Exploring the central and peripheral systems controlling appetite, food intake and digestion in Atlantic halibut (*Hippoglossus hippoglossus)* larvae- Tissue distribution, ontogeny and effects of food attractants

Thesis submitted for partial fulfilment of the degree

Master of Aquaculture

by Endre Lygre

Department of Biological Sciences

University of Bergen, Norway

June 2019

Blank page

TABLE OF CONTENTS

Acknowledgements iv

Abstract v

1 Introduction 1

1.1 Atlantic halibut larvae production 1

1.2 Feeding behaviour 2

1.3 Attractants and their role in feed intake 2

1.4 Appetite control 3

1.4.1 Neuropeptide Y and peptide YY 4

1.4.2 Cholecystokinin and its receptors 6

1.4.3 Current knowledge of appetite control in Atlantic halibut larvae 7

1.4.4 Aims 7

2 Materials and methods 9

2.1 Rearing of larvae 9

2.1.1 Stripping and Incubation 9

2.1.2 First feeding 10

2.2 Feed extract preparation and analysis 11

2.3 Experimental setup and sampling 11

2.4 Staging and feed intake analysis 13

2.5 Dissection 14

2.6 Gene expression analysis 15

2.6.1 RNA isolation 15

2.6.2 DNase treatment 17

2.6.3 RNA quantification and integrity analysis 18

2.6.4 cDNA synthesis 19

2.6.5 Quantitative Polymerase Chain Reaction (qPCR) 20

2.7 Statistics 21
3 Results 23
3.1 Feed intake quantification 23
3.2 Extracts composition analysis 23
3.3 mRNA expression analysis 24
3.3.1 Tissue distribution 24
3.3.2 mRNA expression during ontogeny 25
3.3.3 Response to feeding 27
3.4 Methodological considerations 32
4 Discussion 33
4.1 Effects of attractant extracts on feed intake 33
4.2 Tissue expression profile 34
4.2.1 cck and cckr 34
4.2.2 pyy and npy 35
4.3 Expression during ontogeny 36
4.3.1 cck and cckr 36
4.3.2 npy and pyy 37
4.4 Response to feeding 38
4.4.1 cck and cckr 38
4.4.2 *npy* and *pyy* 39
4.5 Methodical considerations 41
4.5.1 Sampling 43
4.5.2 Dissection 44
4.6 Summary of hypothesis 45
5 Conclusions and future perspectives 46
References 48
Appendix 1 – Ninhydrin detection 54

Ninhydrin detection results 54
Ninhydrin detection protocol 56
Appendix 2 – Methods testing 62
Appendix 3 – Rin values 65
Appendix 4 – Atlantic Halibut Larva dissection 66
Appendix 5 – Statistical tests 67
Shapiro-Wilk normality test 67
Cyst intake 68
mRNA expression during ontogeny 68
Response to feeding 69

ACKNOWLEDGEMENTS

This master's thesis would not have been completed without crucial help from several people, of which I would like to extend my gratitude:

First of all, I would like to thank my supervisors at University of Bergen; Professor Ivar Rønnestad and Dr. Ana Gomes, and at Institute of Marine Research; Senior scientist Torstein Harboe and Dr. Kristin Hamre for giving me the opportunity to work on this project, supporting me and sharing their knowledge throughout this period.

Secondly, I would like to thank the staff at the Marine Development Biology group at Department of Biological Sciences (BIO) for creating a pleasant social atmosphere in and around the laboratories: especially Dr. Ann-Elise O. Jordal for mentoring in the lab and methodological discussion. Also, the staff at Austevoll research station: especially Margareth Møgster and Ragnfrid Mangor-Jensen for providing halibut larvae of excellent quality and Dr. Perrichon Prescilla for aiding me in sampling of larvae. Finally, I would like to thank Dr. Rolf Edvardsen at IMR for providing access to the Atlantic halibut genome database.

Lastly, but not least, I would like to thank my fellow students, our student union "Linjeforeningen for Fiskehelse og Havbruk", family and, for an excellent and joyful study period in Bergen.

Endre Lygre

ABSTRACT

Atlantic halibut (*Hippoglossus hippoglossus*) is a cold-water species belonging to the Pleuronectidae family and is native to Norwegian waters. Halibut is a high valued food species that have been overfished and wild-stock collapses, creating, therefore, a large potential species for the aquaculture industry. However, problems in the production cycle still exists as farmers have to rely on the use of live feed for ~40 days, which is expensive, labour intensive, and poses a risk of introducing bacterial contamination to the halibut larvae. In this context, he main aim of this thesis was to test if feed intake of inert particles (mimicking dry feed) can be stimulated in halibut larvae by adding attractant extracts in the rearing tanks. We used three different sources of attractants: water soluble extracts of Otohime, Gemma Micro and Northern prawn. Attractant extracts were given to halibut larvae together with food particles (*Artemia* nauplii cysts). Feed intake results, determined based on gut fullness visualization, showed no significant differences between the different attractant extracts and control groups of Atlantic halibut larvae at 10 and 20 days past first feeding (dpff). Subsequently, we decided to analyse the mRNA expression of genes known to be involved in control of feed intake, appetite and digestion in teleost species. We investigated the spatial (brain and gut) and temporal (10 and 20 dpff) expression profile of *cholecystokinin* paralogues (*cck1* and *cck2)* and its receptors (*cck1r, cck2r1* and *cck2r2*), *neuropeptide y* and *peptide yy* paralogues (*pyya* and *pyyb*). In addition, we also analysed how their expression profile was affect by feeding. Our results suggest that *cck1* is involved in the regulation of digestion and acts as peripheral anorexigenic and central orexigenic factor, while *cck2* seems to behave as a feed-forward signal, regulating digestive processes before feed ingestion. *Npy* mRNA expression indicated a central orexigenic role. The expression profile of *pyyb* indicate that this hormone is involved in the regulation of digestion and act as a peripheral anorexigenic factor. We hope that the results of this master's thesis will contribute to a greater understanding of appetite, feed intake and digestion control of fish larva. When the key attractants for Atlantic halibut larvae have been identified, this will form a basis to formulate diets that optimize early weaning or enable formulated feeds from onset of exogenous feeding.

1 INTRODUCTION

1.1 ATLANTIC HALIBUT LARVAE PRODUCTION

Atlantic halibut (*Hippoglossus hippoglossus*) is a cold-water species, native to Norwegian waters, that is valued for its high-quality fillet, and in low supply from capture fisheries, creating large potential as a species for the aquaculture industry. The first cultivation attempts of Atlantic halibut started in Norway in the 1980s, however, its industry has yet to experience the same growth as the salmonid aquaculture industry. Halibut larvae production is one of the major bottlenecks, hindering growth in the industry, as a constant and reliable larvae production is necessary to ensure a constant, or increased, production volume of juveniles and adults. The current feeding protocol for halibut larvae is to supply the larvae with enriched *Artemia* (*A.*) nauplii for 40-45 days, and then wean them off to dry feeds. However, the use of live feed can be problematic as it is expensive, labour intensive, and poses a risk of introducing disease due to bacterial contamination (Nicolas et al., 1989). In addition, the fatty acid enriched gut content of *A*, nauplii may be evacuated before they are ingested by the halibut larvae, making the feed less nutritious for the larvae (Evjemo et al., 1997).

Atlantic halibut larvae are relatively large at onset of exogenous feeding (12 mm in body length, (Pittman et al., 1987)), compared with other marine larvae. Therefore they can be fed *A.* nauplii (Harboe and Mangor-Jensen, 1998), skipping the rotifer feeding stage which is the common start-feed for other marine teleost species larvae. Unfortunately, no formulated diet has shown satisfactory results for larval survival of halibut during the first days of exogenous feeding (Hamre et al., 2019). Attempts to feed the larvae with formulated diets have resulted in high mortality rates, simply because the larvae will not ingest the pellets (T. Harboe 2018, pers. comm.). The *A.* nauplii are enriched with micro and macro nutrients to better match the dietary requirements of marine fish larvae (Watanabe, 1993). This approach has shown to reduce abnormalities, like malpigmentation and incomplete eye migration, in flatfish species (Dickey-Collas, 1993; Næss et al., 1995). Recently, such abnormalities have become less common, as many small improvements of rearing practices since the 90s have reduced the nutrient requirement and/or increased their utilization in Atlantic halibut larvae (Hamre et al., Submitted). Despite recent improvements, the use of *A.* nauplii is still costly, increases the bacterial load in the rearing unit (Grotkjær et al., 2016) and may cause slow growth during the larval stages (Hamre et al., 2019). Thus, rearing practices of halibut larvae would greatly benefit

from a formulated diet which can be utilized earlier than current practice (40-45 days past first feeding (dpff)) or at the start of exogenous feeding.

1.2 FEEDING BEHAVIOUR

Feeding behaviour is initiated by detection of food, and olfaction plays a key role in this process for several fish species (Pavlov and Kasumyan, 1990). Olfaction has the longest detection range of all the senses in many teleost species. Fish larvae will often detect the presence of food by sensing small water-soluble molecules (also called attractants), which leaks from the food to the water. Attractants disperse by diffusion, but more importantly these molecules follow the water current that can be sensed by the lateral line/free neuromasts. Fish can combine this information with the olfactory response to locate the attractant/stimuli source (Valentinčič, 2005). Vision is also often involved in localizing the food particles, while gustation and touch are essential in the close localisation and ingestion and swallowing food.

1.3 ATTRACTANTS AND THEIR ROLE IN FEED INTAKE

Attractants is a term that describes water-soluble low-molecular weight compounds, such as free amino acids (FAA), that stimulate feeding behaviour and allow fish to recognise feed particles as food items. The importance of chemical stimuli (attractants) was demonstrated for marine fish larvae using 20-day old seabream (*Sparus auratus*) larvae (Kolkovski et al., 1997). The seabream larvae increased their feed intake about 35 % in the presence of attractants added to the water, such as arginine, alanine, glycine and betaine. Feeding behaviour evoked by olfactory stimuli, using attractant solutions of various compounds, have been reported for several fish species, including; goldfish (*Carassius auratus*) (Grimm, 1960), Atlantic cod (*Gadus morhua*) (Døving and Selset, 1980), and Atlantic halibut (Yacoob and Browman, 2007a). Attractants are species-specific, and the stimulatory effect may vary with mixture (Harada Katsuhiko, 1986), type and concentration of attractants (For review see Hamre et al., 2013). Despite the benefits of utilising attractants to stimulate feed ingestion, the optimal mixture of key attractants has only been identified for a few species (Hamre et al., 2013). The swimming activity of Atlantic halibut larvae increases in the presence of certain attractant substances. This has been observed during weaning trails, where the larval activity increased in the presence of a commercial feed (Otohime) when compared to other formulated diets (T. Harboe, 2018 pers. comm.). However, the increase of activity in the tank was not correlated with a higher feed intake. In juvenile Atlantic halibut, nerve recording showed that the olfactory system is stimulated in the presence of amino acids, with the highest response to methionine

and glutamine, and the lowest response to proline and aspartic acid (Yacoob and Browman, 2007b). Feeding behaviour has also been invoked in halibut juveniles with attractant extracts from shrimp and squid (Yacoob and Browman, 2007a). Despite these findings, a synthetic mix of attractants, designed to mimic that of crustaceans failed to induce feeding behaviour in halibut juveniles (Yacoob and Browman, 2007a). This emphasises the complexity and importance to identify which key attractants and the mixing ratio that stimulate feeding behaviour in Atlantic halibut larvae.

In this study three different extracts were tested to compare the effect of different attractant compositions on halibut larvae feeding. The extracts consisted of water-soluble compounds from two formulated diets (Gemma Micro (Skretting, Stavanger, Norway) and Otohime (Reed Mariculture, California, US)) and Northern prawn (*Pandalus borealis*; shrimp). Otohime and Gemma Micro are two commercially available feeds for marine fish larvae. Northern prawn was included because crustaceans are a well-known prey of halibut (Bowman et al., 2000). The Northern prawn was the only natural prey commercially available during the time of the year we did the experiments (April, May) we conducted the experiments. Gemma Micro and Otohime have previously been tested in weaning trials of Atlantic halibut larvae (Hamre et al., 2019). The halibut larvae fed with Otohime achieved the highest feeding rate, and successful weaning 28 dpff (Hamre et al., 2019).

1.4 APPETITE CONTROL

In vertebrates, food intake is regulated by a complex system involving central (brain) and peripheral signals (e.g. signals from gastrointestinal tract (GI-tract), liver and muscle), which includes orexigenic factors, i.e., hormones that boost appetite, and anorexigenic factors, i.e., hormones that inhibit appetite (for review see: Rønnestad et al., 2017). The hypothalamus is regarded as the appetite controlling centre in the brain (Figure 1). The peripheral signals include GI-tract hormones which primary act to stimulate/supress (control) digestion, but several of these also have secondary roles in affecting appetite control in the brain by circulating the bloodstream or via neuronal afferent pathways. In the brain, these peripheral signals are integrated together with external sensory signals, e.g. olfaction, by the hypothalamus, which express/supress central neuropeptides that control food intake and also affect the digestive processes.

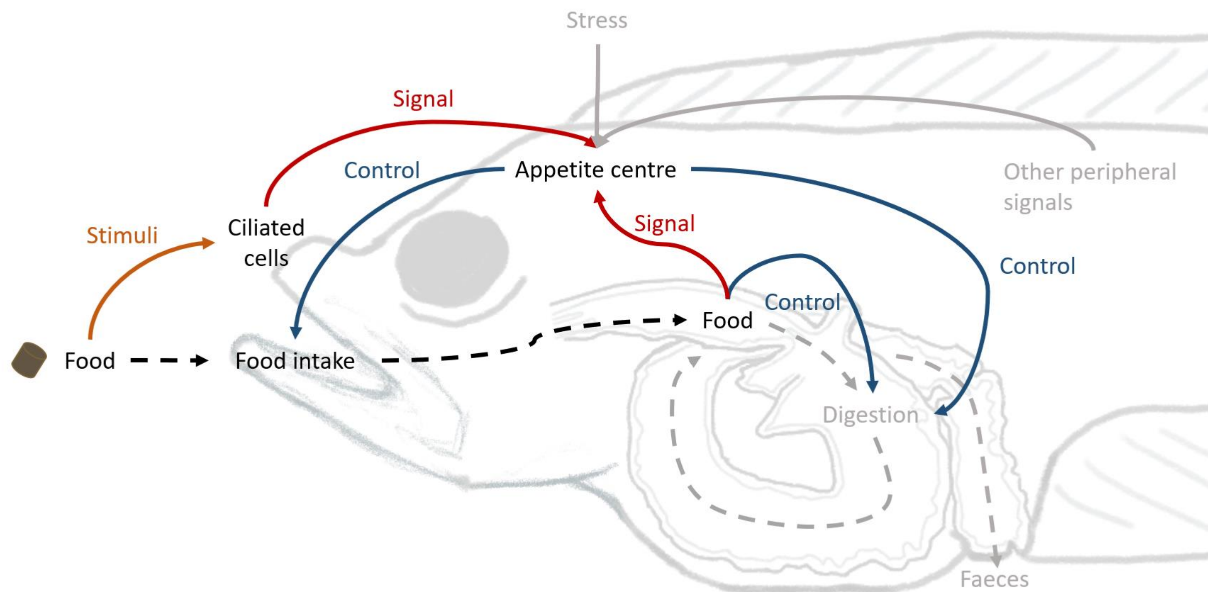


Figure 1. Simplified overview of signalling pathways involved in appetite control, food intake and digestion. Dashed lines (- - -) illustrate the path of food, while solid lines (—) illustrate signalling pathways. Peripheral signals (hormones circulating bloodstream and neuronal signals), are integrated with external stimuli (e.g. attractants trough the olfactory system) in the appetite centre (hypothalamus) which controls food intake (feeding behaviour) and digestive processes. The presence of food in the gut stimulate and control digestion and also provide signals to the appetite centre. Modified from (Rønnestad et al., 2017).

Even though the system controlling appetite is considered relatively well conserved among vertebrates, several studies have shown that in fish the hormones and neuropeptides that control appetite in mammals may differ in their functions in fish (e.g. NPY, discussed in the section below). In addition, it has been observed that within teleost fish the genes involved in appetite control responds differently between species to feeding/food deprivation, suggesting that appetite control might be species-specific (Volkoff, 2015). In addition, the transition between different life stages within the same species may also reflect changes in feed resources and feeding behaviours, and, thus, the appetite control system may change through development.

This thesis focuses on two key gastrointestinal anorexigenic hormones, cholecystokinin (CCK) and peptide YY (PYY), and one of the most abundant orexigenic hormones in the brain, the neuropeptide Y (NPY).

1.4.1 **Neuropeptide Y and peptide YY**

Neuropeptide Y (NPY) is known as a centrally expressed, and strongly orexigenic hormone in mammals (Schwartz et al., 2000), while peptide YY (PYY) is a peripheral expressed hormone which promotes digestive processes (e.g. motility of the intestine (Lundberg et al., 1982)) and acts as a peripheral expressed anorexigenic hormone (Batterham et al., 2002). In teleost fish, because of the teleost-specific whole genome duplication event (Ts3R) (Meyer and Van de

Peer, 2005), the NPY family generally consists of *npya, npyb, pyya* and *pyyb* (Sundström et al., 2008). However, exceptions may exist, e.g. *npyb* seems to have been lost in zebrafish (*Danio rerio*) (Sundström et al., 2013). In Atlantic halibut, two *npy* genes have been previously identified by Gomes et al., (2015). However, the authors have misidentified *npyb* as *pyy* due to the small size fragment that was amplified (A. S. Gomes 2019, unpublished data). Here, we only analysed the mRNA expression of *npyb*, which from now on it is referred to as *npy*.

1.4.1.1 Neuropeptide Y

In juvenile and adult teleost fish, *npy* seems to mainly act as an orexigenic factor (see review by Rønnestad et al., 2017). There are, however, few studies that have targeted the larval stages and analysed expression in individual organs/tissues. The role of *npy* as an orexigenic factor have been confirmed in zebrafish larvae at 25 days post fertilization (dpf), where fasting increased total (whole body) *npy* expression (Opazo et al., 2019). Contradictory results have been found in Senegalese sole larvae (*Solea senegalensis*) where feeding increased total *npy* expression at 16 days post hatching (dph) (Bonacic et al., 2016). In Atlantic halibut larvae at 49 days post first feeding (dpff), fasting decreased mRNA expression of *npya* ("*npy*" in the article) in the brain after 5 hour, while brain *npyb* ("*pyy*" in the article) increased after 4 hours in fed larvae (Gomes et al., 2015).

1.4.1.2 Peptide YY

Feeding and food deprivation studies in teleost fish have yielded different results in terms of *pyy* expression levels, indicating a species-specific role of PYY in appetite control (for review see: Rønnestad et al., 2017). However, few studies have analysed and compared *pyya* and *pyyb*, in teleost fish (Yan et al., 2017), and limited information is available on the role of *pyy* in the larval stages (Rønnestad et al., 2013). In adult grass carp (*Ctenopharyngoodon idellus*) (Chen et al., 2013) and Nile tilapia (*Oreochromis niloticus*) (Yan et al., 2017). *pyya* was mainly expressed in the central nervous system (CNS) whereas *pyyb* was mainly found in the gastrointestinal (GI-) tract (Chen et al., 2014). In both species, *pyya* and *pyyb* acted anorexigenic: Expression levels increased in grass carp for brain *pyya* and gut *pyyb* after feeding (Chen et al., 2013, 2014), and in tilapia Nile tilapia brain *pyyb* and *pyya* increased in fed fish at 1 and 6 hours respectively, while foregut *pyyb* increased 1 h after feeding (Yan et al., 2017). In addition. 7-days food deprivation decreased the mRNA expression of *pyya* in the brain and *pyyb* in the foregut in Nile tilapia. Anorexigenic effect of *pyya* have also been found in 34 dph Senegalese sole larvae, where *pyya* mRNA expression in the head increased 0.5 hour after feeding, however no significant changes were found for *pyyb* (Bonacic et al., 2016).

1.4.2 Cholecystokinin and its receptors

Cholecystokinin (CCK) is a well-known gastrointestinal hormone which promotes digestive processes (e.g. inhibition of gastric emptying, stimulation of emptying of the exocrine pancreas and gallbladder (Raybould, 2007)) and acts as an peripheral expressed anorexigenic factor in mammals (Gibbs et al., 1973). In teleost fish, *cck1* and *cck2* have been identified in several species such as spotted river puffer (*Tetraodon nigroviridis*), Japanese flounder (*Paralichtys olivaceus*) (Kurokawa et al., 2003), white sea bream (Micale et al., 2012) and Atlantic salmon (*Salmo salar*) (Murashita et al., 2009b). However, few studies compared the role of *cck1* and *cck2* in feed intake control (Micale et al., 2012; Murashita et al., 2009b).

