,299).

In an attempt to observe possible differences in serotonin concentrations between experimental triplicates, they were plotted separately (Figure 16). The first and second experimental replicates (A and B in Figure 16) were observed to have similar levels of serotonin throughout the time of incubation (3 hours). On the other hand, the third experimental replicate (C in Figure 16) showed higher levels of serotonin for most of the time points, especially for 90 to 150 minutes, not following the same modulation pattern than the other two experimental replicates.

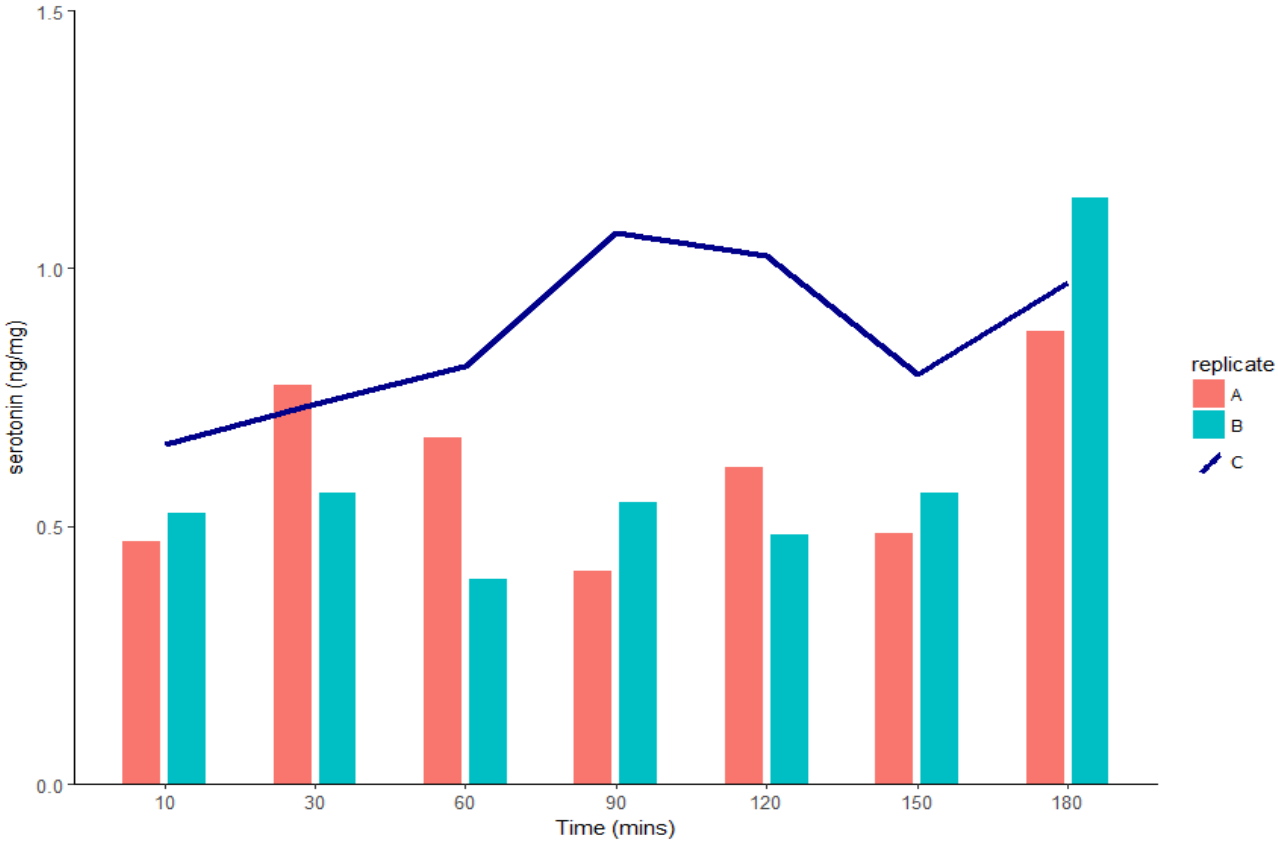

Figure 16. Serotonin concentration (ng/mg) in tissue for the three experimental different replicates (A, B, and C correspond to replicate 1, 2 and 3). The third experimental replicate (c) showed a concentration of serotonin twice as large as the first and second experimental replicates at 90 and 120 minutes.

3.3.3. Correlation analyses

Pearson's product-moment correlation test (Figure 17) showed not significant correlation between serotonin in tissue (ng/mg) and the expression of any of the genes (DDC r (19) = -0.005, p=0.98; MAO A r (19) = -0.41, p = 0. 066; MAO B r (19) = -0. 26, p=0. 26; SLC6A4 r (19) = -0.39, p= 0.081; TPH 1 r (19) = -0. 1, p= 0.66; TPH 2 r (19) = 0.29, p= 0.2). TPH2 expression seemed to be the only positively correlated with serotonin in tissue while the rest of genes tend to lower their expression when serotonin in tissue increases (negative correlation).

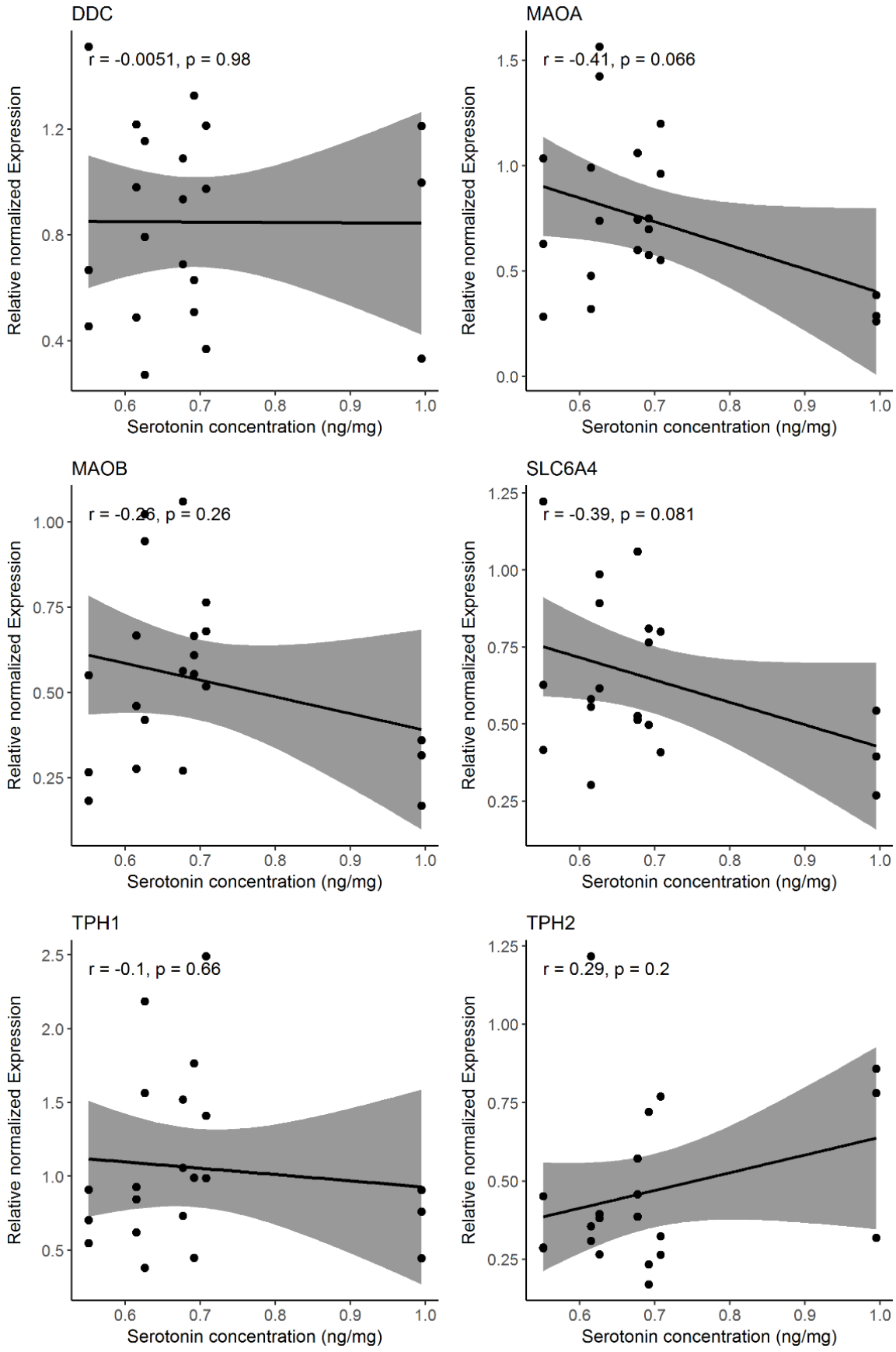

Figure 17. Pearson correlation coefficient, *r*, and p values for each gene. Values corresponding to the three timelines are plotted for each given time.

4. Discussion

4.1. Methodological considerations

4.1.1. In vitro analyses

Gene expression analyses together with 5-HT measurements were investigated in intestinal tissues under *in vitro* conditions representing a novel method. *In vitro* experiments using gut tissues have previously been reported for example by (Velarde et al. 2010) that evaluated the effect of 5-HT on the intestinal motility of goldfish. However, this current *in vitro* study analyses modulation of gene expression in response to diet using RT-qPCR methodology.

4.1.2. Sample recollection and gene expression repercussions

The RNA integrity of a sample is determined by the RNA integrity Number (RIN) that follows a numbering system from 1 to 10, with 1 being the most degraded RNA, 5 being highly degraded, and 10 being the best intact. It has been previously reported that the gut tissue is especially fragile and RNA degrades rapidly if not treated adequately (Heumüller-Klug et al. 2015). The RNA integrity of the gut samples analysed in this study cannot be considered as optimal (RIN values varied between 6 and 7,5) but still acceptable and following gene expression analyses were therefore trusted (Heumüller-Klug et al. 2015). The life span of intestinal epithelial cells (IECs) is short (3–5 days), and its regulation is thought to be important for homeostasis of the intestinal epithelium (Park et al. 2016). This high cell-turnover ratio would lead to the presence of degraded RNA which could lower the RIN values in intestinal tissue. There were several steps throughout the experiments that could potentially have damaged the gut tissue affecting cell function and gene expression. Firstly, guts were flushed several times with Ringer's solution prior to feeding in order to wash out food remains. The intestinal tissue was also cleaned after incubation for the same purpose. This procedure, together with the fact that the gut was kept out of the body during sampling and incubation, might have caused damage and degradation of the tissue.