In Japanese flounder, cck1 and cck2 were both highly expressed in the brain, but cck1 expression in the intestine was higher than cck2 (Kurokawa et al., 2003). In Atlantic salmon, cck1 and cck2 ("CCK-N" and "CCK-L" in the article) were mainly expressed in the brain (Murashita et al., 2009b). Expression of both genes were also found in the pyloric caeca, but cck2 was the only gene expressed in the midgut. Six days of feed deprivation led to lower cck1 and -2 mRNA levels in the Atlantic salmon brain (Murashita et al., 2009b) and cck2 expression increased immediately after feeding while cck1 decreased 24 h after feeding (Valen et al., 2011). In white seabream, intestinal cck1 expression increased, and cck2 expression decreased in starved fish (Micale et al., 2012). This suggests that in white seabream cck paralogues may have different functions, while in salmon the available results suggest anorexigenic function for both cck1 and cck2. Kamisaka et al. (2001) studied the ontogeny of CCK-immuno reactive (IR) cells in the gut of halibut larvae and demonstrated that single cells first appear at around 45 dph and increases in number as development continues.

Three *cck* receptors (*cck1r, cck2r1* and *cck2r2*) have been identified in Atlantic salmon (Rathore et al., 2013). *cck1r* and *cck2r1* was mainly expressed in the digestive system, and *cck2r2* was mainly expressed in the brain. Two *cck* receptors have been found in goldfish (*cckar*, and *cckbr* in the article) (Tinoco et al., 2015) where *cckar* was mainly expressed across the whole GI-tract, while *cckbr* was mainly expressed in the brain and posterior intestine. In yellowtail (*Seriola quinqueradiata*), *cck1r* was mainly expressed in the digestive system (Furutani et al., 2013) and increases in the digestive system after feeding, indicating an anorexigenic role for *cck1r* (Furutani et al., 2013). In the light of these observations, *cck1r* seems to be primarily located in the digestive system, while *cck2r* is primarily located in the CNS across teleost species.

1.4.3 **Current knowledge of appetite control in Atlantic halibut larvae**

Atlantic halibut larvae are capable exogenous feeding at 260-290 °d after hatching (Harboe and Mangor-Jensen, 1998), but an adult type functional digestive system is still not fully developed (Ana S. Gomes et al., 2014b; Pittman et al., 1990). The pyloric sphincter is functional at first feeding and the presumptive stomachs main function is suggested to store ingested food (Ana S. Gomes et al., 2014b). Prophylactic function and acid production in the stomach occurs later at metamorphic climax. The first feeding halibut larvae also seems to have a poor or non-developed appetite controlling system (Gomes et al., 2015), which is supported by observations of undigested or only partly digested *A.* nauplii in the faeces (T. Harboe 2018, pers. comm., Own observations). This is particularly evident when food is given in excess, limitation in digestive capacity and continued feeding results in nutrient losses and consequently suboptimal growth (Boehlert and Yoklavich, 1984; Werner and Blaxter, 1980). The observed continuous feeding suggests that a lack of satiation signals from the gut to the brain may exist in these early developmental stages (Ana S. Gomes et al., 2014b; Gomes et al., 2015; Kamisaka et al., 2001).

1.4.4 **Aims**

The main aim of this thesis was to test if feed intake can be stimulated in halibut larvae by adding attractant extracts in the water. We used three different sources of attractants: water soluble extracts of Otohime, Gemma Micro and Northern prawn. To quantify feed intake, we used *Artemia* cysts. This represent an inert food particle that mimics formulated pellets and are known to be ingested by Atlantic halibut larvae (T. Harboe, 2018 pers. comm.). An additional aim was to identify key attractants for Atlantic halibut larvae by analysing cyst uptake in different experimental groups given different attractant extracts. Finally, the aim was to explore how the different experimental conditions affected expression of some of the key neuropeptides and hormones involved in appetite control. When the key attractants for Atlantic halibut larvae have been identified, this will form a basis to formulate diets that optimize early weaning or enable formulated feeds from onset of exogenous feeding. Therefore, the objectives of this study were:

Objective 1 – Examine the content of each extract to identify potential attractants:
$H0_1$: There is no difference in the FAA content of attractant extracts from Otohime, Gemma Micro or Northern prawn.

Objective 2 – Determine which attractant extract stimulates halibut larvae to ingest *Artemia cyst* at the highest rate based on gut fullness analysis:
$H0_2$: Different attractant extracts do not affect feed intake in terms of gut fullness.

Objective 3 – Investigate differences in expression of appetite controlling factors, particularly *cck1, cck2,* and their receptors (*cck1r, cck2r1* and *cck2r2*), and *npy, pyya* and *pyyb* in halibut larvae when exposed to different attractants:
$H0_3$: Different attractant extracts do not affect gene expression of *cck1, cck2, cck1r, cck2r1, cck2r2*, *npy pyya* or *pyyb.*

Objective 4 – Tissue expression profile:
$H0_4$: Expression of *cck1, cck2, cck1r, cck2r1, cck2r2*, *npy, pyya* or *pyyb* do not vary between brain or gut tissue.

Objective 5 – Expression during ontogeny:
$H0_5$: Expression of *cck1, cck2, cck1r, cck2r1, cck2r2*, *npy, pyya* or *pyyb* do not vary between 10 and 20 dpff larvae.

Because $H0_2$ was confirmed, $H0_1$ was not examined in detail, and $H0_3$ replaced with the following objective and hypothesis:

Objective 6 – Analyse the effects of food intake on the mRNA expression levels of the selected appetite controlling genes:
$H0_6$: There is no difference in expression of *cck1, cck2, cck1r, cck2r1, cck2r2*, *npy, pyya* or *pyyb* in either feeding or not-feeding larvae.

2 MATERIALS AND METHODS

Attractant extracts from Otohime, Gemma and shrimp were prepared and analysed for water-soluble compounds (possible attractants). These results were used to determine the attractant dose administered into the experimental tanks and also identify possible key attractants for Atlantic halibut larvae. During the experiment, attractant extracts and *A*. nauplii cysts were given to 10 and 20 dpff Atlantic halibut larvae reared at the institute of Marine Research's (IMR) facilities at Austevoll (Norway). Larvae samples were collected for analysis of gut content and gene expression. Relative mRNA expression of candidate genes involving appetite control were analysed using quantitative polymerase chain reaction (qPCR) using gene specific primers.

2.1 REARING OF LARVAE

The Atlantic halibut larvae were reared at IMR Austevoll, where the parental broodstock is located. Three larval group were produced during the spring of 2018. However, due to high mortality and deformities observed in the third group, only the two first groups were used in the experimental setup.

2.1.1 Stripping and Incubation

Eggs and sperm were stripped from the broodfish and fertilized by mixing eggs from one female with sperm of 1-2 males to supply one batch of experimental larvae in 1 mL sperm/ L eggs. This approach ensured that genetic differences would not affect the results between experimental groups in each batch. Sperm is activated by mixing with filtered seawater (3 L/L eggs). Fertilization was performed in a sterilized container and completed in 10 minutes. Fertilized eggs were then transferred to incubation tanks (250L) (Mangor-Jensen et al., 1998) for ~43 days at 6 °C (260 °d) (Harboe and Mangor-Jensen, 1998). In the incubation tanks the eggs were kept in complete darkness (Mangor-Jensen and Waiwood, 1995), with high saline water (32-34 ‰ salinity) on the bottom which created a saline gradient where the eggs rest at neutral buoyancy. Eggs were kept in darkness for 15 days then exposed to light which induced synchronized hatching (Helvik and Walther, 1993). Dead or empty eggs and dead larvae sank to the bottom and were removed from the system when needed.

2.1.2 **First feeding**

After the incubation period (total 350 d°), the larvae were transferred and distributed into four first feeding tanks (Harboe et al., 1998). The tanks (1100 L) were made of fiberglass with a height of 1 m and 1.5 m in diameter. The water inlet was at the surface and positioned to the side to ensure circulation in the tank by the constant inflowing water. The outlet was in the centre of the bottom of the tank and was connected to three valves, which stand vertical in relation to each other. The highest valve was always open and set the maximum water level inside the tank. The valve in the middle was opened to flush the rearing unit, while the lowest was at the bottom and to ensure that the unit could be easily emptied. The tank was constantly aerated through a hose attached to a steel cylinder with 0.3 mm holes lowered close to the outlet. The lid had a hole in the centre that allowed a 18 W fluorescent white light bulb (Osram, Munich, Germany) mounted 55 cm above the water surface, to light up the centre of the tank, creating a shade towards the edges where light conditions were favourable for larvae to hunt for prey. The intensity of the aeration was chosen so that the larvae would aggregate in the shaded area.

The tanks were prepared in advance by cooling the water, to 9 °C, adding clay, and approximately 1 million *A.* nauplii (1000 nauplii per tank litre). Before transporting the halibut larvae from the silos to the first feeding tanks, the lid of brackish water was removed, and a light source placed on the top of the incubation silo. This ensured that the halibut larvae aggregated near the surface where they could easily be collected. The larvae were transported from the incubation tank to the first feeding tanks using clean 10 L plastic buckets (approximately 500 larvae per bucket) and were systematically added to the tanks ensuring an even number of larvae per tank. This process was repeated until the silo was empty and the first feeding tanks contained approximately 5000 larvae each. After the transfer, the water temperature was increased to 12 °C over a period of 24h. Larvae were fed twice a day (morning and evening), and meal size was adjusted according to larvae appetite which was based on the remaining *A.* nauplii in the tank. Before feeding, 30 g of ceramic clay was added to the tank to keep turbidity high during feeding (2NTU). Tanks were illuminated from 07.00 to 24.00, with a light intensity of 5 μEcm^{-2}.

The first larval group was given *A.* nauplii, prophylactically treated with tribrissen (2.4 g of tribrissen for 1 h at 20 °C) for the first five days to reduce the risk of bacterial growth. However, this resulted in a high halibut larvae mortality. Therefore, the next two larval groups were fed *A.* nauplii not treated with tribrissen.

2.2 FEED EXTRACT PREPARATION AND ANALYSIS

To determine the effects of the different attractants in halibut larvae feed intake based on gut fullness (Objective two), three attractant extracts were made from Gemma Micro (Skretting, Stavanger, Norway), Otohime (Reed Mariculture, Redmond, US) or Northern prawn (*Pandalus borealis),* following the same protocol described by Yacoob and Browman, (2007a). Briefly, 50 g of feed material was mixed with 300 mL of water and homogenized with a hand blender for 10 minutes. The homogenate was centrifuged at 4000 rpm, 4 ºC, for 10 min. The supernatant was then decanted and stored at -20°C until use. Note that the shrimps were pealed before use.

A normal weaning dose (10-20 g formulated feed) was used as a baseline to determine the attractant dose strength. Based on ninhydrin analysis of the extracts the following doses were set:

- 25g weaning feed = 150 mL attractant extract (shrimp and Otohime)
- 17,5g weaning feed = 75 mL attractant extract (Gemma)

The Gemma extract contained twice as much water-soluble low molecular weight nitrogen compounds as the Otohime and shrimp extracts (Figure 6), thus the Gemma was set to ½ of shrimp and Otohime.

The different extracts were analysed for FAA and other low molecular weight nitrogen compounds at the IMR facilities at Nordnes. The extracts were analysed by ninhydrin detection following the protocol of ninhydrin detection (2015) described in Appendix 1. Briefly, Otohime and shrimp extracts were prepared by filtering through a syringe filter (0,45µm) into Eppendorf tubes. Gemma extract was filtered through a folded filter (4-7 µm). Two 500 µL sample were taken from each filtered extract and an internal standard was added to all samples in a ratio of 1:1 and then vortexed. The samples rested for one hour at room temperature (RT) before being vortexed for 30 min. Samples were then centrifuged for 30 min at 8000 rpm. Next, 20 µL of each sample was injected into a Boiochrom 30+ (Biochrom Ltd., Cambourne, UK) for analysis.

2.3 EXPERIMENTAL SETUP AND SAMPLING

To accomplish our main goals (objectives two to six), the experimental setup described in Figure 2 was performed. Four experimental groups (Otohime, Gemma, Shrimp and control) were performed in separated tanks. Each batch was sampled at 10 and 20 dpff. At each sampling day, six larvae were collected at four different time points; 0.7 h before feeding and 0.5, 1 and 3 h after feeding for each experimental group. Before the trial, larvae were deprived of food for 12 hours. Dissolved clay was added to the tank before feeding and 1 million *A.* nauplii cysts

were given together with the attractant extract of interest at 0 h. To the control group it was given 150 mL filtered seawater instead of the attractant extract. The solutions were poured around the aerator in the surface of the tank and was spread evenly in the tank after about one minute. New A. nauplii cysts (approximately 500 000) were given at 1 and 2.3 h after first feeding. At 3 hours after the first feeding 20 larvae were fixated on 4 % formalin for gut fullness analysis.

Larvae were randomly sampled using a hand net. A clean “squeezing bottle” with filtered seawater was used to carefully transfer the fragile larvae from the net to a petri dish containing a lethal dose of anaesthetics, MS 222 (Pharmaq, Overhalla, Norway). From the samples, six well-developed larvae were individually photographed and stored on RNAlater (Invitrogen, Carlsbad, California) for gene expression analysis. Samples stored in RNAlater were incubated over night at 4 ^{o}C before being stored at -80 ^{o}C. In addition, 20 larvae from each group from the last sampling point were fixated in formalin to determine gut fullness.

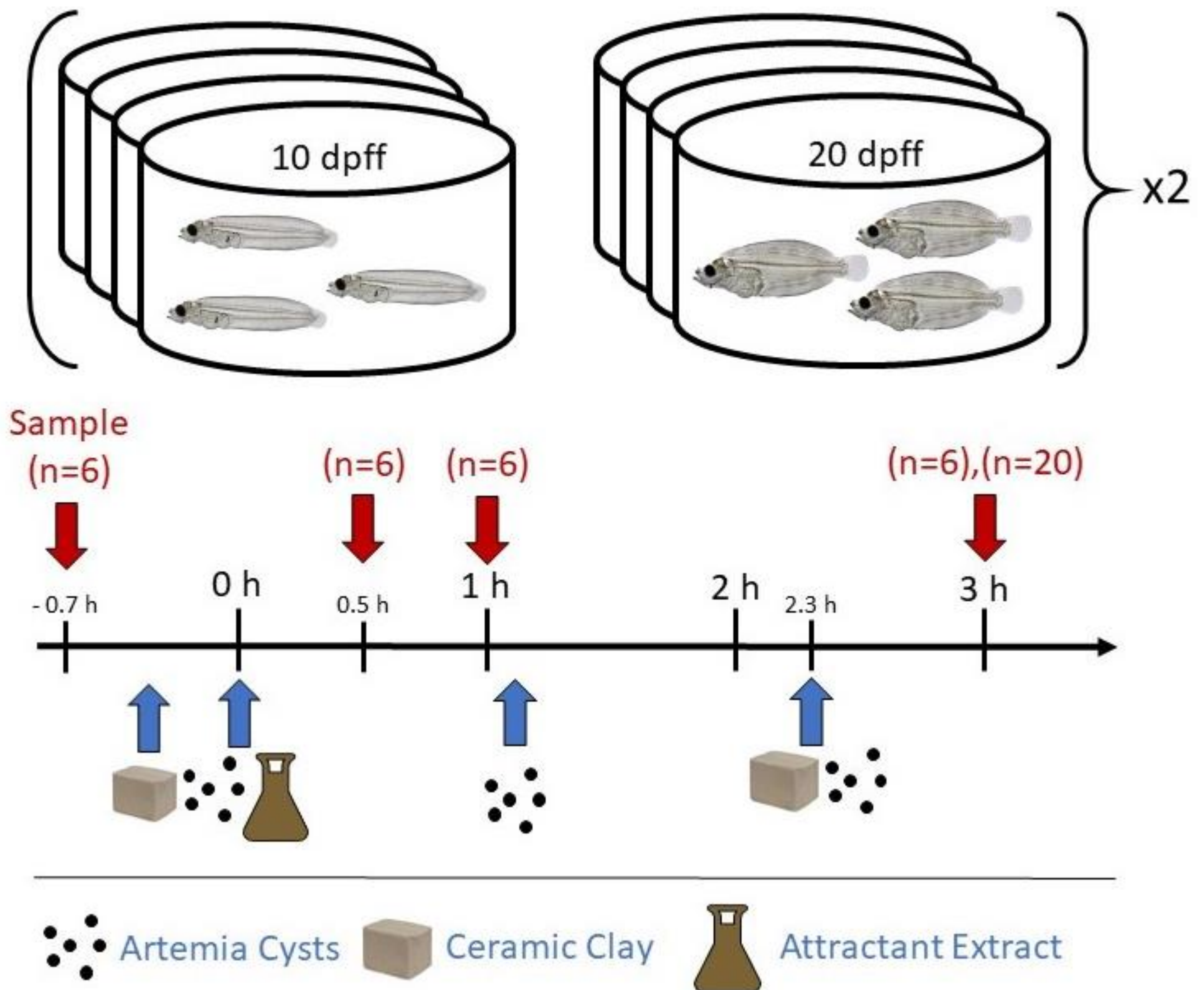


Figure 2. Experimental design. Six larvae were sampled at -0.7, 0.5, 1 and 3 h. Clay (represented as a cube) dissolved in water was added before feeding. Larvae were first fed at 0 h, with A. nauplii cysts (black circles) and attractant extracts (Erlenmeyer shape). At 1 h and again at 2.3 h another administration of only cysts were given to the larvae. At 3 h additional 20 larvae were sampled and fixated in formalin for gut fullness analysis.

2.4 STAGING AND FEED INTAKE ANALYSIS

All the larvae sampled for gene expression analysis were photographed with an Olympus S251 stereo microscope (Olympus, Tokyo, Japan) using a Moticam 1080 (Motic, Kowloon Bay, Hongkong) with Motic Images Plus 3.0 software, before being stored in RNAlater. Using ImageJ (Ver. 1.52a), the pictures were analysed and the myotome height calculated to stage the larvae according to Sæle et al. (2004). Food intake was registered by counting the number of *A.* cysts and nauplii in the gut.

Before analysing the larvae fixated in formalin for gut fullness, the larvae were washed with 70% ethanol. The pectoral fin was carefully removed using forceps, making it easier to observe the gut content trough the transparent skin and gut tissue (Figure 3). Some larvae required the removal of the skin covering the gut. This was done in one swift motion, grabbing skin at the

base of the pectoral fin and pulling towards the hindgut. The larvae were analysed one at the time under an Olympus S251 stereo microscope and the exact number of cysts observed in the gut was registered.

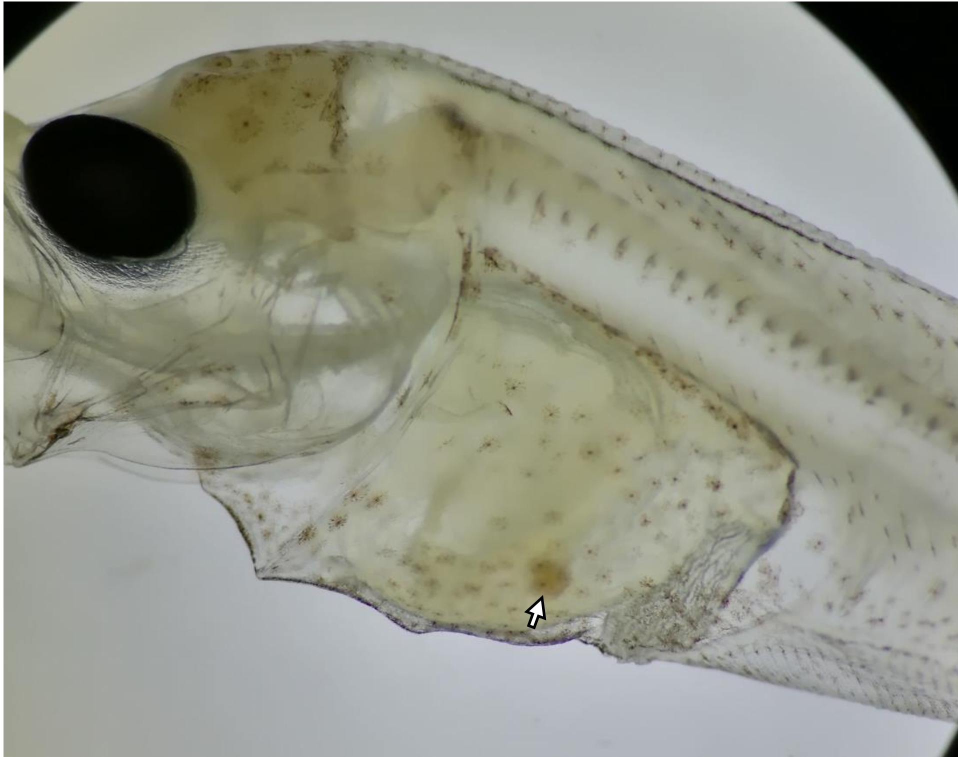


Figure 3. Atlantic halibut larvae (stage 5, 10 dpff) with one ingested Artemia cyst, which can be observed as a round brown object (see white arrow) inside the transparent gut. The pectoral fin has been removed. Photo: Endre Lygre

2.5 DISSECTION

To perform the mRNA expression analysis in individual tissues we first had to dissect the halibut. The braincase (brain) and abdominal cavity (gut) were dissected from the larva using an Olympus S251 stereo microscope following the steps described below (Figure 4): First, both eyes and pectoral fins were carefully removed, using forceps and cutting around the edge with a syringe needle (1). The brain was extracted by a diagonal cut through the eye socket (2), a horizontal cut ventral to the braincase (3), a horizontal cut dorsal to the braincase (4) and then a vertical cut posterior to the braincase and anterior to the abdominal cavity (5). The gut was removed by a horizontal cut dorsal to the cavity (6) and a posterior cut vertical to the cavity (7). All tissue was kept in RNAlater during the dissection, and then stored at -80°C.

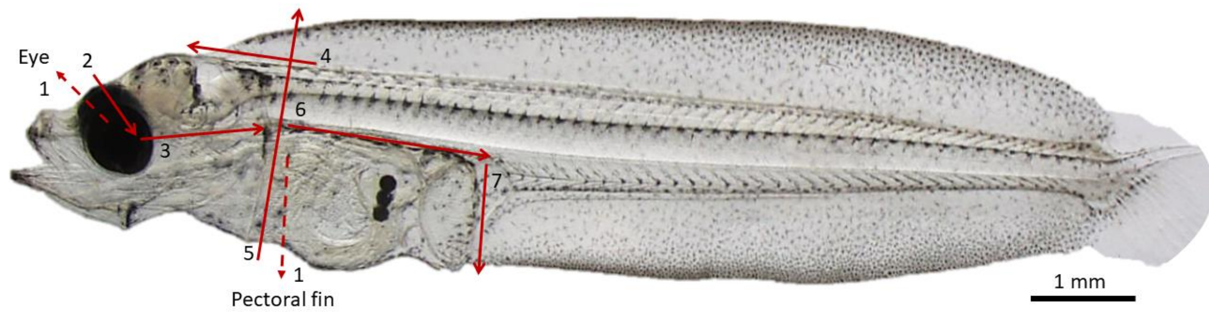


Figure 4. Dissection of halibut larvae. The dashed lines illustrate tissue that was removed to get access to the braincase (brain) and abdominal cavity (gut), and the full lines illustrate the cuts. First, both eyes and pectoral fins were removed, plucking them out with forceps and cutting around the edge with a syringe needle (1). The brain was extracted by a diagonal cut through the eye socket (2), a horizontal cut ventral to the braincase (3), a horizontal cut dorsal to the braincase (4) and a vertical cut posterior to the braincase and anterior to the abdominal cavity (5). The gut was removed by a horizontal cut dorsal to the cavity (6) and a posterior cut vertical to the cavity (7). Photo: Endre Lygre

2.6 Gene expression analysis

The analysis took place at the Marine Development Biology laboratory at the Department of Biological Sciences, University of Bergen.

Total RNA was isolated from brain and gut tissue and treated for possible genomic DNA contamination. cDNA was synthesised using DNase treated total RNA. mRNA expression analysis was performed by quantitative polymerase chain reaction (qPCR) using gene specific primers. The primers were designed spanning an exon-exon junction to avoid any possible genomic DNA amplification.

2.6.1 RNA isolation

Due to the small tissue, i.e., halibut larvae brain and gut, three different RNA isolation methods were tested to determine which was the most adequate to retrieve enough high-quality total RNA for the downstream steps. The methods used were TRI Reagent (Sigma-Aldrich, St. Louis, Missouri US), RNeasy Mini Kit (Qiagen, Venlo, Netherlands) and NucleoSpin RNA XS (MACHEREY-NAGEL, Düren, Germany). The RNeasy Mini Kit was the chosen method.

2.6.1.1 TRI Reagent

All work involving TRI-reagent was performed in a fume hood. The tissues were thawed on ice, and then swiftly but gently rubbed on Kimtech wipes (Kimberly-Clark professional, Milsons Point, Australia) to remove crystalized RNA-later, before adding it to a centrifuge tube containing 0,6-0,7 g of ceramic spheres (1.4 µm) and 0.5 mL of TRI-reagent. After 5 min of incubation on ice the samples were homogenized using a Precellys 24 homogenizer (5000 rpm

for 15 s). The samples were then incubated for 5 minutes at room temperature (RT). 100 µL of chloroform was added to each sample, vortexed for 1 min and then centrifuged for 15 min at 4 °C at 13200 rpm in an Eppendorf Centrifuge 5415R. The aqueous phase containing the total RNA was transferred to a new 1,5 mL Eppendorf tube, avoiding all contact with the interphase. The total RNA was precipitated by adding 250 µL of isopropanol. The tubes were inverted 5 times and incubated at RT for 10 min. Then, samples were centrifuged for 10 min at 4 °C at 13200 rpm. The supernatant was decanted, making sure not to lose the pellet, and 1 mL of cold 80% ethanol was added to wash the RNA pellet.

The samples were centrifuged for 5 min at 4 °C at 13200 rpm, supernatant decanted and the RNA pellet air dry for 5-10 min, until no trace of ethanol was left. The RNA pellet was dissolved in 25 µL of DEPC (diethyl pyrocarbonate) water and quantified using nanodrop (Thermo Scientific NanoDrop One Microvolume UV-Vis Spectrophotometer).

2.6.1.2 RNeasy Mini Kit

QIAGEN RNeasy Mini Kit combined with RNase-free DNase Set was used to isolate RNA and remove genomic DNA contamination.

All work involving β-Mercapoethanol was performed in a fume hood. Total RNA was extracted following the manufacturer's instructions. All centrifugation steps were performed at 13200 rpm, at RT using an Eppendorf Centrifuge 5415R. First, 6 µL of β-Mercaptoethanol was mixed with 600 µL Buffer RTL in a centrifuge tube containing 0.6-0.7 g of ceramic spheres (1.4 µm). Tissue was prepared as mentioned in section 2.6.1.1 before being homogenized using a Precellys 24 homogenizer (5000 rpm for 15s) After, samples were centrifuged for 3 min. Approximately 565 mL of lysate was obtained and transferred to a clean 1.5 mL Eppendorf tube. The same volume of 70% ethanol was added and mixed immediately by pipetting. Following, ½ volume (565 mL) was transferred to a spin column, centrifuged for 15 s and flow-through discarded. The remaining lysate-ethanol mix (565 mL) was added to the spin column and centrifuged for 15 s, and flow-through discarded. To the spin column it was added 350 µL Buffer RW1 and centrifuged for 15, and flow-through discarded. 70 µL of Buffer RDD mas mixed with 10 µL DNase and added to the centre of the spin column. After incubation at RT for 15 min, 350 µL Buffer RW1 was added to the spin column and centrifuged for 15 s, and the flow-through discarded. In two washing steps, 500 µL (x2) Buffer RPE was added to the spin column and centrifuged for 15 s and 2 min, respectively. After discarding the flow-through the column was centrifuged for 1 additional min to remove residual wash buffer (RPE). After, the

spin column was transferred to a clean 1.5 mL Eppendorf tube and 25 µL DEPC water added and centrifuged for 1 minute. The total RNA was quantified as previously described.

2.6.1.3 NucleoSpin RNA XS

Total RNA extraction using NucleoSpin RNA XS kit was performed accordingly to the manufacturer's instructions. All centrifugation steps were carried out at 11 000 x g at RT. Working in a fume hood, the tissue was prepared as mentioned in section 2.6.1.1, and added to centrifugation tubes containing 0.6-0.7g ceramic spheres (1.4 µm), 200 µL Buffer RA1 and 4 µL of reducing agent TCEP (0.14 mg/µL). The samples were vortexed twice for 5 s and homogenised using a Precellys 24 homogenizer (5000 rpm for 15s). 5 µL of pre-prepared carrier RNA (400ng/µL) working solution was added to each tube and vortexed two times for 5 s. The lysate was transferred to a NucleoSpin filter placed in a 2 mL collection tube and centrifuged for 30 s. The spin column was discarded, and 200 µL 70% EtOH added to the homogenized lysate and mixed by pipetting. NucleoSpin RNA XS column was placed in new 2 mL collection tubes and the lysate transferred into the column, and centrifuged for 30 s. The spin column was then placed in a new 2 mL collection tube, and 100 µL of Membrane Desalting Buffer was added to the column and centrifuged for 30 s. 25 µL of rDNase reaction mix (3 µL of rDNase in 27 µL Reaction Buffer) was carefully pipetted directly into the centre of the column membrane and incubated for 15 min at RT. For the first wash, 100 µL Buffer RA2 was added to the spin column, incubated for 2 min at RT and centrifuged for 30 s. The spin column was transferred to a new 2 mL collection tube for the second wash, 400 µL Buffer RA3 added to the column. and centrifuged for another 30 s. The flow-through was discarded and the spin column put back into the same collection tube, 200 µL Buffer RA3 was added to the column and centrifuged for 2 min. After, the column was placed in a new 1.5 mL collection tube and 10 µL of DEPC water was added and centrifuged for 30 s. The total RNA was quantified as previously described.

2.6.2 **DNase treatment**

DNase treatment was conducted to eliminate possible genomic DNA contamination and therefore, ensuring that we would only amplify cDNA by qPCR. When DNase treatment was not included in the RNA extraction protocol, as it is for NucleoSpin RNA XS and RNeasy Mini Kit, or in the cDNA synthesis protocol, as is for Super Script IV VILO (see below for detailed information), the Ambion TURBO DNA-*free* Kit (Invitrogen, Carlsbad, California US) was used.

Briefly, 5 µg (gut) or 3 µg (brain) of total RNA was added into a final reaction volume of 22.4 µL containing 0.1 volume of 10X DNase I Buffer and 2 U of TURBO DNase enzyme. This reaction was incubated at 37 °C for 30 min in a 2720 Thermic Cycler (Applied Biosystems, California, US). After, 0.1 volume of DNase Inactivation Reagent was added to each tube and mixed well by pipetting. Samples were incubated at RT for 2 min and mixed occasionally by flicking the tubes. Samples were centrifuged at 10 000 x g for 1.5 min at RT and the supernatant transferred to a new PCR tube. The total RNA concentration was measured using the NanoDrop and samples stored at -80 °C until further use.

2.6.3 **RNA quantification and integrity analysis**

The total RNA was quantified in order to normalise the input value for DNase treatment and cDNA synthesis and, therefore, ensure an equal amount of cDNA into the qPCR reaction. The integrity and purity of RNA was analysed ensure accuracy in the mRNA expression analysis.

A NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Scientific, Waltham, Massachusetts US) was used to measure total RNA concentrations and the A260/A280 and A260/A230 ratios for purity. Nucleic acids absorbed light at 260 nm, proteins at 280 nm and other contaminants, like phenol, at 230 nm. The absorbance ratios of A260/A280 and A260/A230 can therefore indicate possible protein and phenol contamination. Values above 2.0 for A260/280 and 2.2 for A260/230 is recognised as "pure" (Desjardins and Conklin, 2010).

The integrity of the total RNA was measured in an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, California US) using the Agilent RNA 6000 Nano kit accordingly to the manufacturer's instructions. The gel was prepared by pipetting 550 µL of RNA gel matrix into a spin filter and centrifuged at 1500 x g for 10 minutes at RT. 1 µL of RNA dye concentrate, equilibrated to RT for 30 minutes was vortexed, spun down and added to 65 µL of the filtered gel. The mix was vortexed well and centrifuged at 13000g for 10 min at RT. All samples and ladder were heat denatured at 70 °C for 2 min to avoid secondary structures like hairpins, and then immediately cooled on ice. For the halibut larvae gut only 25 % of the samples were analysed. However, for the brain, all samples were analysed because in the first 25% we obtained RIN (RNA integrity number) value below 7 (scale: 0-10) (Figure 18, Appendix 3).

The RNA chip (Figure 5) was loaded in the priming station, and 9 µL gel-dye mix was pipetted into well nr. 3 in the 4th column. The plunger was set to 1 mL, then closed and gently pressed down until it was held by the clip. The clip was released after 30 s and the plunge slowly pulled back to its initial position after 5 s. The prime station was then opened and 9 µL of gel-dye mix pipetted to the 1st and 2nd wells of the 4th column. 5 µL of RNA marker was pipetted into the 12 sample wells and the ladder well (Figure 5). 1 µL of RNA ladder was added into the ladder well in the 4th column. 1 µL of each sample was added into each of the 12 sample wells. The chip was vortexed for 1 min at 2400 rpm and then analysed in the Agilent 2100 Bioanalyzer instrument (Assay class: Eukaryote Total RNA Nano, version 2.6).

Figure 5. RNA chip. Well 1-12 in column 1-3 are used for samples and the 4th column is used for gel-dye mix and ladder.

2.6.4 **cDNA synthesis**

Two protocols: SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, California US) and Super Script IV VILO Master Mix (Invitrogen, Carlsbad, California US) were tested for cDNA synthesis, as described below. Based on the results obtained in this test, SuperScript III Reverse Transcriptase was the selected protocol to process our samples.

2.6.4.1 SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, California US)

cDNA was synthesised from 1.2 µg (gut) and 0.7 µg (brain) DNase treated total RNA in a final volume of 20 µL consisting of 1 µL of 50 µM oligo(dT)$_{20}$, 1 µL of 10 mM dNTP mix and nuclease-free water in a PCR tube. The reactions were heated to 65 °C for 5 minutes in a PCR machine followed by 1 min incubation on ice. Tubes were then briefly centrifuged to collect all contents before adding 4 µL of 5x First-Strand Buffer, 1 µL of 0.1 M DTT, 1 µL RNaseOUT (40 U/µL) and 1µL SuperScript III RT (200 U/µL). A no reverse transcriptase control (NRT) reaction was made by replacing SuperScript III RT with nuclease free water. After mixing gently, by pipetting, the reaction was incubated in a PCR machine at 50 °C for 50 minutes, and head inactivated at 70°C for 15 minutes. The cDNA was stored at -20 °C until further use.

2.6.4.2 SuperScript IV VILO Master Mix (Invitrogen, Carlsbad, California US)

2,5 µg of DNase treated total RNA in a total volume of 10 µL was gently mixed by pipetting with 1 µL 10x ezDNase Buffer and 1 µL ezDNase enzyme in a PCR tube. The reactions were

incubated at 37 °C for 2 minutes. Then, 6 µL of nuclease free water and 4 µL of SuperScript IV VILO Master mix was added to each sample. A NRT was made by replacing SuperScript IV VILO Master mix with SuperScript IV VILO No RT Control. The 20 µL final volume reactions were gently mixed by pipetting, and incubated at 25 °C for 10 minutes, 50°C for 10 minutes and heat inactivated at 85 °C for 5 minutes. The cDNA was stored at -20ºC until use.

2.6.5 **Quantitative Polymerase Chain Reaction (qPCR)**

The initial work involving the search of genes of interest in the Atlantic halibut databases (Transcriptome (Alves et al., 2016; Ana S. Gomes et al., 2014a) and Genome databases (IMR)), cloning and sequence verification was performed by Dr. Ana S. Gomes (unpublished results). Specific primers for our target genes (Table 1), were designed spanning an exon-exon junction to avoid genomic DNA amplification. Standard curves for each gene were generated using a 10-fold dilution series from plasmids (gene of interest cloned in TOPO4 vector (Invitrogen)). The Cq (quantification cycle) value was plotted against the number of copies and the slope used in the following equation to calculate primer efficiency (E (%)) for each target gene.

$$E\ (\%) = 10^{\left(-1/_{slope}\right)} - 1$$

Each qPCR reaction was performed in a final volume of 20 µL containing 25 ng of cDNA, 10 µL iTaq universal SYBR Green Supermix (Bio-Rad, Hercules, California US), 0.4 µM forward primer, and 0.4 µM reverse primer, in a hard-shell 96-well PCR plate (Bio-Rad, Hercules, California US) sealed with Microseal "B" (Bio-Rad, Hercules, California US). All the qPCR assays were carried out in a CFX 96Real Time System (Bio-Rad, Hercules, California US). All reactions were performed in duplicates, except standard curves, which were performed in triplicates, using the following PCR conditions:

95 °C for 30 s followed by 40 cycles at 95 °C for 5 s to 60 °C for 25 s. The absence of non-specific products and primer dimers was verified in all qPCR assays by melting curve analysis: 65 °C to 95 °C (increment of 0.5 °C for 2 s).

Three controls were added to each qPCR plate: No template control (NTC), to analyse for possible general contamination of the reaction and primer-dimer formation. No reverse transcriptase control (NRT), to analyse for possible genomic DNA contamination. Between plate control (BPC), consisting of a mix of all cDNAs to evaluate for possible differences between runs. Duplicates with a standard deviation (STD) greater than 0.5 were repeated. Copy number was calculated by using the mean Cq value of each duplicate reaction, slope and y-axis intersection of the following formula:

$$Copy\ number\ = 10^{((Cq-intersection)/slope)}$$

Relative copy number was calculated by dividing the copy number of the gene of interest by the reference gene elongation factor 1 alpha (Ef1a) copy number. Ef1a was the chosen reference gene because its expression was stable between samples regardless of the type of treatment (fed/fasted) or tissue (brain/gut), as also previously reported for this species (Ana S. Gomes et al., 2014a; Gomes et al., 2015; Infante et al., 2008). Normalising the data to a reference gene is a simple method for controlling for internal errors in the qPCR, such as the amount of starting material and variation of reverse transcription efficiencies.

2.7 STATISTICS

The statistical analysis and plots were conducted and rendered in RStudio (Version 1.1.419 – RStudio, Inc. http://www.rstudio.com/), using R (Version 3.5.1 – R Core Team, http://www.R-project.org/) with the following packages: ggplot2 (Wickham, 2016), ggpubr (Kassambara, 2018) and dplyr (Wickham et al., 2018). All the experimental groups were first tested for normality, using Shapiro-Wilk test, and visualised by histograms. Since the data mostly followed a Poisson distribution the differences between treatments were analysed using a Poisson regression model; a generalized linear model which uses the logarithms of the probability of each observation to find the best suited model for the data. Codes of significance ($p < 0.001$ (***), $p < 0.01$ (**), $p < 0.05$ (*), $p \geq 0.05$ (ns)) were added to statistical plots in Power Point (Microsoft, Washington, US). Statistical test results can be found in Appendix 5.

Table 1. Sequence of the specific primers used in qPCR gene expression analysis. Primer sequence, amplicon size, primer efficiency and R^2 is shown.

Gene	Sequence (5'→3')	Amplicon (bp)	E (%)	R^2
npy	F1: TTATAATGATTCGCTCGAGCTAC	111	98	0,999
	R1: GGGGACTGACCGGCTTGACT			
pyya	F2: GTGTGTCTGGGAACGCTGGC	140	100	0,997
	R2: TTTCCATACCTCTGCCTTGTGAT			
pyyb	F2: TCATCACCAGACAGAGGTATG	81	97	0,999
	R2: GGCTTGAATCGCCTCCGAAC			
cck1	F1: CCCTCTGAAGCCCTCCTTGAG	158	99	0,999
	R1: AGGAGATGAGTCTGGCCAGC			
cck2	F1: AGCTCGCCAACTACAACCAA	102	101	0,999
	R1: CCTGGTGGGGAGAGCCTTT			
cck1r	F1: AAGCCTTTTACCCGCCTCAA	96	96	1,000
	R1: CAACAGAGACACGTACCAGGA			
cck2r1	F2: CTCTGCAGGCGGTGAATGGGAG	144	105	0.995
	R2: GAGTCCATCTCTCTGACTCTGG			
cckrl2r2	F3: ACACGCTCCGGGGTAACGGG	162	103	0,997
	R3: GCAGATTCCCAAACACACTC			
ef1a	F2: CGCAGAAACACCGCAACTACAA	180	96	0,997
	R2: GCCCTTGCCCATCTCGGCAG			

3 RESULTS

3.1 FEED INTAKE QUANTIFICATION

No significant differences in cyst intake was found between experimental groups ($p \geq 0.05$). The majority of larvae sampled at 3 hours after feeding had 0 cysts in the gut (Table 2). The highest ingestion rate observed was six cysts in the gut of one larva given Otohime extract, and one larva given Gemma extract. On average, 85 % of larvae analysed had zero *A*. nauplii cysts in the gut, 13% had one *A*. nauplii cysts in the gut and 2% had two or more *A*. nauplii cysts in the gut (Table 2)

Table 2. Overview of cyst intake in larvae sampled at 3 h. n= 80.

Extract	Cysts in gut = 0	Cysts in gut = 1	Cysts in gut ≥ 2
Otohime	86%	12%	2%
Gemma	84%	11%	5%
Shrimp	84%	14%	2%
Control	83%	15%	2%
Mean	85%	13%	2%

3.2 EXTRACTS COMPOSITION ANALYSIS

Ninhydrin detection analysis showed that Gemma extracts contained approximately two times more the amount of water-soluble molecules than Otohime and shrimp extracts (Figure 6).