4.1.3. Serotonin quantification by ELISA kit

ELISA serotonin analyses have been reported as an efficient method to measure serotonin in serum, plasma, cell culture supernatants, and urine (Nichkova et al. 2012; Lee et al. 2014). Most of the literature testing the efficiency of ELISA kits refers to serotonin from body fluids in either humans or mice (Kim and Camilleri 2000; Gershon and Tack 2007). A recent study focused on 5-HT quantification from the gut tissue of two fish species (Atlantic salmon and Coho salmon) was performed by Mardones et al. (2018) using HPLC for quantification instead ELISA. The 5-HT concentration seemed to vary

considerably between the two investigated species (from 1 to 1, 5 ng/mg of 5-HT in the midgut of A. salmon and 2,5 to 3 ng/mg in the midgut of C. salmon). This gives a perception of the variation of 5-HT between species. Ballan wrasse belonging to a completely different order of fishes, had 5-HT values from 0, 5 to 1, 1 ng/mg over the post prandial incubation time (Figure 15) being close to those values showed by salmon. Although the 5-HT levels from salmon and Ballan wrasse are not comparable (different species, one with empty guts and the other with lipid infusion before 5-HT measurements), the similarity in the 5-HT concentration found in the gut of the previous mentioned species could confirm the suitability of ELISA analysers used in these *in vitro* experiments**.**

4.1.4. Statistical considerations

By looking at the data from gene expression and serotonin quantification (Figures 9-13 and 15) large variances were observed although outliers were not found in any case. In order to test the possible effect of variances the data were evaluated for homoscedasticity by plotting weighted regression in which variances were normalized, and comparing them to linear (unweighted) regressions. Weighted regressions did not show any significant lack of fit at the 95 % confident level which meant that the data were heteroscedastic (significant differences in the variances) (Miller and Miller 2018). For this reason and according to Araujo et al. (2014) , weighted regressions were chosen as the most suitable modelling approach for showing the time dependant modulation of gene expression after feeding.

Non-constant variance (heteroscedasticity) in repeated measurements is a frequent problem in biological data (K. Jensen, personal communication, 2017) especially when the number of replicates is low. Interestingly enough, the current study counted with three experimental replicates (n=3) which is proposed to be the main reason for the big variation in our data. In an attempt to add more information to the weighted regression models and define significances, a non-parametric statistical test was applied with its corresponding decrease in sensitivity. In this case Generalized Linear Mixed Models for multiple random effects (glmmPQL) which accounted for large variances was used.

4.2. Transcriptomes analyses

The principal component analysis together with the heat map of gut transcriptomic patterns of the control (c) and intact fish oil groups (TG) revealed larger differences than expected. Only digested oil (hTG) in the form of free fatty acids can be absorbed and therefore trigger a transcriptomic response. Thus, intact fish oil dietary groups (TG) was expected to not trigger transcription of genes and cluster closer to the control than it did. It can be hypothesized that either the low amount of free fatty acids in the intact oil (TG) used in the experiment was enough to initiate the transcription of the differentially expressed genes, or there are enzymes left for lipolysis in the *in vitro* gut. However, the intact fish oil

dietary group (TG) still clustered closer with the control group than with the group given hydrolysed fish oil (hTG).

Lipid diets, *in vivo*, are a strong stimuli for the activation of digestion mechanisms and gene expression (Sæle et al. 2018). The hydrolysed dietary group (hTG) which is mainly free FAs (available for absorption) did not cluster with the control group concluding that hTG diet evokes significantly different expression of genes than TG and control. Lie et al. (2018) demonstrated that the first three segments of the Ballan wrasse gut act as one continuous short tube with high expression of genes associated with digestion and absorption of nutrients as well as appetite regulation. In the same paper they revealed the loss of all known genes (except for one) related to stomach function suggesting the necessity of formulated diets with higher levels of easily digestible protein than those for gastric species.

4.3. Modulation of gene expression following lipid administration

4.3.1. Lipid transport and absorption in the gut of Ballan wrasse

The genetic expression of lipid transporters (SLC27A4, CD36, and APOA-IV) were investigated in the gut of Ballan wrasse after administering a lipid-rich meal *in vitro*. The *in vitro* diet used in these experiments was cod oil rich in omega-3 polyunsaturated fatty acids (90% PUFAs: EPA and DHA and only 10 % saturated fatty acids) (NIFES 2018).

SLC27A4 also known as FATP4 is specifically expressed in enterocytes and directly related to the uptake of fatty acids specially LCFAs, generation of energy and biosynthesis of lipids (Milger et al. 2006; Anderson and Stahl 2013). Results showed a slight down-regulation of SLC27A4 after feeding. Yan et al. (2015) also observed downregualtion of SLC27A4 with fish oil diet and suggested that the role of FA transporters may differ under various dietary lipid levels. Supported by this, it is hypothesized that SLC27A4 might lack direct implication in PUFAs transport in the gut of Ballan wrasse.

Schwartz (2011) reported the fatty acid translocase CD36 as the main molecule involved in the absorption of lipids with a likely important secondary role in chylomicron formation. Results did not show a significant response of CD36 to feeding. A close related enzyme, Apo A-IV, is primarily synthesized in the enterocytes of the gut during fat absorption and further incorporated into the surface of nascent chylomicrons and high-density lipoproteins (HDL) (Stan et al. 2003). As for CD36, results showed not significant response of Apo A-IV to feeding. Apo A-IV synthesis and secretion have been reported to be actively involved in fat absorption in the gut of rats (Kalogeris et al. 1994) and zebra fish (Otis et al. 2015) with diets rich in TAG. It is well known that Apo A-IV gene expression is up-regulated in response to lipid ingestion (Otis et al. 2015; Sæle et al. 2018).

The lack of time dependant modulation of CD36 and specially, Apo A-IV by feeding is difficult to discuss. According to (Schwartz 2011) and (Otis et al. 2015) the high content of long chain fatty acids (LCFA) in the diet would trigger up-regulation of both genes and therefore, a higher response of Apo A-IV and CD36 was expected in the gut of Ballan wrasse as fish oil was used as dietary treatment.

4.3.2. Lipid droplets formation

Perilipin (PLIN) proteins constitute an ancient family important for the assembly and biogenesis of cytosolic lipid droplets (CLD) which typically store neutral lipids and play diverse roles in metabolism and signalling (Demignot et al. 2014; Granneman et al. 2017). Results showed a significant upregulation of PLIN2 in the gut of Ballan wrasse corroborating its response to lipid diets. Most work addressing PLIN protein function has been performed in mammals and relatively little on non-mammalian vertebrates. In accordance with our results, Lecchi et al. (2013) hypothesized that n3 LC- PUFAs (EPA and DHA) may modulate lipid droplet formation by upregulating PLIN2 and PLIN3 mRNA expression. Besides, upregulation of PLIN2 in fish has been shown to correlate with the inclusion of dietary TAG (Sæle et al. 2018). However, unlike the rest of the studied genes, PLIN2 was strongly expressed only 2 hours after feeding suggesting that droplet formation mechanisms in Ballan wrasse act more actively after transport and absorption have taken place.

4.3.3. Lipid metabolism

Genes associated with re-synthesis of TAG were up-regulated in the gut of zebrafish larvae with TG rich diets (Sæle et al. 2018). Genes involved in the re-synthesis of TG in Ballan wrasse did not show the same up-regulated expression pattern.

PLPP3 expression in the gut of Ballan wrasse was not significantly modulated by feeding. PLPP3 is a member of the phosphatidic acid phosphatase (PAP) family with a crucial role in de novo synthesis of glycerolipids such as TGs and DAGs (Tocher et al. 2008). It is well known that different genes act specifically in the synthesis of different glycerolipids. Sæle et al. (2018) showed that genes involved in DAG synthesis were higher expressed compared to those involved in TAG synthesis when TAG rich diets were supplied. The lack of time-dependant TAG-rich diet modulation of PLPP3 in the gut of Ballan wrasse suggest the likely presence of other genes with a higher commitment in glycerolipids synthesis.

ELOVL1 has been suggested to be involved in the elongation of both saturated and monounsaturated fatty acids (Jakobsson et al. 2006). Fish oil-enriched diets repressed elongase activity in livers of rats (Wang et al. 2005). Interestingly, also diets rich in monounsaturated fatty acids (oleic and erucic) have been reported to inhibit endogenous ELOVL1 (Sassa et al. 2014). The expression of ELOVL1 mRNA in Ballan wrasse only started to decrease significantly one hour after feeding probably indicating that the feed used in these trials (fish oil rich in PUFAs) did not trigger any specific response and it was the time

the main factor for the decreased expression of ELOVL1. The slightly up-regulated pattern of ELOVL1 right after feeding could still be explained by the presence of few SFAs in fish oil (Jakobsson et al. 2006). This is supported by (Sassa et al. 2014) that dismissed the role of PUFAs (EPA and DHA) at inhibiting ELOVL1 activity.

PPARG also known as PPARγ, is a regulator of adipocyte differentiation and lipid storage (Morais et al. 2012). PPARγ transcriptional activity is regulated by fatty acids availability. In the absence of FAs, PPARγ is transcriptionally silent whereas it is highly expressed when fatty acids are added to the diet (de Paula et al. 2013). Unexpectedly and similar to ELOVL1, results in Ballan wrasse showed a significant downregulation of PPARG with fish oil diet. Interestingly, Morais et al. (2012) claimed that PPARG was strongly up-regulated by diets rich in vegetable oil compared to fish oil diets. It is then suggested that a lower adipocyte synthesis and lipid storage occurs when fish oil rich in PUFAs is used instead of vegetable oil, which is rich in monounsaturated fatty acids (MUFAs). The latter is supported by (Morais et al. 2012).

The last gene investigated was Phospholipase D (PLD1) which catalyses the hydrolysis of the phospholipid phosphatidylcholine (PC) and its metabolites participate in multiple cellular activities including cell proliferation, differentiation, migration, and survival (Zeng et al. 2009). The lack of response of PLD1 to feeding was expected as fish oil used in this diet did not have phosphatidylcholine.

Although most of the target genes involved in lipid metabolism did not show as strong response to feeding as expected, there is a tendency of changing patterns in their expression during the in vitro experiments which are triggered by “feeding”. This implies the presence of active cellular mechanisms in the gut of Ballan wrasse that efficiently respond to nutrients *in vitro*. According to Bellono et al. (2017), EC cells have receptors and transduction mechanisms that detect ingested chemicals and contribute to other sensory or neural signalling systems. Based on the fact that dietary lipids modulated expression of genes involved in lipid metabolism, it is suggested that serotonergic EC cell will also respond to diet and further discussion will be therefore focused on the regulation of 5-HT by lipid diets in the gut of Ballan wrasse.