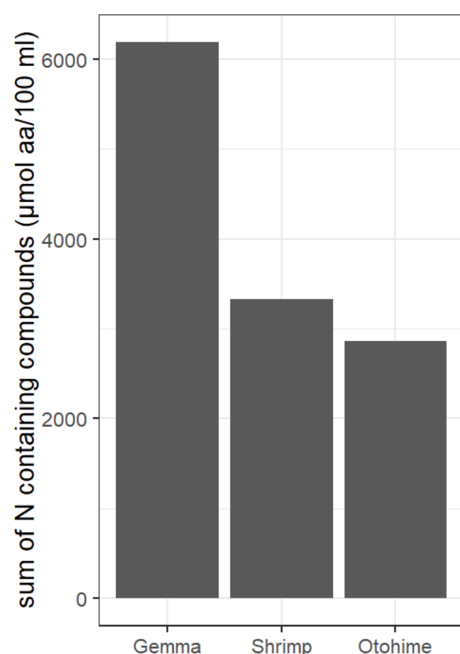


Figure 6. Sum of water soluble low molecular weight nitrogen compounds in three attractant extracts made from Gemma Micro, Otohime or Shrimp extracts. Data is shown as the total sum of compounds.

The extracts obtained from formulated feeds contained two (for Otohime) to four times (for Gemma) as much dry matter than shrimp (Table 3). The Otohime and Gemma dry weight per dose was approximately equal, while a single shrimp dose weighed half of Otohime and Gemma.

Table 3. Dry weight of the different extracts. Note that Shrimp and Otohime doses are 150 mL while Gemma is 75 mL.

Feed resource	Dry weight (g) per 100 mL	Dry weight (g) per dose
Otohime	3,6	5,3
Gemma	8,2	6,1
Shrimp	1,7	2,6

3.3 mRNA EXPRESSION ANALYSIS

3.3.1 Tissue distribution

To compare the levels of expression of the genes of interest between brain and gut tissue, we used unfed larvae at 20 dpff. *cck1* was highly expressed in the brain, while *cck2* was equally expressed in brain and gut (Figure 7, A). *cck1r* was mainly expressed in the gut, while *cck2r1* was more expressed in the brain (Figure 7, B). Clearly, *cck2r1* was more abundant than *cck2r2* in both tissues analysed. *pyyb* was predominately expressed in the gut (Figure 7, C). For *pyya,* mRNA expression levels were highest in brain and gut (Figure 7, C). The levels of *npy* mRNA expression was highest in the brain but much lower when compared to *pyya* in brain and gut and *pyyb* in the gut *(*Figure 7, D).

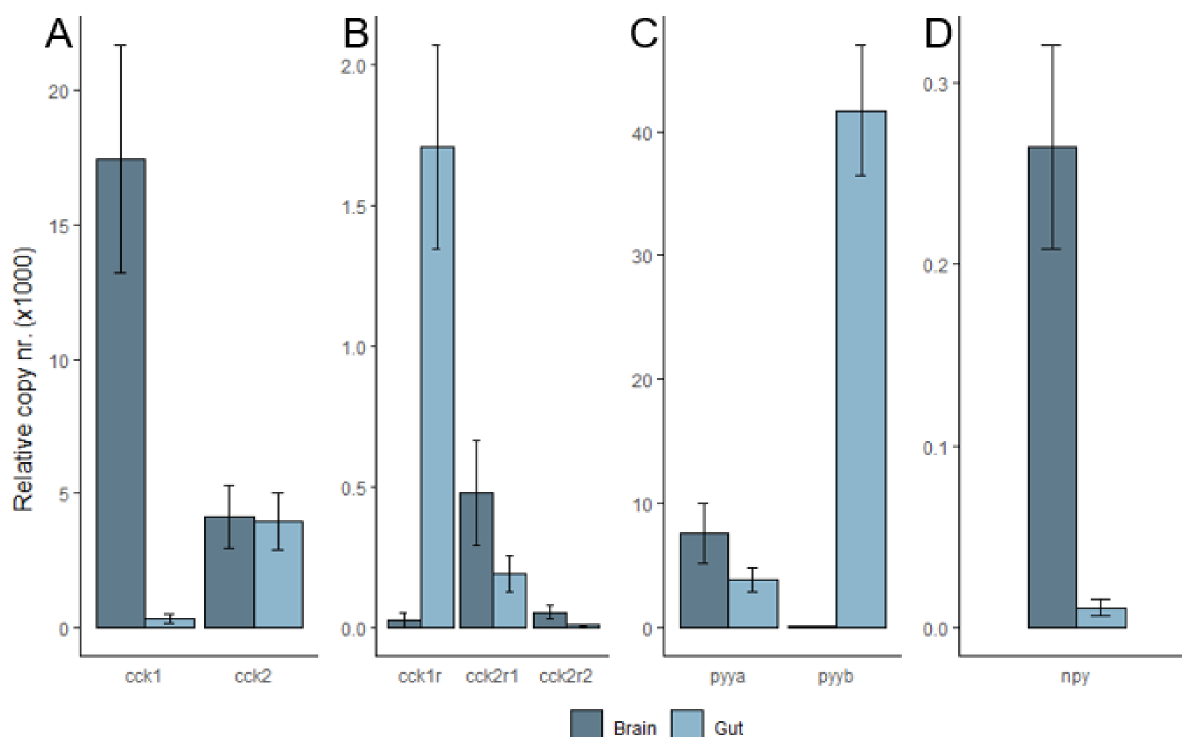


Figure 7. Relative tissue mRNA expression (x 1000) profile of cck1 and cck2 (A), cck1r, cck2r1 and cck2r2 (B), pyya and pyyb (C) and npy (D) of unfed larvae at 20 dpff. The number of amplified transcripts is presented in relation to ef1a1 copy number (n=12). Data are presented as mean +/- standard error.

3.3.2 **mRNA expression during ontogeny**

During development, the relative mRNA expression increased significantly in both brain and gut tissue for *cck1* (Figure 8)($p < 0.05$). *cck1r*, *cck2, pyya* and *pyyb* mRNA expression levels also increased in the gut ($p < 0.05$, $p<0.001$, $p<0.001$ and $p<0.001$ respectively). However, *cck2r1* mRNA expression decreased from 10 to 20 dpff in the halibut larvae gut ($p < 0.05$).

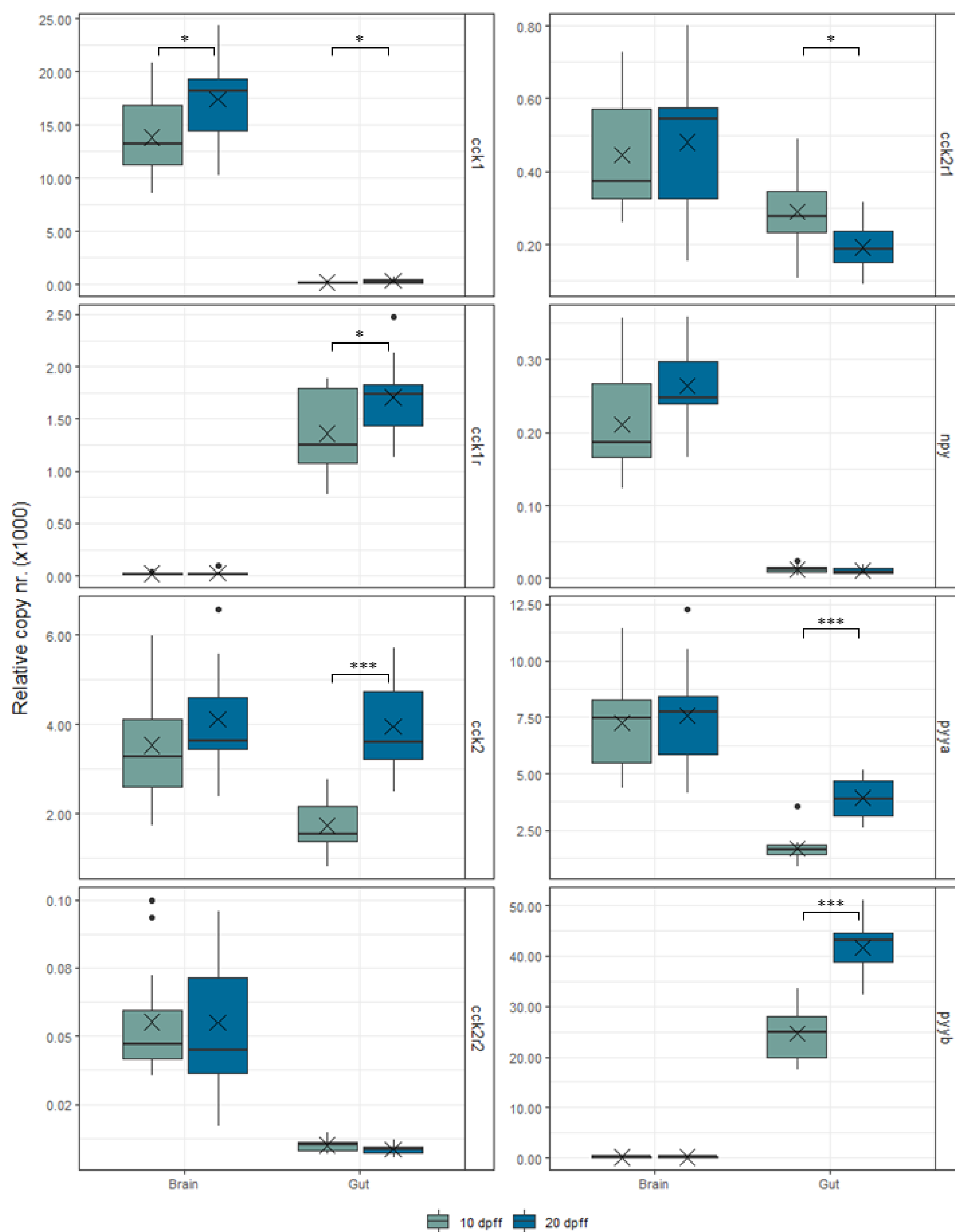


Figure 8. Relative mRNA expression (x 1000) of cck1, cck2, cck1r, cck2r2, cck2r1, npy, pyya and pyyb in brain and gut tissue from Atlantic halibut larvae at 10 and 20 dpff. The number of amplified transcripts is presented in relation to ef1a1 copy number (n=12 for all except for brain 20dpff (n=11)). Significant differences: $p<0.001$ (***), $p<0.01$ (**), $p<0.05$ (*) are marked. Boxes spans the 1st to 3rd quartile, horizontal lines mark the median, crosses mark the mean, whiskers mark variation outside 1st and 3rd quartile and dots mark outliers.

3.3.3 **Response to feeding**

Gene expression in response to feeding was compared between three groups: Unfed larvae; were sampled at “-0.7 h” prior to feeding (0 h), feeding larvae; were sampled after feeding at 0.5, 1 and 3 h and had cysts in the gut and not-feeding larvae; were also sampled after feeding at 0.5, 1 and 3 h but did not have cysts in the gut. The mRNA expression levels of the selected genes of interest to feeding at 10 and 20 dpff is described in the following sections:

In the brain, *cck1* mRNA expression levels were significantly lower in the feeding larvae compared to unfed larvae at both 10 and 20 dpff (Figure 9)(10 dpff: 0.5 h ($p<0.05$), 1 h ($p<0.05$), 3 h ($p<0.01$) and 20 dpff: 0.5 h ($p<0.01$), 1h ($p<0.05$) and 3 h ($p<0.01$)). In addition, at 10 dpff, the *cck1* levels of feeding larvae at 1 h after feeding was significantly lower than not-feeding larvae ($p<0.05$). The same pattern was observed for 20 dpff larvae, but at 3h after feeding ($p<0.05$).Opposite to what was observed in the brain, in the gut *cck1* mRNA expression was significantly lower in not feeding larvae at 3 h after feeding at 10 dpff ($p<0.05$), and significantly higher in feeding larvae at 20 dpff compared to unfed larvae (Figure 9)($p<0.05$). Expression level was significantly higher in feeding larvae at 1 and 3 h at 10 dpff, and 3h at 20 dpff ($p<0.05$ and $p<0.01$ respectively), compared to not-feeding larvae ($p<0.01$).

In the brain, *cck2* mRNA expression levels were significantly lower in feeding larvae at 1 h after feeding at 10 dpff and 0.5 h after feeding at 20 dpff when compared to the unfed group (Figure 9)($p<0.05$). At 10 dpff, *cck2* mRNA expression was significantly higher in the gut of not-feeding larvae at 0.5 and 1 h after feeding compared to unfed larvae (Figure 9)($p<0.05$ and $p<0.01$ respectively). The *cck2* expression levels in the gut of not-feeding larvae were significantly higher than in fed larvae at 1 and 3 h after feeding ($p<0.01$ and $p<0.05$ respectively). At 20 dpff, it was observed a significant decrease of *cck2* mRNA levels in the gut of feeding larvae at 0.5 h compared to the unfed group ($p<0.05$).

No significant changes were found for *cck1r* expression in the brain (Figure 10) ($p\geq0.05$), while in the gut of 20 dpff its expression increased significantly in not-feeding larvae at 3 h after feeding compared to the unfed group (Figure 10) ($p<0.05$).

In the brain, *cck2r1* mRNA expression was significantly lower in feeding larvae at 1 h after feeding at 10 dpff ($p<0.05$), and at 0.5 and 1 h after feeding at 20 dpff compared to unfed larvae (Figure 10) ($p<0.05$). In the gut, *cck2r1* mRNA expression only changed for 10 dpff larvae, with significantly lower levels in not-feeing larvae at 0.5 h after feeding ($p<0.05$), and in feeding larvae at 3 h after feeding compared to the unfed group (Figure 10) ($p<0.05$).

Halibut larvae at 10 dpff showed significantly lower levels of *cck2r2* mRNA expression in the feeding group at 1 h after feeding (Figure 10) ($p<0.05$). In the gut, *cck2r2* mRNA expression only changed for 10 dpff larvae, with significantly higher levels in not-feeding larvae 1h after feeding compare to unfed larvae (Figure 10) ($p<0.05$).

No significant changes in the *npy* mRNA expression levels was observed for 10 dpff halibut larvae brain and gut, and for the later tissue also no differences were found at 20 dpff (Figure 11). At 20 dpff, *npy* expression in the brain was significantly higher at 3 h in not-feeding larvae compared to unfed and feeding larvae ($p<0.05$ and $p<0.01$ respectively).

In the brain, *pyya* mRNA expression decreased significantly in feeding larvae at 1 and 3 h after feeding at 10 dpff (Figure 11)($p<0.05$). At 20 dpff, the expression levels of *pyya* in the brain and gut decreased significantly in not-feeding larvae at 0.5 h after feeding ($p<0.05$). No significant changes in *pyya* mRNA expression was found in the gut at 10 dpff (Figure 11)($p\geq0.05$).

The mRNA expression levels of *pyyb* changed significantly in the 10dpff groups as levels decreased in the brain of feeding larvae at 3 h after feeding ($p<0.01$), and increased in the gut of 0.5 and 3 h after feeding compared to unfed larvae (Figure 11)($p<0.01$ and $p<0.05$ respectively).

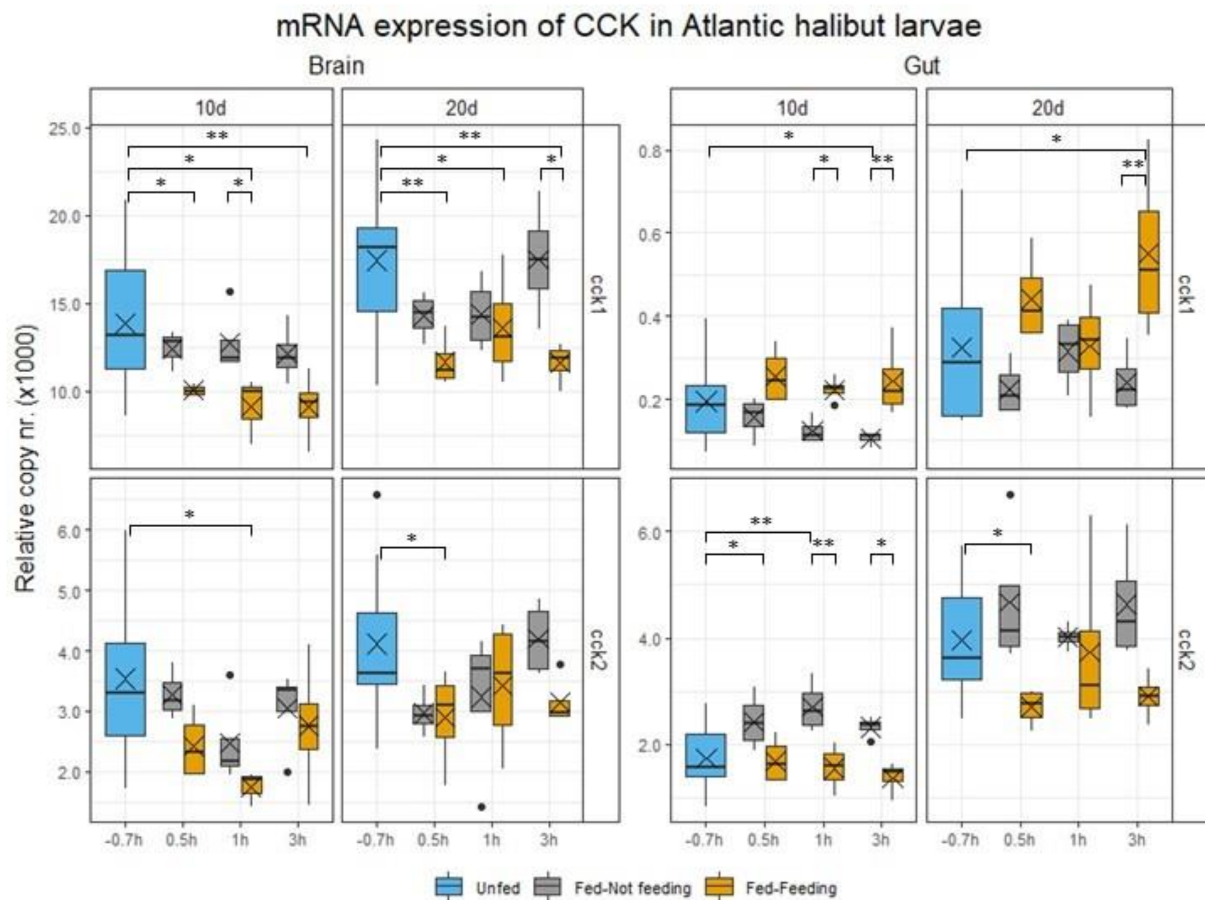


Figure 9. Relative mRNA expression (x 1000) of cck1 and cck2 in the brain and gut tissue from Atlantic halibut larvae at 10 and 20 dpff. Results are presented as copy number relative to gene of reference Ef1a copy number. Unfed larvae n= 12, feeding and not-feeding larvae n=4 each. Significant differences are marked as p<0.001 (***), p<0.01 (**), p<0.05 (*). Boxes spans the 1st to 3rd quartile, horizontal lines mark the median, crosses mark the mean, whiskers mark variation outside 1st and 3rd quartile and dots mark outliers.

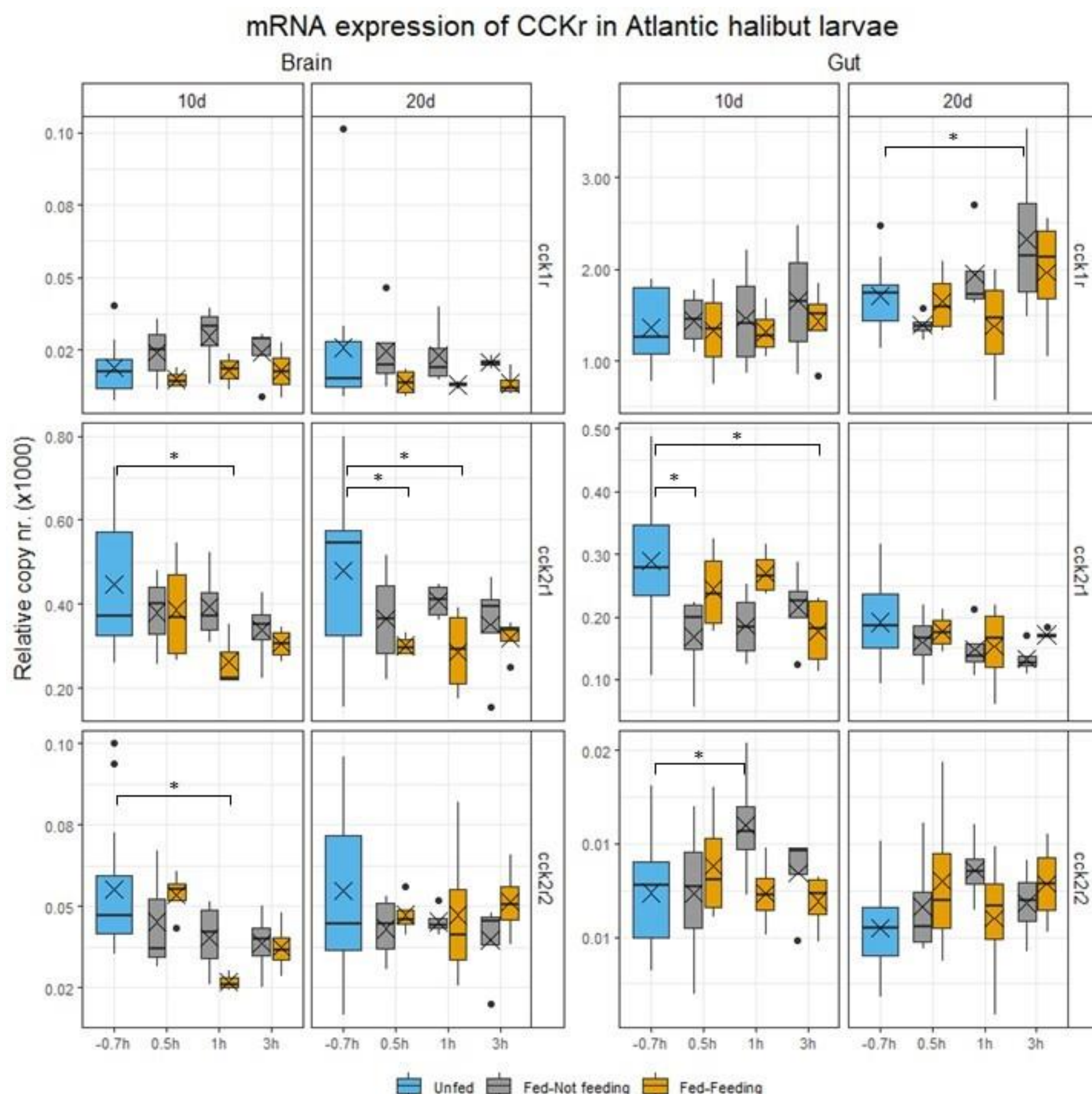


Figure 10. Relative mRNA expression (x 1000) of cck1r, cck2r1 and cckr2r2 in the brain and gut tissue from Atlantic halibut larvae at 10 and 20 dpff. Results are presented as copy number relative to gene of reference Ef1a copy number. Unfed larvae n= 12, feeding and not-feeding larvae n=4 each. Significant differences are marked as p<0.001 (***), p<0.01 (**), p<0.05 (*). Boxes spans the 1st to 3rd quartile, horizontal lines mark the median, crosses mark the mean, whiskers mark variation outside 1st and 3rd quartile and dots mark outliers.

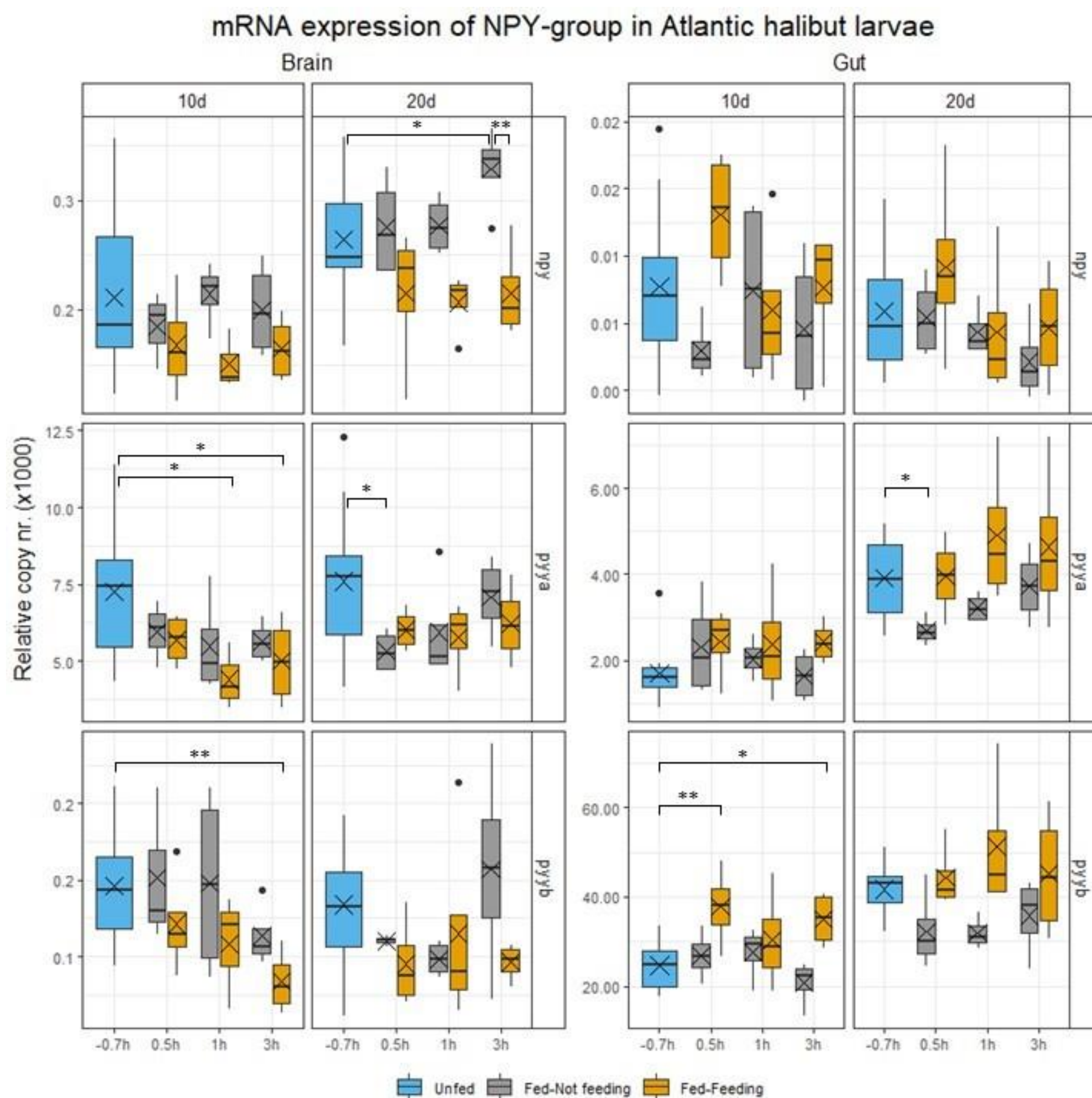


Figure 11. Relative mRNA expression (x 1000) of npy, pyya and pyyb in the brain and gut tissue from Atlantic halibut larvae at 10 and 20 dpff. Results are presented as copy number relative to gene of reference Ef1a copy number. Unfed larvae n= 12, feeding and not-feeding larvae n=4 each. Significant differences are marked as p<0.001 (***), p<0.01 (**), p<0.05 (*). Boxes spans the 1st to 3rd quartile, horizontal lines mark the median, crosses mark the mean, whiskers mark variation outside 1st and 3rd quartile and dots mark outliers.

3.4 METHODOLOGICAL CONSIDERATIONS

RNA isolation using the RNeasy mini kit yielded the highest (Figure 12) and purest (Table 5) total RNA concentration when compared to the results obtained using the Nucleospin kit and the TRI Reagent protocol. This is particularly relevant for the brain tissue, which was the limiting factor. qPCR results showed that Ambion DNA-free kit was the only protocol to successfully in removed genomic DNA contamination (Figure 17, Appendix 2).

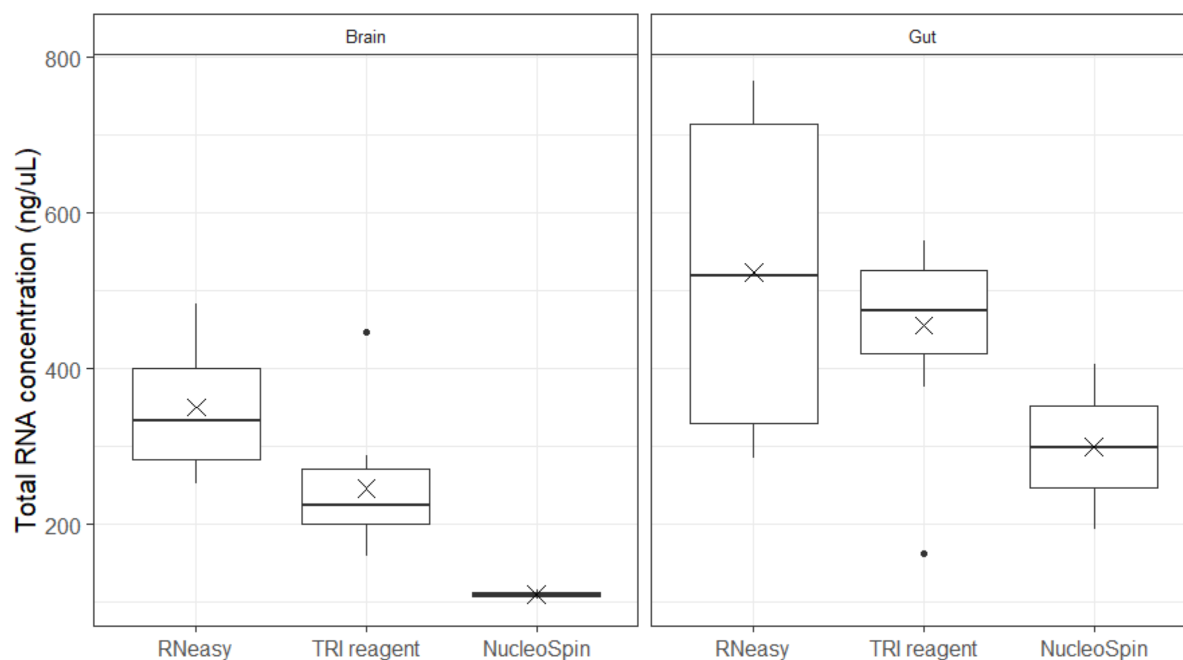


Figure 12. Total RNA concentration (ng/µL) yield from brain and gut tissue, from stage 5 halibut larvae, using three RNA isolation protocols: RNeasy Mini Kit, TRI reagent, NucleoSpin RNA XS. Boxes spans the 1st to 3rd quartile, horizontal lines mark the median, crosses mark the mean, whiskers mark variation outside 1st and 3rd quartile and dots mark outliers. For detailed information, see Table 5, Appendix 2.

4 DISCUSSION

In this study, *A*. nauplii cyst intake in Atlantic halibut larvae at 10 and 20 dpff was not significantly affected by adding into the rearing. To describe the central and peripheral systems controlling appetite, feed intake and digestive processes in Atlantic halibut larvae, we have analysed the mRNA expression of key hormones (*cck, npy* and *pyy*) and *cck* receptors in brain and gut tissue during development and in response to feed intake. The following genes were analysed by qPCR: *cck1, cck2, cck1r, cck2r1, cck2r2, npy, pyya* and *pyyb.* All genes analysed were expressed in both tissues at both sampling days (10 and 20 dpff): *cck1, cck2r1, cck2r2*, *npy* and *pyya* were more abundant in the brain, *cck1r* and *pyyb* in the gut, while *cck2* were expressed at similar levels in both tissues. Throughout development the expression of *cck1*, *cck2, cck1r, pyya* and *pyyb* increased significantly in the gut, which is in line with the ongoing development and growth of the GI-tract. However, the expression levels of *cck2r1* in the gut decreased with development.: Expression of *cck1*, in both the brain and gut tissue, as well as *cck2* and *pyy* paralogues, in the gut, and *npy*, in the brain of 20 dpff larvae responded significantly to introduction of feed particles (by feeding or not feeding) after a period of feed deprivation. This indicates that these hormones might be involved in control of feed intake and promote digestive processes in halibut larvae.

4.1 EFFECTS OF ATTRACTANT EXTRACTS ON FEED INTAKE

The total content of water soluble low molecular weight nitrogen compounds and the dry weight of attractant extracts are presented in Figure 6 and Table 3 in appendix 1. Extracts of Gemma contained approximately twice as much water-soluble N compounds (possible attractants) as Otohime and shrimp. The dry weight was also highest in the Gemma solution. To account for Gemma extract containing approximately twice the amount of water-soluble compounds compared to the other extracts (Figure 6), its dose was reduced to half volume. We expected that the feed intake in larvae exposed to shrimp attractants would be higher than larvae in the control group (added to water), since it has been previously shown that feeding behaviour is evoked in halibut juveniles exposed to shrimp extract (Yacoob and Browman, 2007a). However, statistical analysis showed no significant difference ($p \geq 0.05$) between this two groups. In addition, shrimp extract contained the highest FAA content in total compared to the other attractant extracts (Table 4, Appendix 1). On the other hand, a single amino acid (L-glycine) accounted for ~61% of total content in shrimp attractants, which was ranked as the 12th most potent FAA regarding the olfactory nerve response in halibut juveniles of the 20 FAA

tested by Yacoob and Browman (2007b). Gemma extract, on the other hand, contained the highest amount of the most potent FAA (L-Methionine), in addition to L-Serine and L-Valine (3rd and 4th) and Otohime contained relative high amounts of L- and β-Alanine which was the 5th most potent FAA (Figure 14, Appendix 1) (Yacoob and Browman, 2007b). In seabream larvae, increased feed ingestion was attributed to betaine, arginine, alanine and glycine. Of these, shrimp contained the highest levels of L-glycine and Arginine, while L-Alanine content was higher in extracts from Otohime and Gemma. We know from earlier trials that designing attractant solutions is complicated, as synthetic mixes, designed to mimic extracts with known stimulatory effect, have failed to induce feeding behaviour in Atlantic halibut juveniles (Yacoob and Browman, 2007a). Earlier studies on Atlantic halibut olfactory stimuli (Yacoob and Browman, 2007b, 2007a) was performed on juveniles. Taking into consideration that we have analysed a completely different developmental stage (larvae at 10 and 20 dpff), we could only speculate that the stimuli response could be similar. Because increased feed intake evoked by attractants was possible in 20 day old seabream larvae (Kolkovski et al., 1997), and earlier observations when weaning halibut larvae on to commercial diets (T. Harboe, 2018. pers comm.), we suspect that also halibut larvae might be able to sense and react to the attractants. However as there were no significant difference in feed intake between the experimental groups in this study, it is possible that extracts had no effect on the larvae due to an insufficient attractant concentration or that halibut larvae did not identify the cysts as potential prey without prior exposure.

Consequently, we explored the presence and response of mRNA expression of key factors involved in physiological control of feed intake between unfed larvae (larvae sampled before feeding, not-feeding larvae (fed larvae with no cysts in the gut) and feeding larvae (fed larvae with cysts in the gut).

4.2 TISSUE EXPRESSION PROFILE

4.2.1 cck and cckr

The tissue distribution pattern was analysed in Atlantic halibut larvae at 20 dpff (Figure 7) and showed that *cck1* was highly expressed in the brain compared to cck2. This partially contradicts previous findings in adult Japanese flounder, where both *cck1* and *cck2* were highly expressed in the brain (Kurokawa et al., 2003). In Japanese flounder intestine, *cck1* expression was clearly more abundant than *cck2,* which also differs from our results where expression was higher for *cck2* in the halibut larvae gut than *cck1* (Figure 7).

For the CCK receptors, mammalian CCK1R is primarily located in the GI-tract, while CCK2R is primarily located in the brain. Our analysis confirmed the former for halibut, as *cck1r* was mainly expressed in the gut (Figure 7). Similar findings have been reported in yellowtail, Atlantic salmon and goldfish (Furutani et al., 2013; Rathore et al., 2013; Tinoco et al., 2015), where *cck1r* was mainly expressed in the intestine. In halibut larvae, both *cck2r1* and *cck2r2* were more abundant in the brain. This is contradictory to findings in salmon where *cck2r1* was mainly expressed in the intestine, and *cck2r2* was expressed at similar levels in both tissues (Rathore et al., 2013). In goldfish, *cck2r* (*cckbr* in the article) was mainly expressed in the brain and in the posterior intestine (Tinoco et al., 2015).

4.2.2 pyy and npy

pyyb was mainly expressed in the gut, while *pyya* was slightly more expressed in the brain. Similar findings have been reported in grass carp, where *pyya* (*pyy* in the article) was mainly expressed in the CNS (brain and spinal cord), whereas *pyyb* was mainly expressed in the gut (mostly in the foregut, but also in mid- and hindgut) and low expressed in brain. However, in Atlantic salmon, *pyya* was found to be mainly expressed in the gut (pyloric caeca and midgut) (Murashita et al., 2009b). Differences in expression when comparing different fish species and different developmental stages are, however, not surprising (for review see: Rønnestad et al., 2017). Sundström et al., (2013) found that *pyya* and *pyyb* was mainly expressed the brain, heart and kidney in zebrafish. During the dissection of halibut larvae, the heart was removed by cutting between the pericardium and abdominal cavity (Figure 4, step 5), but other organs, like the kidney, may have been included in the analysis. Thus, we cannot rule out that the levels of expression that we observe in the "gut" in our case are not affected by other organs, such as the kidney.

Our findings show that *npy* expression was highest in the brain of halibut larvae (Figure 7, c) which also have been reported in halibut larvae (Gomes et al., 2015), and in adult salmon (Murashita et al., 2009a). Murashita et al., (2009a) found only very low *npy* expression in the kidney of salmon, and from the tissues analysed by Gomes et al., (2015), *npy* was mainly expressed in the eyes, followed by brain and muscle. This is also in contradiction to Sundström et al., (2013) study, where only minimal *npy* expression levels were found in the eyes of zebrafish. These findings highlight the importance of precisely isolating different tissues as gene expression may vary between species and tissue.

4.3 EXPRESSION DURING ONTOGENY

4.3.1 cck and cckr

Ontogeny had a clear effect on the CCK system. This was particularly evident in the gut, where the mRNA expression of *cck1*, *ck2* and *cck1r,* was significantly higher in the older larvae. In contrast, *cck2rl* levels were significantly lower at 20 dpff halibut larvae. In the brain, only expression of *cck1* was affected by development. In this study, the sampled larvae were in development stage 5 or stage 6 (Sæle et al., 2004), which means that their digestive system is still rudimentary and they lack a functional stomach (Ana S. Gomes et al., 2014b; Pittman et al., 1990). Ontogeny of Cck-producing cells have been previously studied using immunohistochemistry in larvae of Atlantic halibut (Kamisaka et al., 2001), bluefin tuna (*Thunnus thynnus*) (Kamisaka et al., 2002), ayu (*Plecoglossus altivelis*) (Kamisaka et al., 2003) and Atlantic herring (*Clupea harengus*) (Kamisaka et al., 2005). In Atlantic halibut larvae sampled through the first feeding stages from 33 to 66 dph Cck-producing cells were observed in 30 % of larvae at 45 dph and that the number of Cck- producing cells increased through development (Kamisaka et al., 2001). The antibody used by Kamisaka et al., (2001) can bind to both *cck1* and *cck2* (data not shown), and, thus, the cell populations (Cck1 or Cck2-producing cells) identified in the study couldn't be discriminated. Their findings are, however, in agreement with our results, since the expression levels of both *cck1* and *cck2* increased in the gut from 10 to 20 dpff (~53 and 63 dph, respectively) (Figure 8). Cck-producing cells emerge at different timepoints in teleost fish (Kamisaka et al. (2001, 2002, 2003, 2005), e.g., Cck-producing cells are present at hatching in ayu and herring, while they emerged during hatching in bluefin and later in development in halibut. Cck analysis by radioimmunoassay of halibut larvae have also shown that Cck levels increase during development (7-26 dpff) (Rojas-García and Rønnestad, 2002). The expression of *cck* have been studied in whole-body larvae of several species, including: Atlantic cod (Kortner et al., 2011), blunt snout bream (*Megalobrama amblycephala*) (Ping et al., 2014) and rose snapper (*Lutjanus guttatus*) (Moguel-Hernández et al., 2016). Results from the different studies show that there is no correlation of *cck* expression between species for instance, in cod larvae, *cck* mRNA expression levels decreased after hatching, while they increased in blunt snout bream. In rose snapper, *cck* expression increased at first feeding (2 dph) until 10 dph, and then decreased until 20 dph. With the increase in Cck-producing cells during larval development found in other teleost larvae (halibut, bluefin, ayu and herring) (Kamisaka et al., 2001, 2002, 2003, 2005), *cck* expression for cod and rose snapper would be expected to also increase through ontogeny as found for blunt snout bream and

presented in our results (Figure 8). One reason for these ambiguous results might be the use of whole-body larvae. As shown by Rojas-García and Rønnestad (2002), the mRNA or protein expression levels, may vary between development stages and tissues: In the gut of 7 dpff halibut larvae, the total Cck content accounted for about 6% of the whole larva body (Rojas-García and Rønnestad, 2002). An increase to 62% at 26 dpff, demonstrating once again the importance of analysing individual tissues to avoid that the expression of the genes analysed is "diluted" when analysing whole larvae.

We found that *cck2r1* levels decreased significantly in the gut from 10 to 20 dpff (Figure 8) and, therefore, we hypothesize that its function in the gut may change or become less important in this tissue in older larvae.

4.3.2 **npy and pyy**

No significant differences were found for *npy* mRNA expression from 10 to 20 dpff (Figure 8). These results corresponds to a previous study on halibut larvae, where *npy* mRNA expression was not affected by development (stage 5 to stage 9B) (Gomes et al., 2015). Thus, we have strong support that *npy* expression is not affected by development, at least during the first feeding stages. A few studies have also analysed *npy* mRNA expression during larval development, including cod (Kortner et al., 2011), blunt snout bream (Ping et al., 2014) and rose snapper (Moguel-Hernández et al., 2016). In cod larvae, *npy* decreased after hatching and during onset of exogenous feeding (4dph), while it increased in blunt snout bream (3dph) and rose snapper after (2 dph). The changes in expression levels during first feeding might indicate a role of *npy* in appetite control for rose snapper and blunt snout bream, but not for cod larvae, However, as discussed above, the specific expression changes in gut and brain tissue is unclear due to the use of whole larvae in these studies.