4.4. Serotonin in Ballan wrasse

4.4.1. Serotonin quantification in the gut

The surprising lack of the significant effect of dietary lipids post prandial on the amount of 5-HT in tissue might be due to the low number of biological replicates (low n) used in the present experiments

in combination with large variation in the data. The apparent higher concentration of 5-HT at the end of the trial (180 minutes after feeding) (Figure 15 and 16) might suggest the likely presence of a regulatory mechanism that evokes an increase of 5-HT in the gut of Ballan wrasse in response to feed. This however can only be proved with more experimental replicates (higher n). The suggested mechanisms involved in the synthesis of 5-HT in Ballan wrasse will be later discussed.

It was also shown that the amount of 5-HT was similar in the guts dissected at the same time early in the morning but in different months (first and second experimental replicate). Interestingly, the third experimental replicate that was perform later in the afternoon showed in average higher levels of serotonin.

Stress of cultured fish is of great concern as it directly affects growth and health causing fish mortality. Conte (2004) recognized that controlling animal stress is absolutely essential to ensure animal welfare. There are many factors that can potentially lead to stress in fish such as water characteristics (quality, quantity, and temperature), high animal density, and poor feeding regime among others. Poor water quality (low oxygen and high waste accumulation) together with high fish density will overload the system with metabolites thereby leading to stress (Conte 2004). Fish respond differentially to stress and count on mechanisms to cope with disturbances and maintain its homeostatic state. Genetic history of fish appears to be highly affected by stress when trying to cope with it (Barton 2002). Serotonin has been reported to be strongly implicated in stress regulation in fish (Backström and Winberg 2017). Brain 5-HT release is easily affected by multiple stressors such as nutritional status, social interaction, and immune challenges (Winberg and Nilsson 1993). In accordance with this, both synthesis of 5-HT and its metabolism was observed to increase under pathological or stressful circumstances in the brain of trout and the gut of mice (Øverli et al. 2001; Gershon 2013; Winberg and Thörnqvist 2016). As mentioned before fish used in the third experimental replicate was kept in the bucket for longer period implying longer fasting and repeated disturbances with the net to catch fish. These factors might have caused higher stress for the fish of the third experimental replicate compared to the other individuals. I therefore propose stress as the potential reason of the higher concentration of 5-HT in the third experimental replicate.

4.4.2. Synthesis of serotonin and the role of EC cells

Serotonin is synthesized through the actions of two different rate-limiting tryptophan hydroxylases, TPH1 which yield the mucosal synthesis of serotonin and TPH2 (the neural form), which are found respectively, in enterocromaffin cells (EC cells) and neurons (Gershon and Tack 2007; Bornstein 2012). DDC also known as L-AADC, is also involved in serotonin synthesis as a non-rate limiting enzyme

(Bertrand and Bertrand 2010). DDC expression did not show any change after feeding which is consistent with its non-rate limiting action and thus, not large variations were expected.

The gastrointestinal (GI) tract in fish is innervated by sensory neurons that respond to nutrients, chemicals or mechanical stimuli within the gut lumen (Velarde et al. 2010). Stimuli however, do not seem to interact directly with enteric neurons but instead activate specialized cells (EC cells) in the epithelium in a process of sensory transduction (Bertrand and Bertrand 2010; Velarde et al. 2010). Many authors have supported the idea that this sensory system regulates the synthesis and release of 5-HT by EC cells which will act on intrinsic nerves regulating GI tract motility. EC cells have been found in protochordates and most vertebrates, but were previously thought not to be present in teleost. Now it is known that the presence and/or location of EC cells in teleost varies greatly among species being found both in the stomach and/or in the gut (Anderson and Campbell 1988). The presence or absence of EC cells in the gut of Ballan wrasse have not been investigated by terms of immunohistochemistry or fluorescence. However, the analyses of the entire genome as well as the intestinal transcriptome of Ballan wrasse recently available (Lie et al. 2018) has made it possible to shed some light on the previously unreported presence of EC cells as discussed in greater detail later (see 4.3 below).

According to Parmar et al. (2012), TPH1 is the main enzyme involved in 5-HT synthesis within EC cells. TPH1 showed the highest expression among all the studied genes in these experiments. mRNA expression analysed by RT-qPCR evidenced the presence of TPH1, which implies the existence of EC cells in the gut of Ballan wrasse unlike another stomach-less species, the goldfish, that was reported to lack EC cells in the gut mucosa (Velarde et al. 2010). Surprisingly, TPH1 did not show to be regulated by feeding, indicating that there might be another important source of 5-HT besides EC cells in the gut of Ballan wrasse.

4.4.3. EC presence in the gut of Ballan wrasse

The presence of known genes associated with EC cells in the gut of Ballan wrasse (table 3) have been reviewed by investigating the genome and intestinal transcriptome of Ballan wrasse found in the European Nucleotide Archive (accession number: PRJEB13687) (From Additional files.2 (Lie et al. 2018)). This small “meta-study” is based on the use of transcriptomic data and it was done as a supplement for the thesis as it was not part of the original focus on this study.

NK2 homeobox 2 (Nkx2.2) has been reported to be essential for the specification of enteroendocrine cells, especially for serotonergic EC cell linage (Gross et al. 2016). The same authors identified Lmx1a as a novel regulator of the 5-HT signalling pathway in the gut of mice being essential for the expression of TPH1. Genome and transcriptomes analyses in Ballan wrasse revealed the presence of Nka2.2a,

Lmx1a and TPH1 (table 3). Also, TPH1 was identified by means of RT-qPCR. Interestingly, unlike the rest of the specific genes of EC cells, Lmx1a did not show to be differentially expressed between the different parts of the gut despite being present in the analysed transcriptome. Also surprisingly, TPH1 and DDC which are enzymes in charge of 5-HT synthesis showed to be upregulated towards the hindgut unlike the rest of the genes expressed by EC cells.

Two catecholamine receptors (adra2a and TRPC4) have been reported to specifically be present in EC cells from the gut of mice (Bellono et al. 2017). TRCP4 was not found in the Ballan wrasse genome, while according to the transcriptome, adra2a seemed to be present only in the first segment of the gut (table W). It has been suggested that EC cells respond to contents of the lumen through the action of cation channels such as Trpa1. Nozawa et al. (2009) reported the high expression of Trpa1 in human and rats EC cells and its direct implication in regulating 5-HT release as well as gut contraction. Trpa1 was also highly expressed in the gut of Ballan wrasse which would be another evidence supporting the presence of EC cells. However, Trpa1 is not exclusively expressed in EC cells as it has been found in sensory neurons (Penuelas et al. 2007). The source of Trpa1 in the gut of Ballan wrasse (from EC cells or neurons) therefore cannot be defined by transcriptomic analyses and cannot be directly linked with the presence of EC cells.

Table 3: Genes expressed by EC cells in the gut of Ballan wrasse. Differentially expressed genes between the third and the fourth segment and the first and the fourth segment. Transcriptomes analysis identified differentially expressed genes (adjusted p< 0, 05) from RNA-sequencing of the gut of Ballan wrasse (From Additional files.2 (Lie et al. 2018)). NA indicates the lack of differential gene expression between segments and the log2Foldchange indicates up/down regulation of the gene expression. All the three genes seem to be higher expressed in the anterior part of the gut indicating a downregulation of the gene expression towards the hindgut.

Segments	***3vs4***			***4vs1***		
Gene	***accession no.***	***log2FoldChange***	***p-value***	***log2FoldChange***	***p-value***	***Function***
Nkx2.2a	*LABE_00038162*	*1,143486*	*0,025603*	*-1,83043*	*1,73E-05*	*Enteroendocrine cell specification*
Trpa1	LABE_00003169	1,435816	0,001067	-2,43727	2,85E-10	Cation channel in EC cells
adra2a	LABE_00053697	NA	NA	-1,05611	0,043905	Catecholamines sensitivity in EC
TPH1	*LABE_00005595*	-1,99856	0,000505	1,426946	0,015247	5-HT synthesis (Rate-limiting)
DDC	*LABE_00062252*	-1,0669	0,001347	NA	NA	5-HT synthesis (Non rate-limiting)

The expression of genes that characterize EC cells (table 3) is a good indicator of the presence of EC cells in the gut of Ballan wrasse. Interestingly, the fact that TPH1 and DDC are the only up-regulated genes towards the hindgut seems to be a bit of contradictory. Regardless, based on the previous

transcriptome analyses, I hypothesize the presence of EC cells in the gut of Ballan wrasse and more specifically, the higher presence of EC cells in the anterior part of the gut compared to the hindgut. It is also proposed that the higher transcriptomic expression of TPH1 and DDC in the hindgut might be promoted by microbiota which has been observed to stimulate 5-HT synthesis in the colon (see 4.8) (Yano et al. 2015). Only TRCP4 was not observed in the genome of Ballan wrasse. Nonetheless, it is very likely that TRCP4 also exist in the gut and that the gene, for example could have remained undetected in regions difficult to sequence (Lie et al. 2018). The absence of a gen is always more difficult to demonstrate than the presence of one. Further and more detailed genetic analyses together with immunohistochemistry might corroborate the presence of EC cells.

4.4.4. The role of the enteric nervous system in teleost

Serotonin has been shown to induce contractions in isolated gut from a stomach-less teleost, the goldfish (*Carassius auratus*), which gut has a prominent population of 5-HT positive neurons and lack EC cells in the mucosa (Velarde et al. 2010). These enteric neurons have projections to the lamina propria and muscle but do not reach the myenteric plexus lacking direct contact with stimuli from the mucosa.

For decades, it was widely believed that serotonin was mainly produced by EC cells (mucosal sources) (Bornstein 2012) and the contribution of the enzyme TPH2 (neural form) in the production of serotonin in the enteric neurons of the gut was not very clear. This was likely due to the use of low sensitive techniques such as formaldehyde induced fluorescence which did not seem to detect low levels of 5-HT and thus, those neurons with low 5-HT concentration were not detected at all (Anderson and Campbell 1988). The use of a more sensitive technique, immunohistochemistry, revealed the presence of enteric neurons in most of the teleost despite having low concentration of 5-HT (Anderson and Campbell 1988). Interestingly, in the same article they proposed the existence of a correlation between intestinal EC cells and enteric neurons: Fish without EC cells in the mucosa had enteric neurons rich in 5-HT while those rich in EC cells might have neurons with low 5-HT concentration. Results showed that TPH2 was also expressed corroborating the presence of enteric neurons in the gut of Ballan wrasse although its expression remained lower than the expression of TPH1 (TPH1 compared to TPH2 had a fold-change varying from 2 after feeding, to 4 after two hours, and 1 fold-change after 3 hours). Gershon (2013) reviewed the substantially smaller 5-HT content of the enteric nervous system in comparison to EC cells which seems consistent with the higher expression of TPH1 (from EC cells) compared to TPH2 (from enteric neurons) in our data. However, small does not mean unimportant. Although results showed low TPH2 expression, TPH2 was observed to increase significantly after feeding suggesting a possible stronger implication of TPH2 in response to feed compared to TPH1.