Our results show that *pyya* and *pyyb* expression increased significantly in the gut from 10 to 20 dpff. This is in agreement with findings in sea bass (*Dicentrarchus labrax*), where Pyy-producing cells appeared in the intestine in "phase II" (9-15 dph) and in the stomach at "phase IV" (55-60 dph) and increased trough development (García Hernández et al., 1994). Pyyb (*py* in the article) producing cells were found in the anterior intestine of Japanese flounder larvae at 3 dph, when the larvae start exogenous feeding (Kurokawa and Suzuki, 2002), and increased, covering a larger area of the gut, until 30 dph. No *pyya* (*pyy* in the article) mRNA was detected in the intestine, and both Pyya and Pyyb producing cells was found in the brain (Kurokawa and Suzuki, 2002). In whole-body grass carp larvae, *pyya* (*pyy* in the article) expression increased from onset of exogenous feeding (5 dph) to 8 dph (Chen et al., 2013) while *pyyb* expression

increased from hatching until 5-8 dph (Chen et al., 2014). The increased expression of *pyy* paralogues during start of exogenous feeding in sea bass and grass carp and in Atlantic halibut larvae from 10 to 20 dpff suggests that pyy paralogues are involved in regulation digestion and appetite control, but may become more relevant in older larvae due to the increase of expression. This support the hypothesis that fish larvae have a rudimental regulatory system of both digestion and appetite (Ana S. Gomes et al., 2014b; Gomes et al., 2015).

In order to understand the tissue-specific functions of *pyy* and *cck* paralogues, *cck* receptors and *npy*, more studies are necessary, including analysing the expression changes over a longer developmental period and also targeting individual tissues by using a more precise dissection method.

4.4 RESPONSE TO FEEDING

The original plan of this thesis to measure differences in cyst intake between experimental groups administered attractant extracts of different feeds that are known to stimulate feed intake in Atlantic halibut larvae. However, as described above, ingestion of cysts was low in all experimental groups, even though we have administered cysts to the larvae three points in time several times (Figure 2, 0h, 1 h and 2.4 h).This also means that we couldn't determine when the larvae ate the cysts i.e. whether the larvae sampled at 3 h ate the cysts at 3 h, 2 h or 20 min prior to sampling. Therefore, our data, cannot be used to discuss the differences between short- and long-term effects of feed intake in appetite control or digestive functions.

4.4.1 cck and cckr

The response of *cck1* expression to feeding was inverse in gut and brain tissue (Figure 9). In the brain, *cck1* expression decreased significantly at 0.5, 1 and 3 h after feeding in larvae at both 10 and 20 dpff. In addition, expression differed significantly between feeding and not-feeding larvae at 1 h (10 dpff) and 3h (20 dpff). In the gut, *cck1* expression decreased significantly in not-feeding larvae at 3 h at 10 dpff, while it increased significantly in feeding larvae at 3 h at 20 dpff. Additionally, expression was significantly higher in feeding, compared to not-feeding larvae at 1 h (10 dpff) and 3 h (10 and 20 dpff). These findings suggest that Cck1 acts as an orexigenic factor in the brain, and in the gut as a primary local digestion-promoting factor with a possible secondary anorexigenic role. Different results have, however, been found for other teleost species. For example, food deprivation led to decreased *cck1* expression in the brain of Atlantic salmon (Murashita et al., 2009b) and increased expression in the gut of white seabream (Micale et al., 2012). Feeding of Siberian sturgeon (*Acipener baerii*) resulted in

increased *cck* expression and injection of Cck led to a decrease of feed intake (Zhang et al., 2017), supporting the role of *cck* as a promotor of digestive processes and anorexigenic factor in fish.

At 10 dpff, *cck2* expression increased significantly in the gut of not-feeding larvae at 0.5 and 1 h (Figure 9). In addition, expression was significantly higher in not-feeding larvae compared to feeding larvae at 1 and 3 h. In contrast, at 20 dpff *cck2* decreased significantly in feeding larvae at 0.5 h. Overall, *cck2* expression in the gut was higher in not-feeding larva compared to feeding larvae. Similar results were found for white seabream, where *cck2* expression was lower in fed compared to starved fish (Micale et al., 2012). We suggest that the presence of either feed items or attractants in the water may have stimulated a feedforward response in halibut larvae, which may have led to an increased *cck2* mRNA expression in the gut to prepare the gut for digestion. Both paralogues, *cck1* and *cck2,* showed a reversed expression profile in the gut for 10 and 20 dpff (Figure 9): *cck1* increased while *cck2* decreased in feeding larvae. This suggests that Atlantic halibut *cck* paralogues may have different functions, as found for white seabream (Micale et al., 2012).

cckr1 expression increased significantly in the gut of not-feeding 20 dpff larvae at 3 h, and the same trend (but not significant) was also observed for feeding larvae (Figure 10). In general, no clear response pattern was observed in the gut. In yellowtail, *cck1r* increased significantly in the digestive system after feeding (Furutani et al., 2013). In addition, *in vitro* experiments using pyloric caeca organ culture showed that *cck1r* increased significantly when *Cck* was added (Furutani et al., 2013). In Siberian sturgeon (*Acipenser baerii*), it was found that C*ck* acts mainly through *Cck1r* (Zhang et al., 2017). Overall, we did not observe a clear response to feeding in *cck2r1* and *cck2r2*, and the author of this thesis is not aware of any published reports on feeding experiments that have analysed *cck2r* paralogues. However, we expected an increase in *cck2r* and *cck1r* equal to what observed in Siberia sturgeon (Zhang et al., 2017). Despite the lack of a clear response, the presence of receptors in brain and gut indicates that *cck1* and *cck2* can exert their functional role in these tissues. Future studies should aim to isolate and analyse all organs and brain parts separately to unveil possible distribution patterns and thus possible difference in function of the receptors.

4.4.2 ***npy* and *pyy***

The only significant differences for *npy* were found in the brain of not-feeding larvae at 20 dpff, where mRNA expression was significantly higher at 3 h and was also significantly higher than in feeding larvae (Figure 11). These results are in accordance with our current knowledge of

Npy acting as an orexigenic factor in teleost fish. The same trend, as in 20 dpff, can be observed for 10 dpff larvae. Our results suggest that *npy* acts as an orexigenic factor in the brain of halibut larvae at 10 and 20 dpff. The same profile has been found in zebrafish larvae (Opazo et al., 2019) and 49 dpff halibut larvae (Gomes et al., 2015), while contradictory results have been found when analysing the *npy* levels in the brain of Senegalese sole larvae (Bonacic et al., 2016). No significant differences of *npy* expression were found in the gut, suggesting that Npy may have only a minor functional role in response to feeding in this tissue.

In mammals, PYY is known to act as an inhibitor of gastric and pancreatic secretion, as well as gastric intestinal motility in the GI-tract (W.F. Colmers, 1993). In this study, both *pyy* paralogues were found in the gut, and most probably promote digestion and act as peripheral expressed anorexigenic signals in halibut, as found in mammals (Batterham et al., 2002; Lundberg et al., 1982). It was observed that the expression levels of *pyya* were generally higher in feeding compared to not-feeding larvae. *pyya* expression decreased significantly in the gut at 0.5 h in not-feeding larvae at 20dpff, while no significant changes were observed at 10 dpff (Figure 11). *pyyb* expression increased significantly in the gut of feeding larvae at 10 dpff 0.5 and 3h after feeding (Figure 11). No significant changes were found at 20 dpff. Overall, *pyya* and *pyyb* tend to be higher expressed in feeding than in not feeding larvae. Results in other teleost species are contradictory and, thus, indicate a species-specific role of *pyy* in fish. For instance, *pyyb* expression decreased in the intestine of fasted red-bellied piranha (*pyy* in article) (Volkoff, 2014) and in the anterior intestine in yellowtail (*py* in article)(Murashita et al., 2006), bot representing orexigenic responses. An anorexigenic role in grass carp has been reported as *pyyb* expression increased in the foregut after feeding (Chen et al., 2014). In Atlantic salmon, starving and feeding fish had no effect on *pyya* (*pyy* in the article) expression in the GI-tract (Murashita et al., 2009b). In 16 dph Senegalese sole larvae, *pyyb* levels increased, in response to some of the experimental diets. However, at 34 dph, no significant changes were found in *pyyb,* while *pyya* increased in the “body compartment”. Similarly, our results show that *pyyb* was the only of the two paralogues that increased significantly after feeding in the early stage (10 dpff), and no significant changes were observed at 20 dpff. Increase of *pyyb* mRNA expression after feeding have also been reported in the gut of adult grass carp (Chen et al., 2014) and Nile tilapia (Yan et al., 2017). Contradictory to the findings in Senegalese sole, halibut *pyya* did not increase after feeding (Figure 11), but decreased significantly in not-feeding larva at 0.5 h after feeding. The same response was observed for *pyyb* at 20dpff, although not significant.

In the Atlantic halibut brain, *pyya* expression decreased significantly in fed larvae at 1 and 3 h after feeding at 10 dpff, while it decreased significantly in not-feeding larvae at 0.5 h after feeding at 20 dpff (Figure 11). *pyyb* expression decreased significantly 3 h after feeding in 10 dpff larvae. Significantly decreasing *pyya* mRNA expression in 10 dpff feeding larvae indicates an orexigenic role. However, *pyya* mRNA expression levels also decreases in not-feeding larvae. We can hypothesise, however not prove, that the decrease in expression of *pyya* might be caused by other factors than feed intake, like the presence of food/attractants in the tank.

Overall, *pyyb* expression in the brain decreased after feeding, in feeding larvae, and was less expressed compared to not-feeding larvae. These results suggest an orexigenic role of *pyya* in the brain of halibut larvae. A similar functional role has been found for *pyya* in the brain of adult grass carp (Chen et al., 2013) and goldfish (*Carassius auratus*) (Gonzalez and Unniappan, 2010). However, no changes were found in salmon under feeding/starving experiment (Murashita et al., 2009b). An anorexigenic role for *pyyb* was also found when analysing whole-body larvae of Senegalese sole at 16 dph, but not when analysing body-compartment at 34 dph (Bonacic et al., 2016).

4.5 METHODICAL CONSIDERATIONS

The low intake of *A*. nauplii cyst was highly unexpected and did not represent the actual intake of prey observed in the days before sampling when *A*. nauplii was given. This raises the question if the low feed intake was due to suboptimal rearing/experimental conditions, since previously larvae have been observed to ingest inert non-food particles such as *A*. nauplii cysts and pollen from pinewood (T. Harboe 2018, pers comm.). To avoid stressing the larvae and create suitable feeding conditions for the halibut we used the best-practice systems developed at IMR over several years (T. Harboe 2018, pers comm.). This includes large tanks with a high stocking density (~5000 individuals). During these trails, larvae ingested *A*. nauplii prior and after being fed cyst. In addition, previous observations where halibut larvae that ingested inert non-food particles, such as *A*. nauplii cysts, occurred the same rearing systems as used in the present experiment. Thus, the tank conditions are most likely not the cause for the low feed intake observed.

A. nauplii cysts were administered to halibut larvae only at 10 and 20 dpff (the sampling days). Thus, halibut larvae were adapted to feed on *A*. nauplii and it is uncertain if they recognised cysts as potential prey. To test for this effect, we performed a separate experiment where a group of halibut larvae were fed *A*. nauplii cysts from the first day of feeding. In the 20 larvae

sampled for gut fullness no ingested cysts were detected. This indicates that halibut larvae might need time to accept inert particle as potential food, such as 28 dpff halibut larvae needed 5 days to completely accept and ingest the feed (Hamre et al., 2019).

One explanation for the low cyst intake may be that the cysts used in our trial, and cysts that are consumed during normal feeding behave differently in the water. Normally, *A.* nauplii preparation protocols, cysts are incubated for over 24 hours to hatch and then the *A.* nauplii are enriched. In our experimental setup the cysts were only hydrated for two hours. Consequently, the experimental cysts may have a different physical behaviour compared to the normal *A.* nauplii production protocol (+ 24 h incubation and unhatched), including a faster sinking rate, thereby becoming less available to the halibut larvae. Cysts that have been through the hatching and enrichment protocols may have different properties and give different visual and chemical cues compared to 2 h hydrated cysts, making, therefore the latter less attractive for fish larvae.

A relatively large amount of the sampled larvae still had *A.* nauplii remaining in their gut (Figure 13, A) after the last feeding before the trial (the morning of the day before sampling). One clear advantage of using *A.* nauplii cysts to measure food ingestion, rather than *A.* nauplii, is that *A.* nauplii cysts are distinguishable from the *A.* nauplii residuals from the previous feeding day (Figure 13, B).

To analyse for differences in the behavioural response of Atlantic halibut larvae to different attractant extracts, we attempted to film the larvae when the attractant extract and *A.* nauplii cysts were added to the rearing tanks (Figure 2, 0 h) using a GoPro Hero 7 Black (GoPro, San Mateo, US). The cameras recorded larvae in the tanks five minutes pre and post feeding. However, we were not able to use these records because the visibility in the water was very low due to the clay added into the system.

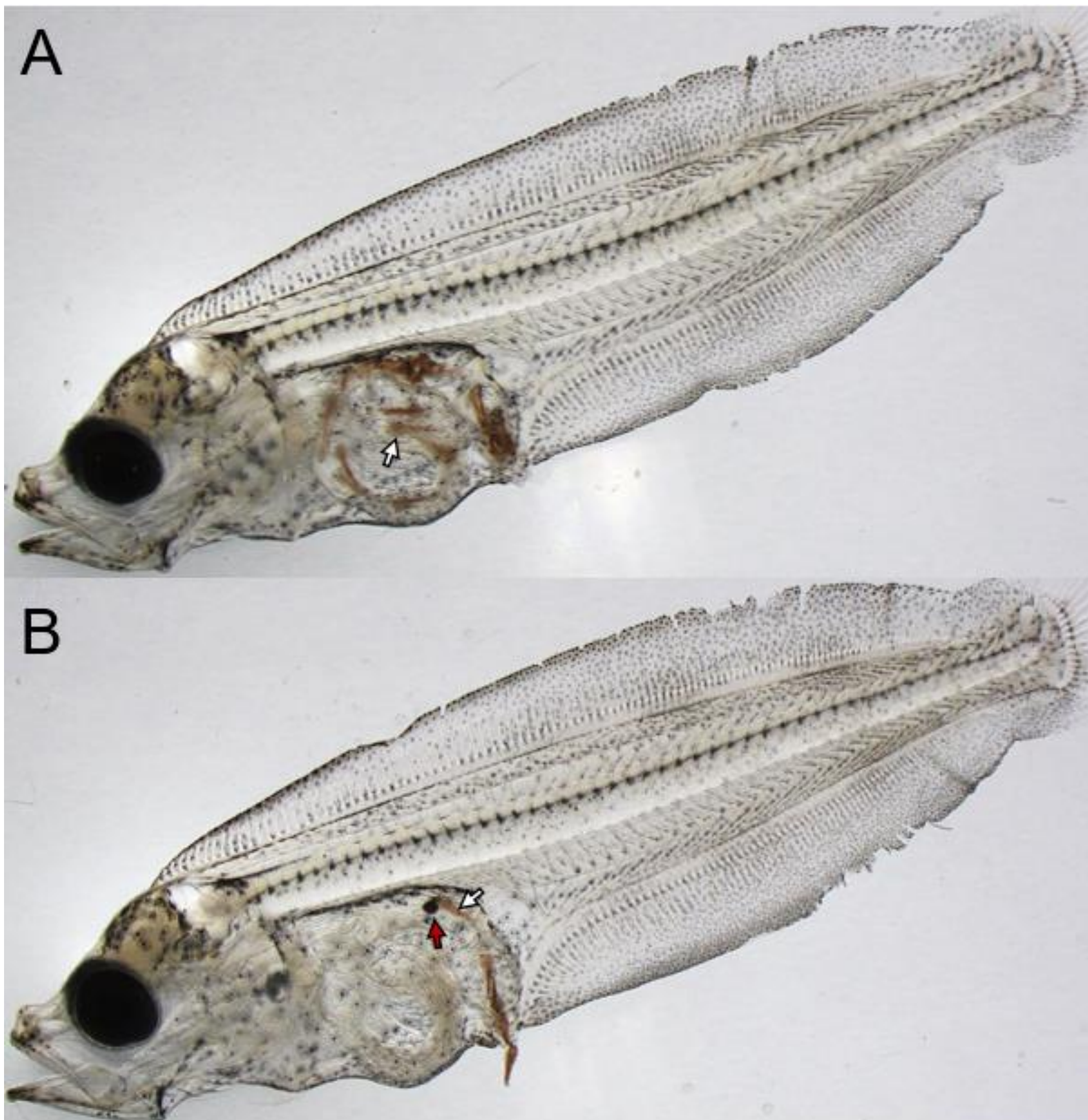


Figure 13. Halibut larva (stage 5) sampled at -0.7 h (A) and 3 h (B) with A. nauplii (elongated brown objects, see white arrow) and A. nauplii cyst (round black object, see red arrow) in the gut. Photo: Endre Lygre.

4.5.1 **Sampling**

Larvae were randomly sampled from all experimental tanks and visually inspected for possible abnormalities. If found, abnormal larvae were discarded and replaced with normal ones for the genes expression analysis. This implies that the sampling was not completely random because we selected only healthy larvae. This was done to avoid measuring the mRNA expression levels of our genes of interest in moribund larvae or small larva with low feeding rates and/or abnormalities, which would not represent the vast majority of larvae and potentially bias the analysis.

Each larvae was individually photographed at each sampling point. Even though this was time-consuming, limiting the number of larvae sampled, it has also provided additional information on the larval state for the downstream analysis. The photographs were used to determine stage/size information of the individual larvae using criteria set by Sæle et al. (2004) and gut fullness.

4.5.2 **Dissection**

Many previous studies on marine fish larvae were unable to extract and analyse isolated tissues because it is very challenging to dissect and obtain enough material from total RNA isolation. Atlantic halibut larvae have a relatively large size compared to most marine fish larvae and allow, therefore, for individual tissue isolation. This provides a well-suited model to study feed intake since it permits the visual inspection of the gut content through the transparent skin and gut tissue.

Even though we isolated the braincase and abdominal cavity in Atlantic halibut larvae, dissection can be further improved. During a pilot dissection protocol, using halibut larvae (stage 5), the gut was successfully dissected and isolated from the abdominal cavity in most cases, but there were exceptions when the GI-tract ruptured in the process of removing the skin (See protocol for Atlantic halibut larva dissection in Appendix 4). Downstream isolation of total RNA gave less material (total RNA) in samples where the gut was damaged. Due to this, and the fact we had limited number of larvae in one experimental group (feeding larvae), we standardized the protocol to include the whole braincase and whole abdominal cavity.

In this study a concentrated effort was made to dissect and isolate key tissues involved in appetite and feed intake control, as it has been for adult fish species (Murashita et al., 2009b, 2009a; Rathore et al., 2013; Yan et al., 2017). Thorough studies like those mentioned, demonstrate that gene expression vary between tissues/organs, implying that a change in expression in one tissue/organ might not be observed if the expression is "diluted" by including other tissues/organs in the analysis. Although the anatomy and development varies between teleost species, the larval gut normally consists of three distinct regions: foregut, midgut and hindgut, which develop into esophagus and stomach, anterior intestine and posterior intestine during metamorphosis (Govoni et al., 1986). The location of *cck* expression differs between the gut regions in Atlantic halibut larvae (Kamisaka et al., 2001) and *npy,* which is expressed in the brain, the eye and muscle (Gomes et al., 2015). The brain also consists of different regions and, therefore, genes involved in appetite control expression may vary between brain regions (Lai et al., 2019). Due to the challenge with isolating enough material for mRNA expression analysis

using qPCR from larval organs/tissues, *in situ* hybridization can be an additional method to visualise the precise location of the genes of interest. Large quantities of total RNA can also be obtained by pooling isolated tissue/organs from several individuals, although this option excludes the ability to account for individual variation.

4.6 SUMMARY OF HYPOTHESIS

$H0_1$ Differences in the FAA composition were found between attractant extracts from Otohime, Gemma Micro and Northern prawn, but this was not statistically tested for as $H0_2$ was confirmed.

$H0_2$ was confirmed because different attractant extracts did not affect feed intake in terms of gut fullness.

$H0_3$ was not tested as $H0_2$ was confirmed.

$H0_4$ was rejected as mRNA expression of *cck1, cck1r, cck2r1, cck2r2*, *npy, pyya* or *pyyb* did vary between brain and gut tissue.

$H0_5$ was rejected as mRNA expression of *cck1, cck2, cck1r, cck2r1, pyya* or *pyyb* did vary between 10 and 20 dpff larvae.

$H0_6$ was rejected as mRNA expression of the genes related to appetite control responded different to feed intake.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis provides novel insights about the complex interplay among feed intake, appetite and digestive processes and their regulation in fish larvae. It also highlights difficulties to wean halibut larvae to inert food particles. Based on the present study, we can conclude that:

- Attractant extracts from feed that is known to stimulate feeding behaviour in Atlantic halibut did not trigger feed intake of inert particles in Atlantic halibut larvae at 10 and 20 dpff. This was independent of the composition of free amino acids in the attractant extracts.
- Analysis of genes known to be involved in the control of appetite and digestion in fish species showed differences in expression levels throughout development. Differences were found both between tissues (gut and brain) and between different feeding groups (unfed, fed not-feeding and feeding).
- Increased mRNA expression of *cck* and *pyy* paralogues and *cck1r* in the gut from 10 to 20 dpff supports earlier observations that the digestive system and satiety signals are still not fully developed at onset of first feeding;
- All analysed genes were detected in brain and gut tissues. However, mRNA expression of *cck1, cck2r1, cck2r2, pyya,* and *npy* were higher in the brain, while *cck1r* and *pyyb* were more abundant in the gut. *cck2* was equally expressed in both tissues.
- mRNA expression of *cck1* in the brain and gut tissue, of *cck2* and *pyy* paralogues in the gut, and of *npy* in the brain responded significantly to feed particles (by feeding or not-feeding) after a short-period of feed deprivation. This suggests that these hormones are involved in the control of feed intake and digestive processes in halibut larvae.
    - *cck1* was highly expressed in the brain and acted as an orexigenic factor. Analysis of isolated brain parts would be beneficial to determine which area of the halibut brain area is involved in appetite control. *cck1* expression in the gut was higher in feeding larvae compared to unfed and not-feeding larvae, indicating a functional role in the regulation of digestive processes, and a possible secondary role as an anorexigenic factor.
    - The high mRNA expression levels of *cck2* in larvae that encountered food but failed to ingest any particles (not-feeding), may indicate a feed-forward mechanism where *cck2* is expressed ahead of feed ingestion to prepare for digestion.
    - The presence of cck receptors in the halibut larvae, particularly high levels of cck1r in the gut and cck2r in the brain, suggest that Cck can exert its functional roles in these two tissues

- Our data suggest that pyy paralogues in the gut locally promotes digestion and acts as a peripheral anorexigenic factor in halibut larvae. Future analysis of Pyy receptors would give new insights about the role of Pyy in these specific tissues (gut and brain).

REFERENCES

Alves, R.N., Gomes, A.S., Stueber, K., Tine, M., Thorne, M.A.S., Smáradóttir, H., Reinhard, R., Clark, M.S., Rønnestad, I., Power, D.M., 2016. The transcriptome of metamorphosing flatfish. BMC Genomics 17, 413. doi:10.1186/s12864-016-2699-x

Batterham, R.L., Cowley, M.A., Small, C.J., Herzogk, H., Cohen, M.A., Dakin, C.L., Wren, A.M., Brynes, A.E., Low, M.J., Ghatei, M.A., Cone, R.D., Bloom, S.R., 2002. Gut hormone PYY 3-36 physiologically inhibits food intake. Nature 418, 728–730.

Boehlert, G.W., Yoklavich, M.M., 1984. Carbon assimilation as a function of ingestion rate in larval pacific herring, *Clupea harengus pallasi* Valenciennes. J. Exp. Mar. Bio. Ecol. 79, 251–262. doi:10.1016/0022-0981(84)90199-0

Bonacic, K., Campoverde, C., Gómez-Arbonés, J., Gisbert, E., Estevez, A., Morais, S., 2016. Dietary fatty acid composition affects food intake and gut-brain satiety signaling in Senegalese sole (*Solea senegalensis*, Kaup 1858) larvae and post-larvae. Gen. Comp. Endocrinol. 228, 79–94. doi:10.1016/j.ygcen.2016.02.002

Bowman, R.E., Stillwell, C.E., Michaels, W.L., Grosslein, M.D., 2000. Food of Northwest Atlantic Fishes and Two Common Species of Squid. National Oceanin and Atmospheric Administration, Woods Hole, Massachusetts.

Chen, Y., Pandit, N.P., Fu, J., Li, D., Li, J., 2014. Identification, characterization and feeding response of peptide YYb (PYYb) gene in grass carp (*Ctenopharyngodon idellus*). Fish Physiol. Biochem. 40, 45–55. doi:10.1007/s10695-013-9822-6

Chen, Y., Shen, Y., Pandit, N.P., Fu, J., Li, D., Li, J., 2013. Molecular cloning, expression analysis, and potential food intake attenuation effect of peptide YY in grass carp (*Ctenopharyngodon idellus*). Gen. Comp. Endocrinol. 187, 66–73. doi:10.1016/j.ygcen.2013.03.029

Desjardins, P., Conklin, D., 2010. NanoDrop Microvolume Quantitation of Nucleic Acids. J. Vis. Exp. 45, e2565. doi:10.3791/2565

Dickey-Collas, M., 1993. The occurremce of juvenile pigmentation abnormalities in plaice (*Pleuronectes platessa*) larvae fed on enriched and unenriched Artemia salina nauplii. J. Fish Biol. 42, 787–795.

Døving, K.B., Selset, R., 1980. Behavior patterns in cod released by electrical stimulation of olfactory tract bundlets. Science. 207, 559–560.

Evjemo, J.O., Coutteau, P., Olsen, Y., Sorgeloos, P., 1997. The stability of docosahexaenoic acid in two *Artemia* species following enrichment and subsequent starvation. Aquaculture 155, 135–148. doi:10.1016/S0044-8486(97)00124-5

Furutani, T., Masumoto, T., Fukada, H., 2013. Molecular cloning and tissue distribution of cholecystokinin-1 receptor (CCK-1R) in yellowtail *Seriola quinqueradiata* and its response to feeding and in vitro CCK treatment. Gen. Comp. Endocrinol. 186, 1–8. doi:10.1016/j.ygcen.2013.02.003

García Hernández, M.P., Lozano, M.T., Agulleiro, B., 1994. Ontogeny of some endocrine cells of the digestive tract in sea bass (*Dicentrarchus labrax*): An immunocytochemical study. Cell Tissue Res. 277, 373–383. doi:10.1007/BF00327785

Gibbs, J., Young, R.C., Smith, G.P., 1973. Cholecystokinin decreases food intake in rats. J. Comp. Physiol. Psychol. 84, 488–495.

Gomes, Ana S., Alves, R.N., Stueber, K., Thorne, M.A.S., Smáradóttir, H., Reinhard, R., Clark, M.S., Rønnestad, I., Power, D.M., 2014a. Transcriptome of the Atlantic halibut (*Hippoglossus hippoglossus*). Mar. Genomics 18, 101–103. doi:10.1016/j.margen.2014.07.005

Gomes, A.S., Jordal, A.E.O., Olsen, K., Harboe, T., Power, D.M., Rønnestad, I., 2015. Neuroendocrine control of appetite in Atlantic halibut (*Hippoglossus hippoglossus*): Changes during metamorphosis and effects of feeding. Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol. 183, 116–125. doi:10.1016/j.cbpa.2015.01.009

Gomes, Ana S., Kamisaka, Y., Harboe, T., Power, D.M., Rønnestad, I., 2014b. Functional modifications associated with gastrointestinal tract organogenesis during metamorphosis in Atlantic halibut (*Hippoglossus hippoglossus*). BMC Dev. Biol. 14, 1–16. doi:10.1186/1471-213X-14-11

Gonzalez, R., Unniappan, S., 2010. Molecular characterization, appetite regulatory effects and feeding related changes of peptide YY in goldfish. Gen. Comp. Endocrinol. 166, 273–279. doi:10.1016/j.ygcen.2009.09.008

Govoni, J.J., Boehlert, G.W., Watanabe, Y., 1986. The physiology of fish digestion in fish larvae. Environ. Biol. Fishes 16, 59–77. doi:10.1007/BF00005160

Grimm, R.J., 1960. Feeding Behavior and Electrical Stimulation of the Brain of *Carassius auratus*. Science. 131, 162–163.

Grotkjær, T., Bentzon-Tilia, M., D'Alvise, P., Dourala, N., Nielsen, K.F., Gram, L., 2016. Isolation of TDA-producing Phaeobacter strains from sea bass larval rearing units and their probiotic effect against pathogenic *Vibrio* spp. in Artemia cultures. Syst. Appl. Microbiol. 39, 180–188. doi:10.1016/j.syapm.2016.01.005

Hamre, K., Erstad, B., Harboe, T., 2019. Early weaning of Atlantic halibut (*Hippoglossus hippoglossus*) larvae. Aquaculture 502, 268–271. doi:10.1016/j.aquaculture.2018.12.060

Hamre, K., Erstad, B., Kok, J. de, Norberg, B., Harboe, T., n.d. Changes in nutrient composition of *Artemia* grown for 3-4 days and effects of feeding on-grown *Artemia* on performance of Atlantic halibut (*Hippoglossus hippoglossus* , L.) larvae. Aquac. Nutr.

Hamre, K., Yúfera, M., Rønnestad, I., Boglione, C., Conceição, L.E.C., Izquierdo, M., 2013. Fish larval nutrition and feed formulation: Knowledge gaps and bottlenecks for advances in larval rearing. Rev. Aquac. 5, 26–58. doi:10.1111/j.1753-5131.2012.01086.x

Harada Katsuhiko, 1986. Feeding Attraction Activities of Nucleic Acid-Related Compounds. Bull. Japanese Soc. Sci. Fish.

Harboe, T., Mangor-Jensen, A., 1998. Time of first feeding of Atlantic halibut, *Hippoglossus hippoglossus* L, larvae. Aquac. Res. 29, 913–118.

Harboe, T., Mangor-Jensen, A., Naas, K.E., Næss, T., 1998. A tank design for first feeding of Atlantic halibut, *Hippoglossus hippoglossus* L., larvae. Aquac. Res. 29, 919–923.

Helvik, J. V., Walther, B.T., 1993. Development of hatchability in halibut (*Hippoglossus hippoglossus*) embryos. Int. J. Dev. Biol. 37, 487–490.

Infante, C., Matsuoka, M.P., Asensio, E., Cañavate, J.P., Reith, M., Manchado, M., 2008. Selection of housekeeping genes for gene expression studies in larvae from flatfish using real-time PCR. BMC Mol. Biol. 9, 1–12. doi:10.1186/1471-2199-9-28

Kamisaka, Y., Drivenes, Ø., Kurokawa, T., Tagawa, M., Rønnestad, I., Tanaka, M., Helvik, J.V., 2005. Cholecystokinin mRNA in Atlantic herring, *Clupea harengus* - Molecular cloning, characterization, and distribution in the digestive tract during the early life stages. Peptides 26, 385–393. doi:10.1016/j.peptides.2004.10.018

Kamisaka, Y., Fujii, Y., Yamamoto, S., Kurokawa, T., Rønnestad, I., Totland, G.K., Tagawa, M., Tanaka, M., 2003. Distribution of cholecystokinin-immunoreactive cells in the digestive tract of the larval teleost, ayu, *Plecoglossus altivelis*. Gen. Comp. Endocrinol. 134, 116–121. doi:10.1016/S0016-6480(03)00242-9

Kamisaka, Y., Kaji, T., Masuma, S., Tezuka, N., Kurokawa, T., Suzuki, T., Totland, G.K., Rønnestad, I., Tagawa, M., Tanaka, M., 2002. Ontogeny of cholecystokinin-immunoreactive cells in the digestive tract of bluefin tuna, *Thunnus thynnus* , larvae. Sarsia 87, 258–262. doi:10.1080/00364820260294888

Kamisaka, Y., Totland, G.K., Tagawa, M., Kurokawa, T., Suzuki, T., Tanaka, M., Rønnestad, I., 2001. Ontogeny of cholecystokinin-immunoreactive cells in the digestive tract of Atlantic halibut, *Hippoglossus hippoglossus*, larvae. Gen. Comp. Endocrinol. 123, 31–7. doi:10.1006/gcen.2001.7653

Kassambara, A., 2018. ggpubr: "ggplot2" Based Publication Ready Plots. R package version 0.2. https://CRAN.R-project.org/package=ggpubr.

Kolkovski, S., Arieli, a., Tandler, A., 1997. Visual and chemical cues stimulate microdiet ingestion in sea bream larvae. Aquac. Int. 5, 527–536. doi:10.1023/A:1018305416501

Kortner, T.M., Overrein, I., Øie, G., Kjørsvik, E., Bardal, T., Wold, P.-A., Arukwe, A., 2011. Molecular ontogenesis of digestive capability and associated endocrine control in Atlantic cod (*Gadus morhua*) larvae. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 160, 190–199. doi:10.1016/j.cbpa.2011.05.033

Kurokawa, T., Suzuki, T., 2002. Development of neuropeptide Y-related peptides in the digestive organs during the larval stage of Japanese flounder, *Paralichthys olivaceus*. Gen. Comp. Endocrinol. 126, 30–38. doi:10.1006/gcen.2001.7774

Kurokawa, T., Suzuki, T., Hashimoto, H., 2003. Identification of gastrin and multiple cholecystokinin genes in teleost. Peptides 24, 227–235. doi:10.1016/S0196-9781(03)00034-2

Lai, F., Kalananthan, T., Berg, G.M., Murashita, K., Gomes, A.S., Handeland, S.O., Rønnestad, I., 2019. Brain Distribution of Key Neuropeptides Involved in Appetite Control in Atlantic Salmon, *Salmo Salar* (L.). FASEB J. 33, 729.3. doi:10.1096/fasebj.2019.33.1_supplement.729.3

Lundberg, J.M., Tatemoto, K., Terenius, L., Hellström, P.M., Mutt, V., Hökfelt, T., Hamberger, B., 1982. Localization of peptide YY (PYY) in gastrointestinal endocrine cells and effects on intestinal blood flow and motility. Proc. Natl. Acad. Sci. 79, 4471–4475. doi:10.1073/pnas.79.14.4471

Mangor-Jensen, A., Harboe, T., Hennø, J.S., Troland, R., 1998. Design and operation of Atlantic halibut, *Hippoglossus hippoglossus* L., egg incubators. Aquac. Res. 29, 887–892.

Mangor-Jensen, A., Waiwood, K.G., 1995. The effect of light exposure on halibut eggs. J. Fish Biol. 47, 18–25.

Meyer, A., Van de Peer, Y., 2005. From 2R to 3R: Evidence for a fish-specific genome duplication (FSGD). BioEssays 27, 937–945. doi:10.1002/bies.20293

Micale, V., Campo, S., D'Ascola, A., Guerrera, M.C., Levanti, M.B., Germanà, A., Muglia, U., 2012. Cholecystokinin in White Sea Bream: Molecular Cloning, Regional Expression, and Immunohistochemical Localization in the Gut after Feeding and Fasting. PLoS One 7. doi:10.1371/journal.pone.0052428

Moguel-Hernández, I., Peña, R., Andree, K.B., Tovar-Ramirez, D., Bonacic, K., Dumas, S., Gisbert, E., 2016. Ontogeny changes and weaning effects in gene expression patterns of digestive enzymes and regulatory digestive factors in spotted rose snapper (*Lutjanus guttatus*) larvae. Fish Physiol. Biochem. 42, 1319–1334. doi:10.1007/s10695-016-0220-8

Murashita, K., Fukada, H., Hosokawa, H., Masumoto, T., 2006. Cholecystokinin and peptide Y in yellowtail (*Seriola quinqueradiata*): Molecular cloning, real-time quantitative RT-PCR, and response to feeding and fasting. Gen. Comp. Endocrinol. 145, 287–297. doi:10.1016/j.ygcen.2005.09.008

Murashita, K., Kurokawa, T., Ebbesson, L.O.E., Stefansson, S.O., Rønnestad, I., 2009a. Characterization, tissue distribution, and regulation of agouti-related protein (AgRP), cocaine- and amphetamine-regulated transcript (CART) and neuropeptide Y (NPY) in Atlantic salmon (*Salmo salar*). Gen. Comp. Endocrinol. 162, 160–171. doi:10.1016/j.ygcen.2009.03.015

Murashita, K., Kurokawa, T., Nilsen, T.O., Rønnestad, I., 2009b. Ghrelin, cholecystokinin, and peptide YY in Atlantic salmon (*Salmo salar*): Molecular cloning and tissue expression. Gen. Comp. Endocrinol. 160, 223–235. doi:10.1016/j.ygcen.2008.11.024

Næss, T., Germain-henrybt, M., Naasa, K.E., 1995. First feeding of Atlantic halibut (*Hippoglossus hippoglossus*) using different combinations of *Artemia* and wild zooplankton. Aquaculture 130, 235–250.

Nicolas, J.L., Robic, E., Ansquer, D., 1989. Bacterial Flora Associated with a Trophic Chain Consisting of Microalgae, Rotifers and Turbot Larvae: Influence of Bacteria on Larval Survival. Aquaculture 83, 237–248.

Opazo, R., Plaza-Parrochia, F., Cardoso dos Santos, G.R., Carneiro, G.R.A., Sardela, V.F., Romero, J., Valladares, L., 2019. Fasting Upregulates *npy*, *agrp*, and *ghsr* Without Increasing Ghrelin Levels in Zebrafish (*Danio rerio*) Larvae. Front. Physiol. 9, 1–13. doi:10.3389/fphys.2018.01901

Pavlov, D.S., Kasumyan, A.O., 1990. Sensory principles of the feeding behavior of fishes. J. Ichthyol. 30, 720–732.

Ping, H.C., Feng, K., Zhang, G.R., Wei, K.J., Zou, G.W., Wang, W.M., 2014. Ontogeny expression of ghrelin, neuropeptide Y and cholecystokinin in blunt snout bream, *Megalobrama amblycephala*. J. Anim. Physiol. Anim. Nutr. (Berl). 98, 338–346. doi:10.1111/jpn.12084

Pittman, K., Berg, L., Naas, K., 1987. Morphological development of halibut (*hippoglossus hippoglossus*) larvae with special reference to mouth development and metamorphosis. Int. Counc. Explor. oj Sea 22.

Pittman, K., Skiftesvik, A.B., Bergs, L., 1990. Morphological and behavioural development of halibut , *Hippoglossus hippoglossus* (L .) larvae. J. Fish Biol. 37, 455–472.

Rathore, R.M., Angotzi, A.R., Jordal, A.E.O., Rønnestad, I., 2013. Cholecystokinin receptors in atlantic salmon: Molecular cloning, gene expression, and structural basis. Physiol. Rep. 1, 1–14. doi:10.1002/phy2.69

Raybould, H.E., 2007. Mechanisms of CCK signaling from gut to brain. Curr. Opin. Pharmacol. 7, 570–574. doi:10.1016/j.coph.2007.09.006

Rojas-García, C.R., Rønnestad, I., 2002. Cholecystokinin and tryptic activity in the gut and body of developing Atlantic halibut larvae: Evidence for participation in the regulation of protein digestion. J. Fish Biol. 61, 973–986. doi:10.1006/jfbi.2002.2113

Rønnestad, I., Gomes, A.S., Murashita, K., Angotzi, R., Jönsson, E., Volkoff, H., 2017. Appetite-controlling endocrine systems in teleosts. Front. Endocrinol. (Lausanne). 8, 1–24. doi:10.3389/fendo.2017.00073

Rønnestad, I., Yúfera, M., Ueberschär, B., Ribeiro, L., Sæle, Ø., Boglione, C., 2013. Feeding behaviour and digestive physiology in larval fish: Current knowledge, and gaps and bottlenecks in research. Rev. Aquac. 5, S59–S98. doi:10.1111/raq.12010

Sæle, Solbakken, J.S., Watanabe, K., Hamre, K., Power, D., Pittman, K., 2004. Staging of Atlantic halibut (*Hippoglossus hippoglossus* L.) from first feeding through metamorphosis, including cranial ossification independent of eye migration. Aquaculture 239, 445–465. doi:10.1016/j.aquaculture.2004.05.025

Schwartz, M.W., Woods, S.C., Porte, D., Seeley, R.J., Baskin, D.G., 2000. Central nervous system control of food intake. Nature 404, 661–671.

Sundström, G., Larsson, T.A., Brenner, S., Venkatesh, B., Larhammar, D., 2008. Evolution of the neuropeptide Y family: New genes by chromosome duplications in early vertebrates and in teleost fishes. Gen. Comp. Endocrinol. 155, 705–716. doi:10.1016/j.ygcen.2007.08.016

Sundström, G., Larsson, T.A., Xu, B., Heldin, J., Larhammar, D., 2013. Interactions of zebrafish peptide YYb with the neuropeptide Y-family receptors Y4, Y7, Y8a, and Y8b. Front. Neurosci. 7, 1–8. doi:10.3389/fnins.2013.00029

Tinoco, A.B., Valenciano, A.I., Gómez-Boronat, M., Blanco, A.M., Nisembaum, L.G., De Pedro, N., Delgado, M.J., 2015. Two cholecystokinin receptor subtypes are identified in goldfish, being the CCKAR involved in the regulation of intestinal motility. Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol. 187, 193–201. doi:10.1016/j.cbpa.2015.05.027

Valen, R., Jordal, A.E.O., Murashita, K., Rønnestad, I., 2011. Postprandial effects on appetite-related neuropeptide expression in the brain of Atlantic salmon, *Salmo salar*. Gen. Comp. Endocrinol. 171, 359–366. doi:10.1016/j.ygcen.2011.02.027

Valentinčič, T., 2005. Olfactory Discrimination in Fishes, in: Reutter, K., Kapoor, B.G. (Eds.), Fish Chemosenses. Science Publishers, Inc, Enfield, NH, pp. 65–85.