4.4.5. Has the role of TPH2 been underestimated?

The mechanisms by which serotonin production is stimulated in enteric neurons together with the role of neural 5-HT in the gut still remains unknown. Some light was shed on its role by Li et al. (2011) that knocked out TPH1 and TPH2 in mice. They concluded that serotonin produced by EC cells had a very minor role in the regulation of IG motility, while neural serotonin (5-HT produced by TPH2 in enteric neurons) may have a much more substantial role than previously believed. Consistent with this, Gershon (2013) claimed that the depletion of TPH2 slows the total gastrointestinal transit time, intestinal propulsion and colonic motility while depletion of TPH1 did not interfere with constitutive GI motility. In accordance with this, both the levels of serotonin in tissue and the expression of TPH2 increased at the end of the trial (3 hours after feeding) suggesting that TPH2 might be directly responsible for serotonin synthesis in response to feed in the gut of Ballan wrasse. Also in line with this, analyses of the transcriptomes in Ballan wrasse (From Additional files.2 (Lie et al. 2018)) revealed the presence of Lmx1b (LABE_00014479) which according to (Gross et al. 2016) is a paralog of Lmx1a (LABE_00030204) and it is only expressed in neurons where it regulates TPH2. Lmx1b has been reported in the gut before but only expressed at extremely low concentrations (Gross et al. 2016). Surprisingly, the presence of Lmx1b was found to be higher than Lmx1a in the gut of Ballan wrasse (Lmx1b expression in the first segment showed a fold-change mean of 4.34 ± 2 compared to Lmx1a). The considerably higher expression of Lmx1b that potentially regulates TPH2 would also support the previously suggested important role of TPH2 in 5-HT synthesis. However, significant correlation between serotonin in tissue and TPH2 expression was not found (Figure 6) which could indicate the lack of relation between mRNA expression of TPH2 and its enzyme activity. It is therefore important to remark that gene expression does not determine the action of the enzymes they code for and there can be many other factors modulating the action of TPH1 and TPH2. In spite of the significant increased level of TPH2 over time, results lack evidences to affirm that TPH2 directly synthesize 5-HT in the gut of Ballan wrasse in response to lipid meals.

Generally, there are many reported contradictory evidences with regards to the role and importance of both EC cells and enteric neurons which might not be easily reconciled (Bornstein 2012). Further studies of the role of TPH1 and TPH2 are needed to understand the mechanism of 5-HT synthesis and settle this controversy.

4.4.6. Metabolism of serotonin

Monoamine oxidase (MAO) enzymes has been reported to be the major pathway of 5-HT metabolism in animals (Sjoerdsma et al. 1955). Serotonin released from the EC cells in the submucosa is eventually transported into epithelial cells via SERTs and metabolized by monoamine oxidase A (MAO A) (Figure 3), which is located in all epithelial cells of the gut (Bertrand and Bertrand 2010). Both isoforms of MAO

(MAO A and MAO B) have been reported to metabolize serotonin in different classes of terrestrial vertebrates but not in teleost (Rao et al. 1993), where monoamine oxidase does not fit into the classical MAO A/MAO B binary classification. Following up, Senatori et al. (2002) reported that MAO is exclusively present in one single form in several species of teleost, and this form has usually been classified as MAO A. Opposed to this, MAO B was measured with RT-qPCR and also present in both the genome and transcriptome (Gene ID: LABE_00012540. From Additional files.2 (Lie et al. 2018)) in the gut of Ballan wrasse, supporting the presence of this isoform. Besides, the expression of these two enzymes showed a tendency to decrease after feeding. If the 5-HT system is somehow genetically regulated by feeding, the tendency towards a lower expression of the genes coding for these enzymes after the lipid meal might be in accordance with the up-regulation of TPH2 and the slightly increase of serotonin levels. The mechanisms causing these expression of different genes is regulated by feeding is unknown and further research is needed.

4.4.7. Transport of serotonin.

The serotonin transporter and its gene (SLC6A4 in humans; Slc6a4 in mice, otherwise known in some published papers as 5-HTT or SERT) (Murphy et al. 2008) is one of the major modulators of serotonergic neurotransmission. It is responsible for the reuptake of 5-HT in serotonergic nerve terminals (Figure 3) determining the magnitude and duration of postsynaptic responses to 5-HT (Sookoian et al. 2008). Several articles have reported the polymorphism form of the human SLC6A4 gene as the main cause of intestinal inflammation, IBS and psychiatric syndromes associated with an altered response of the serotonin system (Saito et al. 2010; Margolis and Gershon 2016). Although our results showed that SLC6A4 gene expression did not change significantly post prandial, it tended to decrease 3 hours after feeding suggesting the likely lower uptake of total 5-HT by serotonergic neurons. Tanofsky-Kraff et al. (2013) and (Murphy et al. 2008) reported that individuals with polymorphic SLC6A4 as well as SLC6A4-/- mice showed reduced synaptic levels of serotonin which leads to great disorder of appetite regulation. Further studies in SLC6A4 seem promising to understand the regulation mechanism of 5-HT (Murphy et al. 2008).The reasons causing this apparently diminution of the expression of SLC6A4 in the gut of Ballan wrasse by lipid diet are unknown and complicated as there is not information about the dietary modulation of SLC6A4.

4.4.8. Other known factors modulating serotonin: Microbiota

Recent relevant studies reviewed by O'Mahony et al. (2015) have investigated the role of gut-microbiota in regulating 5-HT levels within the gut. Although microbiota is not a part of this master's thesis, it is important to remark its apparent complex role on the brain-gut axis and behaviour. Yano et al. (2015) showed that microbiota can promote levels of colon and blood 5-HT by producing metabolites capable of stimulating host EC cells to synthesize 5-HT. Whether some bacteria are able

to produce 5-HT de novo or not remains unclear. Microbiota has revealed its important contribution to regulate 5-HT biosynthesis in the gastrointestinal tract which probably affects gastrointestinal motility and homeostasis. Further research on 5-HT-microbiota relationship might elucidate more about its action mechanisms and regulation systems.

5. Conclusions

In response to a "meal" of hydrolysed cod liver oil, genes associated with elongation of fatty acids (ELOVL1), modulation of long chain fatty acid processing (PPARG) and lipid storage in cytosolic lipid droplets (PLIN2) were upregulated. However, APOA-IV, associated with lipid transport from the intestinal cells and well known to be upregulated in response to lipid ingestion *in vivo* was unexpectedly expressed.

The number or experimental replicates conducted in this study was limited (n= 3) and showed a fairly large variation between them. For this reason I suggest that the low n has contribute to the big variances and a higher number of experimental replicates might probably lead to more robust conclusions.

Serotonin in the gut of Ballan wrasse showed a tendency to increase 3 hours after feeding. I also hypothesise that stress induce higher levels of intestinal 5-HT as has been shown to be the case in brain 5-HT in trout. Genes associated to 5-HT metabolism did not respond to the lipid "meal" except for the enteric neuron specific, rate limiting enzyme TPH2 which has been recently suggested to have a much more substantial role in 5-HT production and regulation than previously believed.

However, based on analysis of the genome and transcriptomic data, I propose the presence of enterocromaffin cells (EC cells) in the gut of B. wrasse. EC cells theoretically, produce the majority of intestinal 5-HT synthesized through the enzyme TPH1. Unlike TPH1, its isoform TPH2 (exclusive to enteric neurons, another 5-HT producing cell) was the only gene involved in 5-HT metabolism which was observed to be significantly upregulated by feeding. Although correlation analyses failed to find a close relationship between 5-HT levels in tissue and gene expression, the upregulation of TPH2 by feeding suggest the active role of the latter in nutrient sensing.

Future perspectives

Based on our observations, both 5-HT and lipid mechanism seems to have some sort of response to lipid meals in the gut of B.wrasse. However, this response is not clear and a higher number of experimental replicates (n) might be crucial to elucidate the genetic regulation of 5-HT synthesis after a lipid meal in the gut of Ballan wrasse.

The present results indicate the likely suitability of the novel *in vitro* model used in these experiments for investigating the impact of lipid diet inclusion at the level of gene expression. However, methodological limitations need to be further elucidated.

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Appendix

Table A.1. Ringer's solution composition

	weight (g) in 10 X solution	*Volume stock (L)*	*Volume 10 X solution (L)*
Nacl	15.121	0.2	0.1
*$MgCl_2$ *6H_2O*	0.191	0.2	0.1
Kcl	0.373	0.2	0.1
*$Cacl_2$ *2H_2O*	0.441	0.2	0.1
$NaHCO_3$	0.339	0.2	0.1
NaH_2PO_4	0.116	0.2	0.1
ddH_2O			0.4
Final Volume			1

Table A.2. RT reaction mix: Reagents and concentrations

	Reagents	***Volume (µl)***	***Concentration***
Non enzymatic reagents	ddH_2O	8,9	
	10x RT buffer	5	1X
	10x MgCl2	11	5,5 mM
	10 mM dNTP mix	10	500µM per dNTP
	50µM oligo d(T)16	2,5	2,5 µM
Enzymes	Rnase Inhibitor (20 U/µl)	1	0,4 U/µl
	Multiscribe Reverse Transcriptasa (50U/µl)	1,67	1,67 U/µl

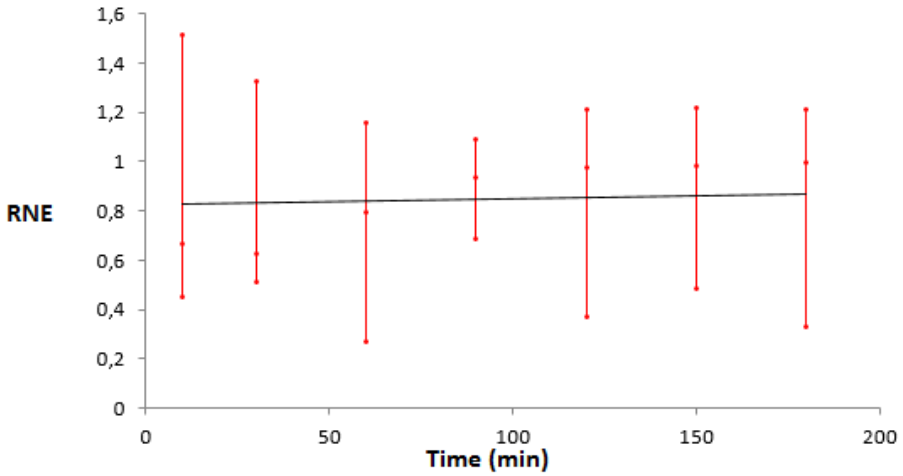
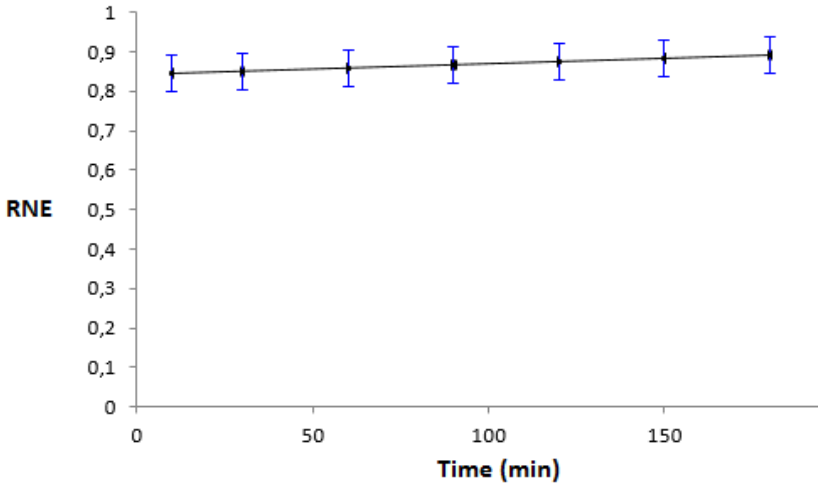
Table A.3. Instrument setup for Reverse Transcription

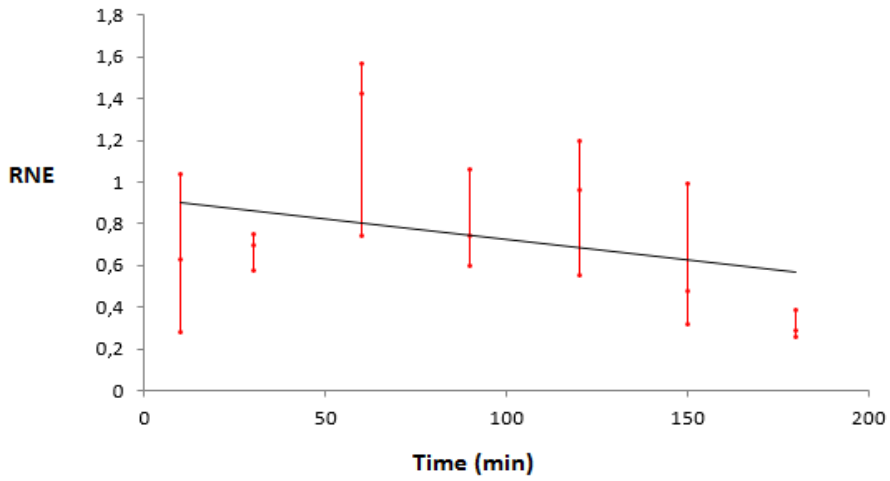
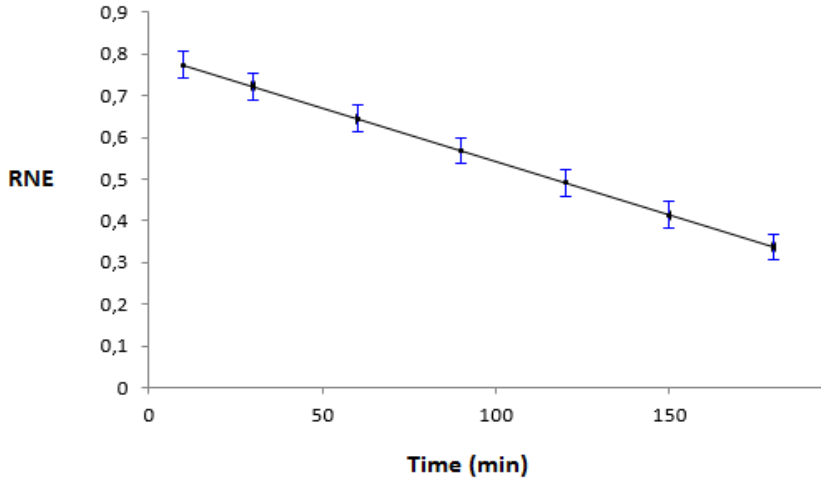
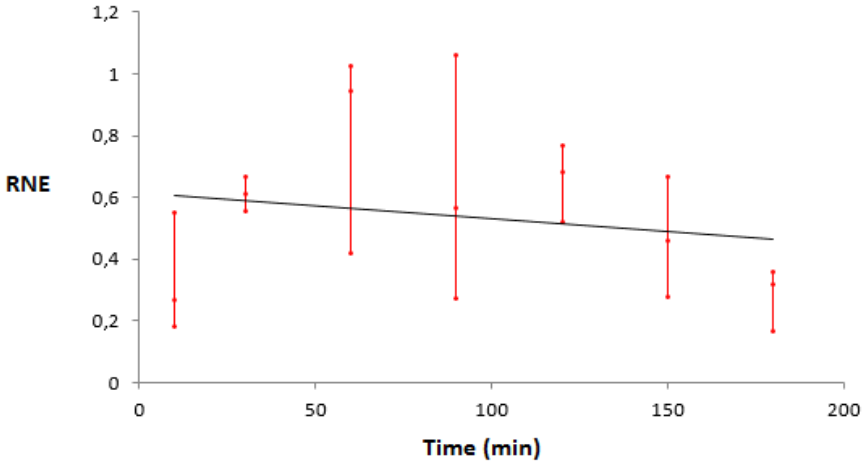
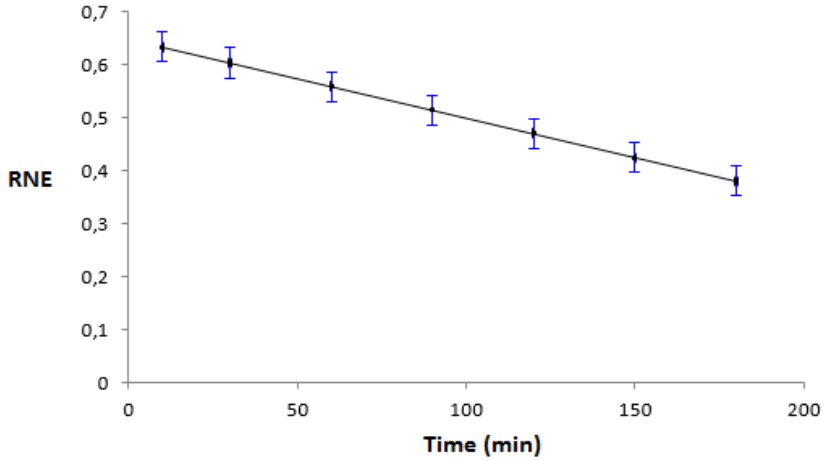
Step	*Temperature (°C)*	*Time (minutes)*
Incubation	25	10
Reverse transcription	48	60
Reverse transcriptase inactivation	95	5
End	4	∞

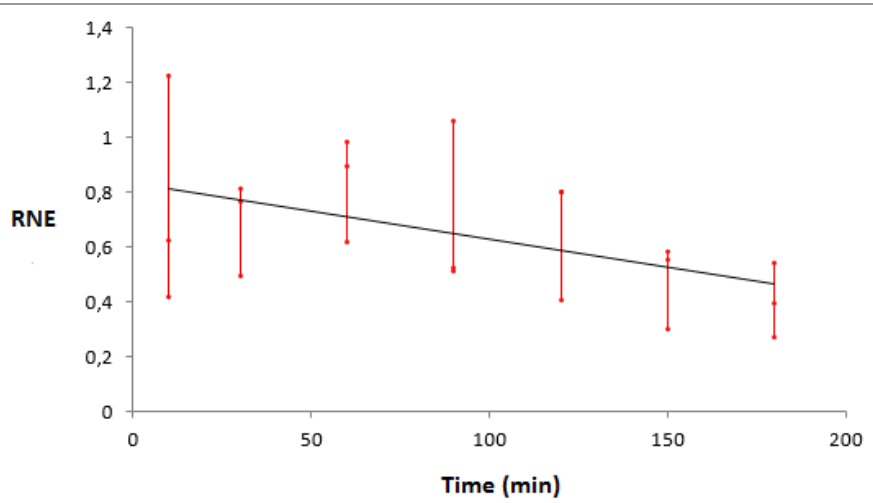
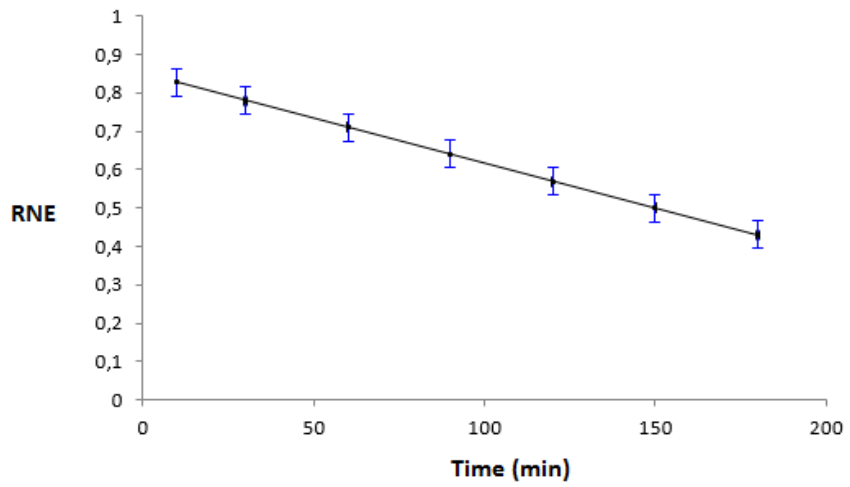
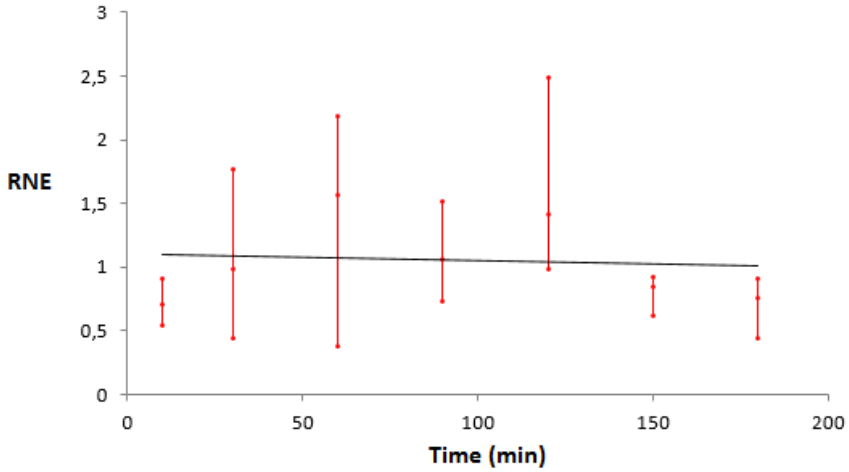
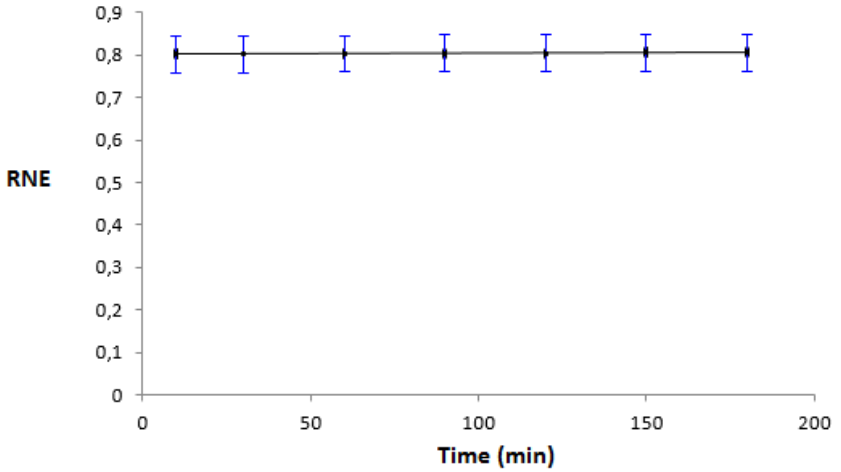
Table A.4. SYBRGreen reaction mix for Light Cycler 480. Expressed volumes correspond to only one well of the plate.

Reagent	*Volume per sample (µl)*
ddH2O	2,8
Primer I (50µM)	0,1
Primer II (50µl)	0,1
SBRY GREEN PCR Master Mix	5

Table A.5: Both linear (unweighted regression) and weighted regression with their corresponding standard deviations (residuals) for all the serotonin related genes are shown. Weighted regressions showed a significant fit at the 95% confidence level for all the genes. TPH 1, TPH 2, and DDC showed a positive slope (weighted m value) which might imply upregulated expression with time. However, TPH1 and DDC exhibited an m value close to 0 suggesting that time hardly had effect on their expression. On the other hand, MAO A, MAO B, and SLC6A4 showed a negative slope (weighted m value) implying downregulated expression with time. This is only a model and it is important to remark that only TPH2 was significantly affected by time (p=6*10-6).

Gen	Unweighted regression	Weighted regression
DDC		

MAOA	 RNE 1,8 1,6 1,4 1,2 1 0,8 0,6 0,4 0,2 0 0 50 100 150 200 Time (min)	 RNE 0,9 0,8 0,7 0,6 0,5 0,4 0,3 0,2 0,1 0 0 50 100 150 Time (min)
MAOB	 RNE 1,2 1 0,8 0,6 0,4 0,2 0 0 50 100 150 200 Time (min)	 RNE 0,7 0,6 0,5 0,4 0,3 0,2 0,1 0 0 50 100 150 200 Time (min)

SLC6A4		
TPH1		

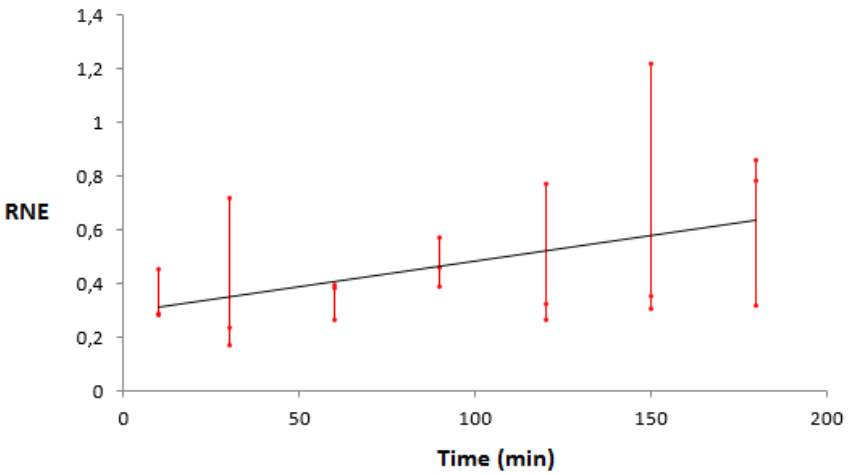
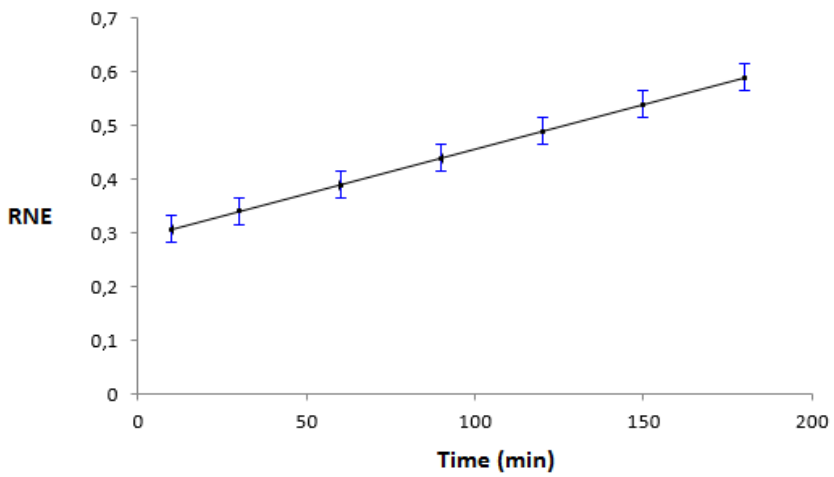
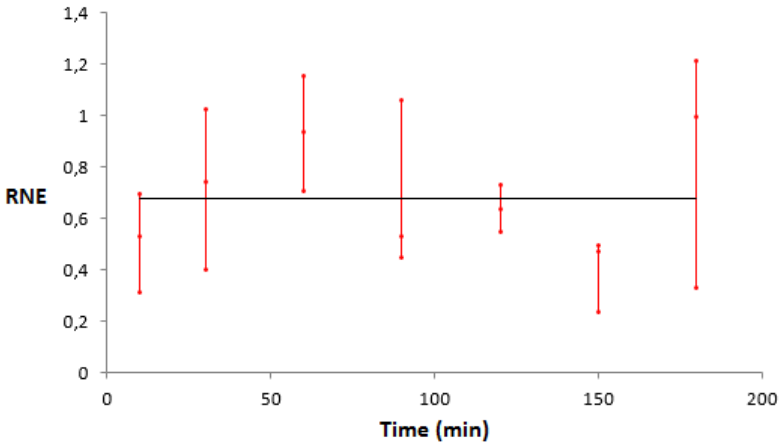
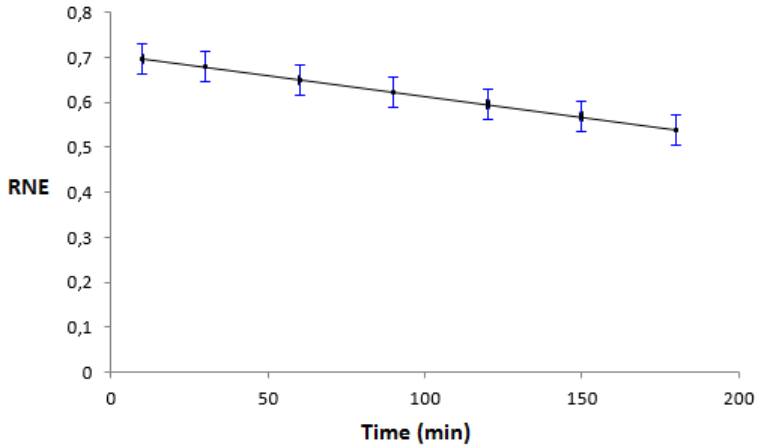
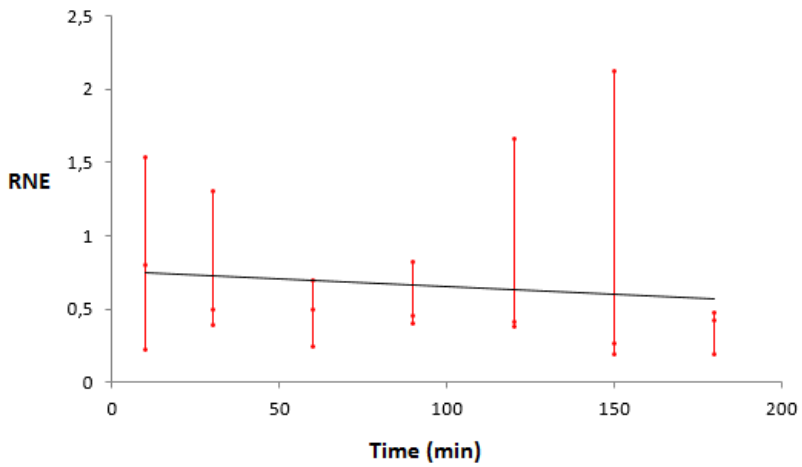
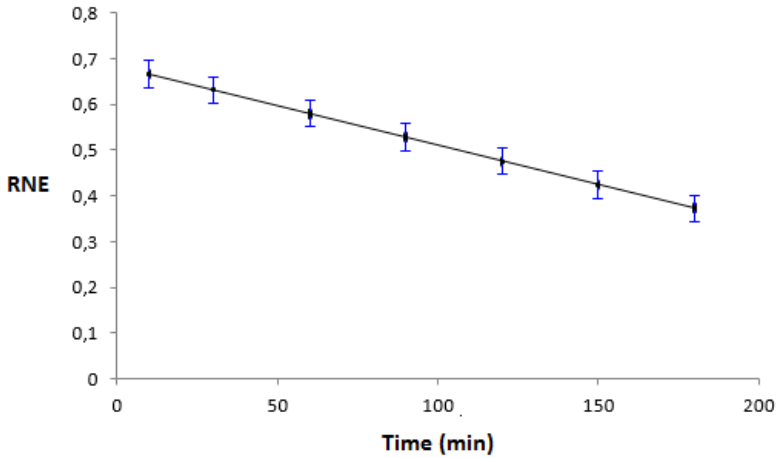
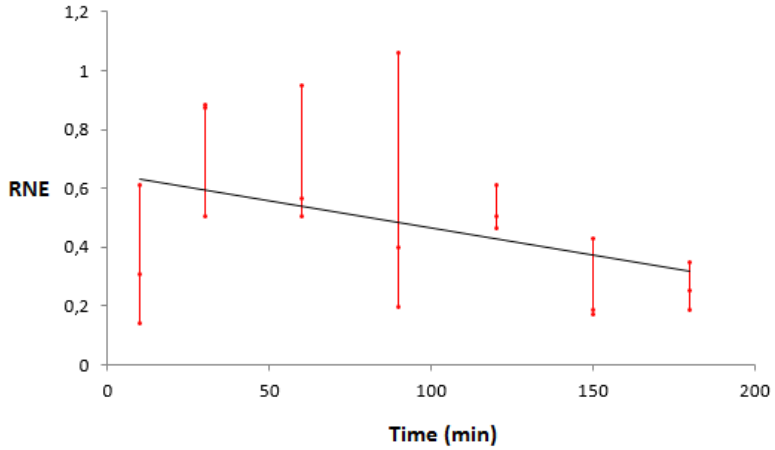
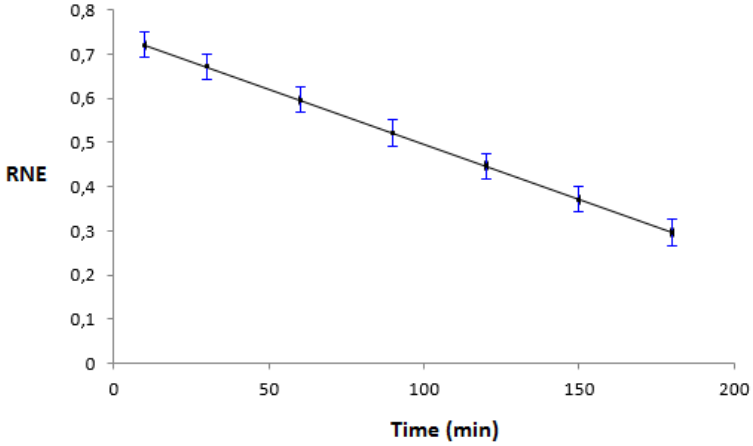
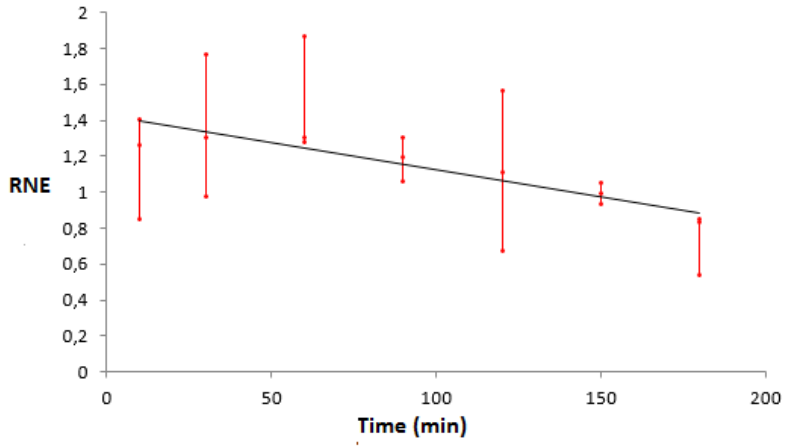
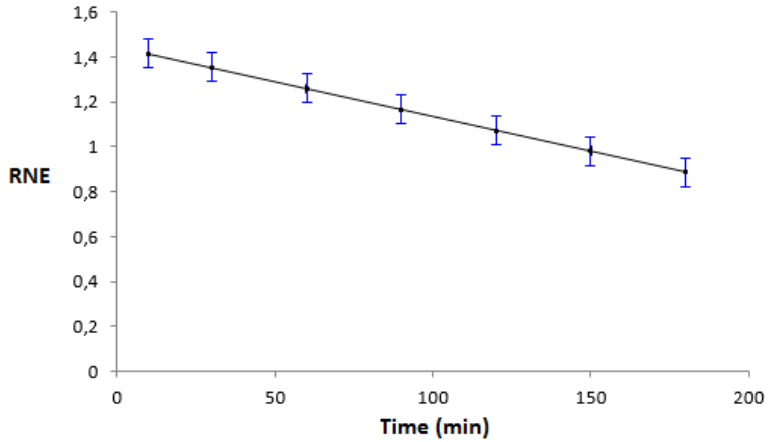
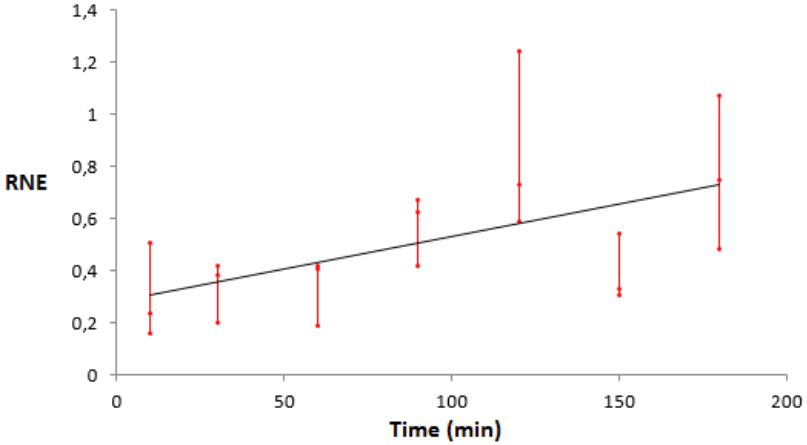
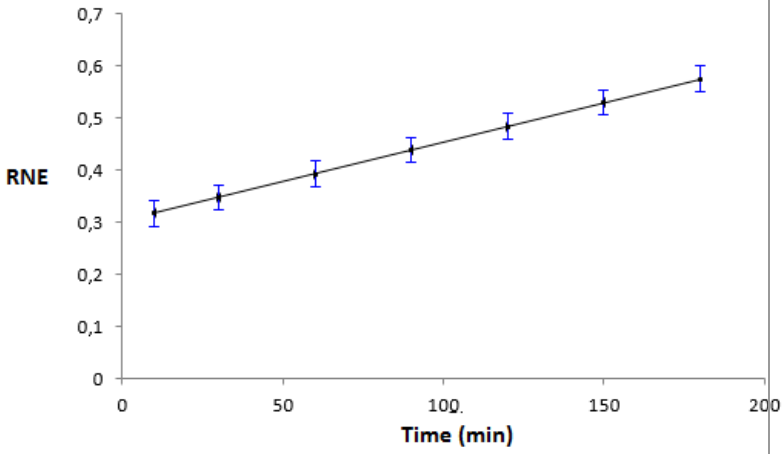
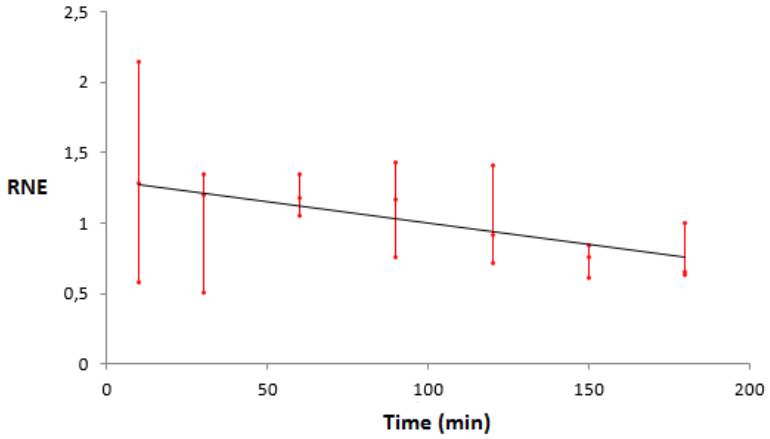
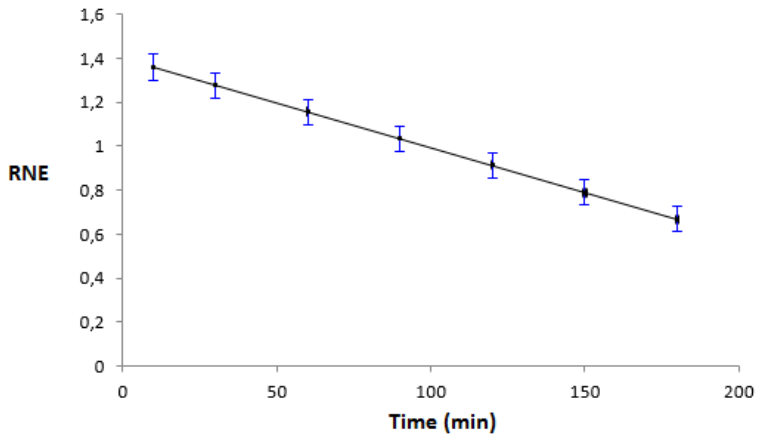
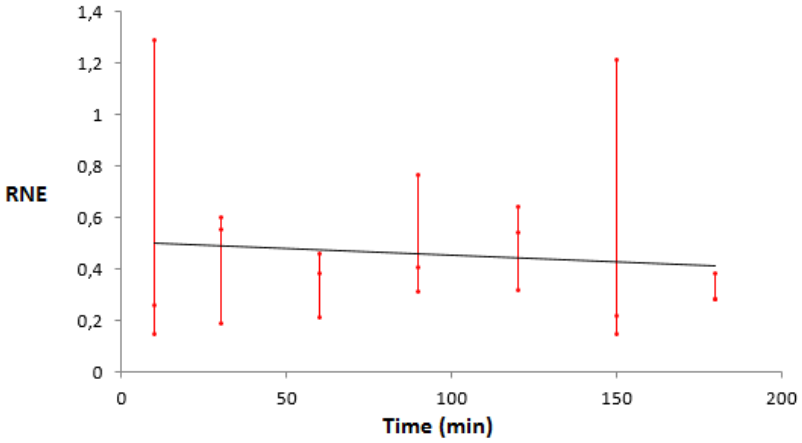
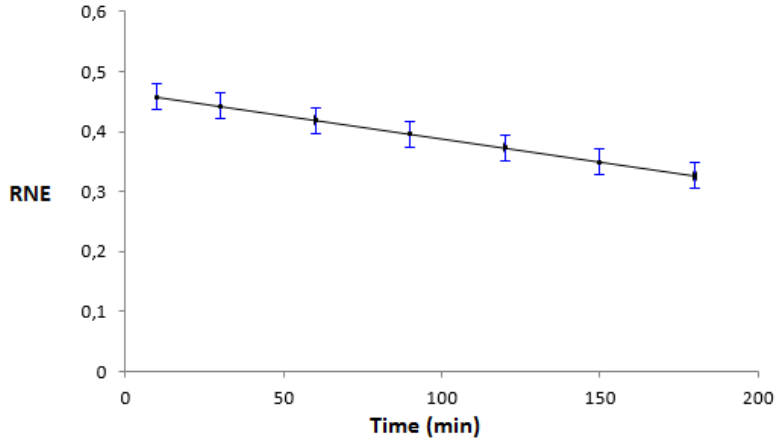
TPH2	 RNE 1,4 1,2 1 0,8 0,6 0,4 0,2 0 0 50 100 150 200 Time (min)  RNE 0,7 0,6 0,5 0,4 0,3 0,2 0,1 0 0 50 100 150 200 Time (min)

Table A.6: Both linear (unweighted regression) and weighted regression with their corresponding standard deviations (residuals) for all the lipid related genes are shown. Weighted regressions showed a significant fit at the 95% confidence level for all the genes. APOA4, SLC27A4, CD36, ELOVL1, PPARG, and PLD1 showed a negative slope (weighted m value) implying downregulated expression with time. PLIN2 and PLPP3 showed a positive slope (weighted m value) which might imply upregulated expression with time. This is only a model and it is important to remark that only ELOVL1, PLIN2 and PPARG were significantly affected by time after feeding (glmmPQL p< 0, 05).

Gen	Unweighted regression	Weighted regression
APOA4		

SLC27A4	 
CD36	 

ELOVL1	 
PLIN2	 

PPARG	 
PLD1	 

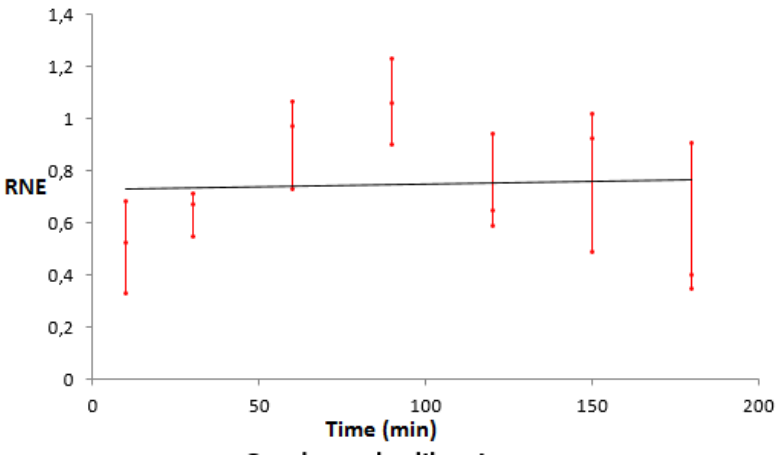
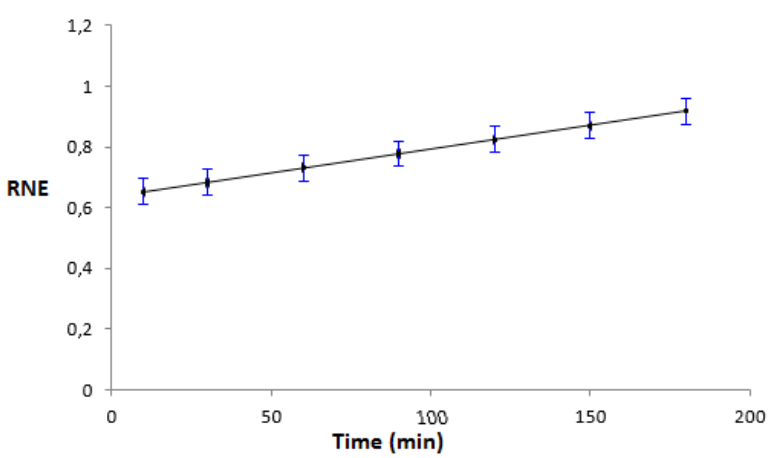
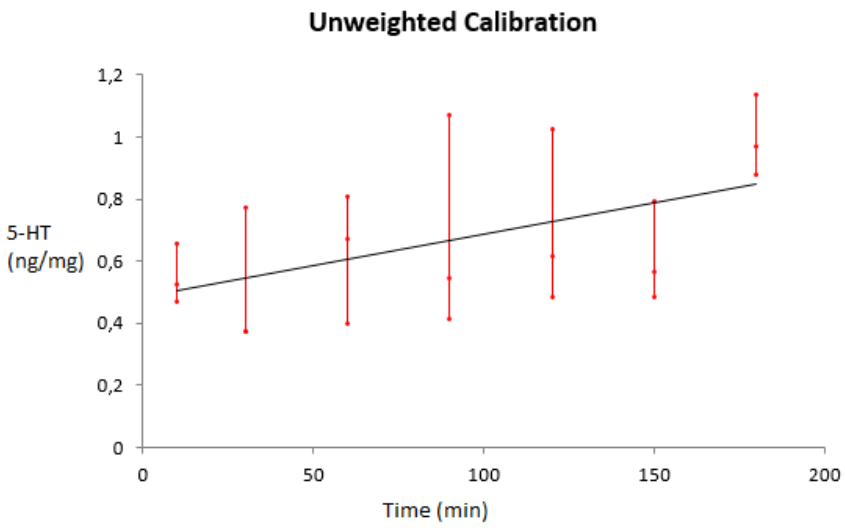
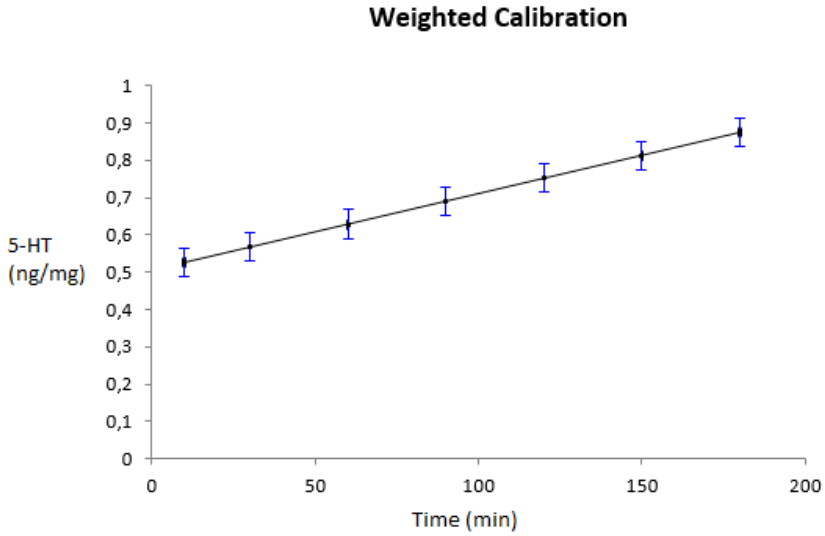
PLPP3	 

Table A.7: Both linear (unweighted calibration) and weighted regression (weighted calibration) with their corresponding standard deviations (residuals) for the concentration of 5-HT in the gut of ballan wrasse at different times. Weighted regressions showed a significant fit at the 95% confidence level. Not significant effect of feeding was found along the time frame (glmmPQL p = 0,299).





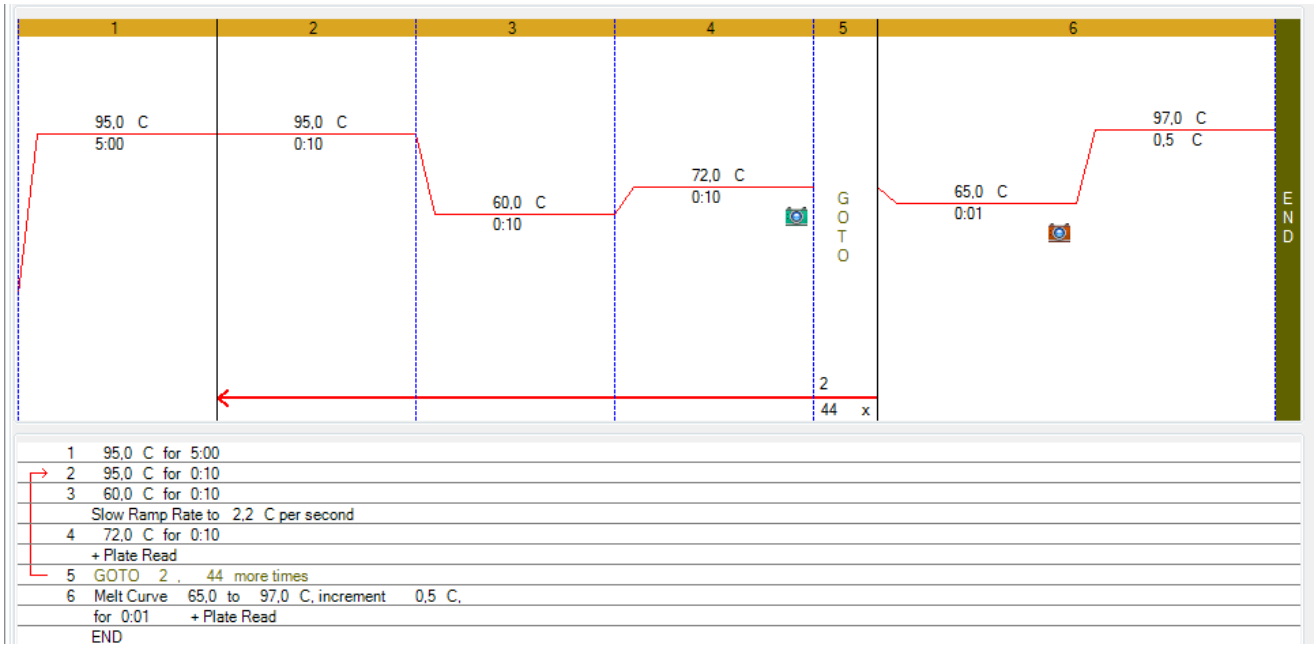


Figure A.1. Standard RT-qPCR protocol for SYBR green 384 plate