Volkoff, H., 2015. Cloning, tissue distribution and effects of fasting on mRNA expression levels of leptin and ghrelin in red-bellied piranha (*Pygocentrus nattereri*). Gen. Comp. Endocrinol. 217–218, 20–27. doi:10.1016/j.ygcen.2015.05.004

Volkoff, H., 2014. Appetite regulating peptides in red-bellied piranha, *Pygocentrus nattereri*: Cloning, tissue distribution and effect of fasting on mRNA expression levels. Peptides 56, 116–124. doi:10.1016/j.peptides.2014.03.022

W.F. Colmers, C.W. (Ed.), 1993. PP, PYY and NPY. Occurrence and distribution in the periphery, in: The Biology of Neuropeptide Y and Related Peptides. Humana Press, Totowa, NJ, pp. 96–157.

Watanabe, T., 1993. Importance of Docosahexaenoic Acid in Marine Larval Fish. J. World Aquac. Soc. 24, 152–161.

Werner, R.G., Blaxter, J.H.S., 1980. Growth and Survival of Larval Herring (*Clupea harengus*) in relation to prey density. Can. J. Fish. Aquat. Sci. 37, 1063–1069.

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

Wickham, H., François, R., Henry, L., Müller, K., 2018. dplyr: A Grammar of Data Manipulation. R package version 0.7.6. https://cran.r-project.org/package=dplyr.

Yacoob, S.Y., Browman, H.I., 2007a. Prey extracts evoke swimming behavior in juvenile Atlantic halibut (*Hippoglossus hippoglossus*). Aquaculture 270, 570–573. doi:10.1016/j.aquaculture.2007.05.003

Yacoob, S.Y., Browman, H.I., 2007b. Olfactory and gustatory sensitivity to some feed-related chemicals in the Atlantic halibut (*Hippoglossus hippoglossus*). Aquaculture 263, 303–309. doi:10.1016/j.aquaculture.2006.11.005

Yan, P., Jia, J., Yang, G., Wang, D., Sun, C., Li, W., 2017. Duplication of neuropeptide Y and peptide YY in Nile tilapia *Oreochromis niloticus* and their roles in food intake regulation. Peptides 88, 97–105. doi:10.1016/j.peptides.2016.12.010

Zhang, X., Tang, N., Qi, J., Wang, S., Hao, J., Wu, Y., Chen, H., Tian, Z., Wang, B., Chen, D., Li, Z., 2017. CCK reduces the food intake mainly through CCK1R in Siberian sturgeon (*Acipenser baerii* Brandt). Sci. Rep. 7, 12413. doi:10.1038/s41598-017-12646-3

APPENDIX 1 – NINHYDRIN DETECTION

NINHYDRIN DETECTION RESULTS

Table 4. Analysis of soluble compounds in feed extracts. Otohime and shrimp results are given as µmol aa/100 mL plasma, while Gemma results are given as µmol aa/50 mL plasma. Results are given as the mean of duplicates.

NAME	OTOHIME	GEMMA	SHRIMP
L-Methionine	11.5	38.1	7.6
L-Glutamine	12.1	5.2	37.2
L-Serine	36.1	48.2	27.2
L-Valine	50.8	93.4	13.8
L-Alanine	218.6	193.7	87.8
L-Glutamic acid	66.6	79.0	14.2
Arginine	187.7	121.6	354.6
L-Leucine	67.0	142.2	12.1
L-Threonine	35.9	62.4	6.0
L-Tyrosine	20.7	52.5	5.7
L-glycine	209.0	185.0	2039.3
L-Lysine	55.0	117.5	12.2
L-Cystine	3.4	1.9	0.0
L-Isoleucine	34.0	57.8	7.1
L-Histidine	69.0	22.3	3.9
L-Phenylalanine	26.4	60.4	4.4
L-Proline	345.3	66.3	284.7
L-Aspartic acid	27.3	30.2	4.0
O-Phospho-L-serine	23.1	11.6	0.0
Taurine	476.6	1164.2	152.1
O-Phosphoethanolamine	2.6	6.0	0.0
Urea	74.0	79.0	102.5
L-Asparagine	23.1	25.0	9.1
L-Sarcosine	224.5	35.0	59.5
L-Citrulline	9.2	11.1	0.0
L-alfa-Amino-n-butyric Acid	2.9	6.0	0.0
Cystathionin1	0.0	2.3	0.0
Cystathionin2	1.7	5.2	0.0
β-Alanine	197.6	20.5	0.0
Gamma-Amino-n-butyric Acid	24.3	6.5	0.0
Ethanolamine	8.9	16.4	4.4
Ammonium chloride	290.8	287.9	47.7
Hydroxylysin2	0.0	4.4	0.0
L-Ornithine	17.6	11.5	31.8
1-Methyl-L-histidine	0.0	8.9	0.0
L-Tryptophan	0.0	6.6	0.0
Anserine	6.3	9.4	0.0

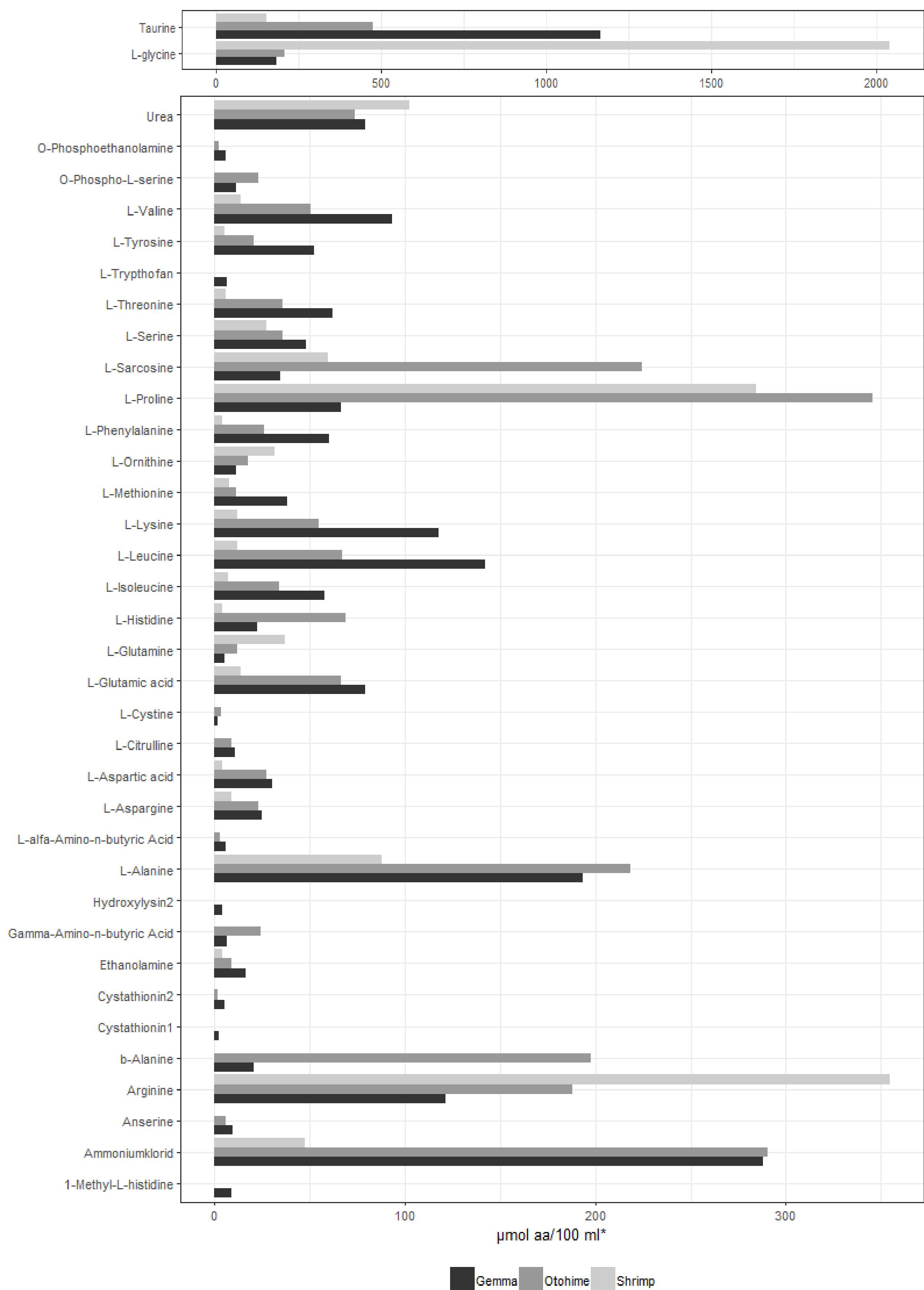


*Figure 14. Water soluble compounds in Gemma, Otohime and Shrimp extracts. *Values from Gemma extract are given as µmol aa/50 ml.*

NINHYDRIN DETECTION PROTOCOL

246 - Fysiologisk aminosyrebestemmelse i plasma, Ninhydrindeteksjon

Ref.id.: MET.NÆR.01-29 Metodebeskrivelse *Side 1 av 6*

1. ANVENDELSESOMRÅDE

Metoden kan brukes til å bestemme fysiologiske aminosyrer i plasma. Kan også brukes til å bestemme fysiologiske aminosyrer i andre flytende prøver som øyekammervæske og urin.

2. PRINSIPP

- Proteinet fjernes v.h.a felling med sulfosalisylsyre.
- Prøvene sentrifugeres og filtreres.
- Innhold av fysiologiske aminosyrer bestemmes v.h.a. ionebyttig - ninhydrin deteksjon.

3. SIKKERHET

Ved tillaging av ninhydrin er det viktig å stå i avtrekk og bruke hansker.

Ved analyse av smitteførende prøver (humane prøver) følges egen sikkerhetsprosedyre. HMS.13.INS-02; Instruks for oppbevaring og håndtering av humane prøver. Prøvene regnes ikke lenger som smitteførende etter behandling med 5% sulfosalisylsyre (8.1). Alle prosjekter blir risikovurdert og analytiker skal sette seg inn i risikovurderingen før analysene startes. HMS.13.ARB-02; Risikovurdering av humane prøver

4. KJEMIKALIER

4.1 Norleucin, DL, Sigma, art. nr N-1398
Sulfosalicylsyre ($C_7H_6O_6S \cdot 2H_2O$) Riedel-deHaën, art.nr 33619
4.2 Saltsyre, 37 % (HCl) Merck, art. nr 1.00317
4.3 Fysiologiske aminosyrer standard A/N, Sigma, art. nr A-6407
Fysiologiske aminosyrer standard B, Sigma, art. nr A-6282
Glutamin, Sigma, art. nr G-3126
4.4 Lithium buffer A, Biochrom, art.nr. 80-2038-15
Lithium buffer B, Biochrom, art.nr. 80-2038-16
Lithium buffer C II, Biochrom, art.nr. 80-2099-83
Lithium buffer D II, Biochrom, art.nr. 80-2097-18
Lithium hydroxide F, Biochrom, art.nr. 80-2038-20
Lithium buffer pH3,55, Biochrom, art.nr. 80-2037-69
Lithium Citrate loading buffer pH 2,2, Biochrom, art.nr. 80-2038-10
4.5 Ultrasolve, Biochrom, art.nr. 80-2110-75
Ninhydrin solution, Biochrom, art.nr. 80-2110-76
4.6 MilliQ-vann
4.7 Is

5. LØSNINGER OG STANDARDER

5.1 Intern standard (1,0 mM Nor) i 10 % Sulfosalicylsyre
Vei inn nøyaktig 0,1312 g Norleucin (4.1). Overfør til 1000 ml målekolbe. Vei inn

Offisiell utskrift
Dok.id: D00286 Versjon: 3.02 Forfatter: ABI Godkjent av: ABO Sist endret: 01.02.2015

100 g Sulfosalicylsyre (4.1) i et begerglass og tilsett litt vann. Overfør fra begerglasset til målekolben og fortynn til merket med vann.
Lagres på blåkorkflaske i kjøleskap. Holdbar 2 år.

5.2 Saltsyre, 6 M
Tilsett 500 ml 37 % Saltsyre (4.2) til vann i 1000 ml målekolbe. Fyll til merket med vann når blandingen er nedkjølt.

5.3 Ekstern standard I (2.5 mM Nor)
Vei inn nøyaktig 0,3280 g Norleucin (4.1). Overfør til 1000 ml målekolbe og løs i 17 ml 6 M Saltsyre (5.2). Fortynn til merket med vann.
Lagres i kjøleskap. Holdbar 2 år.

5.4 2.5 mM glutamin.
Vei inn nøyaktig 0.0365 g Glutamin (4.3). Overfør til 100 ml målekolbe og fortynn til merket med vann.
Lagres i små porsjoner ved -20°C inntil 1 år.

5.5 Arbeidsstandard, 0.625 mM
Pipetter ut nøyaktig og overfør til 4 ml prøveglass 500 µl standard A/N (4.3), 500 µl standard B (4.3), 500 µl ekstern standard (5.3) og 500 µl Loading buffer (4.4). Rør godt på whirlmixer. Lagre ved -20° C inntil 1 år.

5.6 Standard ,0.5 mM.
Pipetter ut nøyaktig 200 µl arbeidsstandard (5.5). Tilsett 50 µl glutamin (5.4).
Rør godt på whirlmixer.
Bruk blandingen samme dag, da glutamin er ustabil i surt miljø.

5.7 Ninhydrinreagens.
Sett Ninhydrin solution (4.5) i ultralydbad i 10 min.
Overfør Ultrasolve (4.5) til en 2 liter lysfiltrert blåkorkflaske (La noen ml være igjen til skylling av Ninhydrin solution) og sett på røring tilført Nitrogen i 10 min.
Overfør den sonikerte Ninhydrin solutionen til Ultrasolven (skyll flasken med rest Ultrasolve) og forsett røring med Nitrogen i maks 10 min.

6. INSTRUMENT OG UTSTYR

6.1 Glassvarer:
Veieskip
Flasker 1000 ml (blåkork)
Reservoarflasker 2000 ml, (Blåkork)
Reservoarflasker 1000 ml, (Blåkork)
Målesylindre: 250 - 500 - 1000 ml
Målekolber: 100 - 1000 - 2000 ml
Chromacol prøveglass: 2SV uten insert
Chromacol prøveglass: 2SV med insert

Urglass
6.2 Eppendorfrør, 1.5 ml
6.3 Finnpipetter:20-200µl, 200µl, 200-1000µl,1000-5000µl
6.4 Pipettetipper: 200-1000-5000 µl finntipper
6.5 Korker:
Korker til Chromasol prøveglass uten insert: 8mm 8-SC-8RT1
Korker til Chromasol prøveglass med insert: 9mm 9-SC-8RT1
6.6 Engangssprøyter: 1ml, 10 ml
6.8 Filter:
Filter: Sprøytefilter 4mm-0.22µm, Millex-GV.
6.9 Analysevekt, 4desimaler
6.10 Sentrifuger:
Wifug 50
Eppendorf Centrifuge 541
6.11 pH - meter, Scott pH Meter CG84
6.12 Whirlmikser
6.13 Biochrom 20 Plus
Kolonne, Biochrom Physological column 200 mm art.nr. 80-2038-20
Midas cooltray-injektor, Spark
BusSAT/IN, Waters
Integreringssystem, Empower 3
6.14 Gass: Nitrogen

7. EKSPERIMENTELT

Flow: 25 ml/h for buffer, 20 ml/h for Ninhydrin
Bølgelengde: 570 nm for Channel 1, 440 nm for Channel 2
Injeksjonsvolum: 20 µl
Midas karusell: 8 °C

Viser ellers til "Short form operation instruktion" for start og stopp av instrumentet.

Instrumentvedlikehold:
Månedlig: Demontere og rense bufferpumpe, ninhydrinpumpe og flowcelle.
Årlig: Skifte Piston seals, coilflush diaphagm, filter catridge for buffer og ninhydrinpumpe og checkvalves. Rense og skifte rotorseal på Midas autosampler.

8. PROSEDYRE

8.1 Opparbeiding
Ved all opparbeiding av fysiologiske aminosyrer skal det jobbes på is. Bruk hansker.
Bland 500 µl plasma og 500 µl sulfosalicylsyre m/nor (5.1) i eppendorfrør.

Bland på whirlmixer. La stå i romtemperatur i 1 time. Sentrifuger i 30 min v/8000 rpm i eppendorfsentrifugen. Overfør supernatant til nye eppendorfrør. Lagre v/ - 20°C fram til kjøring.

8.2 Analyse:
Kjør prøvene på Biochrom-system som styres av Empower.
Kjør med ferdige buffere fra Biochrom. Ninhydrin må lages.
Injiser 20 µl standard og prøve.
Hold karusellen nedkjølt på 8°C
Hent inn og beregn signalene v.h.a. Empower.

9. VALIDERINGSPARAMETERE
Vi har ikke utført full validering på denne metoden. Biochrom som har lang erfaring med analyser av aminosyrer har levert instrumentet og metodeapplikasjonene, og vi har derfor tatt utgangspunkt i deres validering. Vi benytter de samme standarene som Biochrom.

9.1 Selektivitet: Ved innkjøring av instrumentet ble det analysert 6 blankprøver. Disse viste ingen interferende topper unntagen for cystein som får en skulder ved toppen under analyse. Denne skulderen fjernes ved integrering.

9.2 Linearitet: Det er også utført en linearitetsbestemmelse for metoden med godkjent resultat.

9.3 Deteksjonsgrense/Kvantifiseringsgrense:

Deteksjonsgrense (Biochrom): 15 pmol

Øvre grense (Biochrom): 25 nmol

9.4 Riktighet

9.5 Presisjon

9.6 Måleområde

9.7 Måleusikkerhet

9.8 Robusthet

10. KVALITETSKONTROLL

11. BEREGNINGER
Benytt intern standard metode.
Legg inn konsentrasjon av alle komponentene i standard (0,5mM).

Konsentrasjon av intern standard i prøve skal være 50 µmol/100 ml.
(Intern std.verdi 50 µmol/100 ml = 0,5 mM)
Dilution legges inn som 2,0 ml.
Vekt legges inn som 1 ml.

Resultatene beregnes av Empower på følgende måte:

Arealene under toppene måles både for standard og prøver. Responsen korrigeres utfra respons for intern standard.

$$R = \frac{\text{Areal (aa)}}{\text{Areal (is)}} * C(is)$$

Programmet beregner så en standardkurve for hver aminosyre vha. lineær regresjon.
y = ax+b
der
b=0, kurven tvinges gjennno,origo.
y = R
x = C2
a = responsfaktor for den enkelte komponent i standard.

For å beregne innholdet av aminosyrer i prøven brukes følgende formel:

C1 =(C2 x V)/w

R = Korrigert respons for aa, mM
Areal (aa) = Respons for aa (areal i AU)
Areal (is)= Respons for intern standard, (areal i AU)
C(is) = Konsentrasjon av intern standard, mM
C1 = konsentrasjon i utgangsmaterialet, mmol/100 ml
C2 = konsentrasjon i injisert prøve, mM
V = Fortynning, ml
w = innveid mengde, ml

12. RAPPORTERING

Resultatene av prøvene skal oppgis som µmol/100 ml.

13. BEMERKNINGER

Hydroksyprolin og prolin detekteres ved bølgelengde 440 nm på Channel 2 og må beregnes etter egen metode.

14. LIMS

Legge til metoder (analyser)
Se brukerveileding *LIMS.BRUK-02* Prøveflyt for oversikt over hvordan metoder (analyser) legges på prøver.

Legge inn resultat
Se brukerveileding *LIMS.BRUK-02* Prøveflyt, manuell metode.

I Excel-filen må det være komma, ikke punktum. For de verdiene som er angitt med < skal det ikke være mellomrom mellom tegnet og tallet. For å ordne dette markerer du ønsket område (i Excel), velg Rediger, Erstatt. I feltet Søk etter: skriver du . eller < (med ett mellomrom bak) og i feltet Erstatt med: , eller < (uten mellomrom).

Ved overføring av flere resultat fra excel-mal: Marker ønskede resultat og kopier (Ctr+C). Lim inn (Ctr + V) resultatene i aktuelle journalnummer i LIMS Journalnummerfolder i Result Entry by Sample (Parametere nedover og journalnummer bortover) eller i Grid REselected Tests (Journalnummer nedover og parametere bortover).

Nærmere beskrivelse av felt som brukes til resultatene:
Metoden er satt opp med ett felt; gjennomsnitt.

Gjennomsnitt (rapporteres på analysebevis):
Legg inn verdi

15 REFERANSER

15.1	Instrumentreferanse; methode	Biochrom 20 plus, Amino analyser ninhydrine
15.2	Metodereferanse;	Biochrom methode AAAFAQ8

APPENDIX 2 – METHODS TESTING

Our initial work plan consisted of isolating the total RNA using TRI-reagent, and then use SuperScript IV VILO Master Mix (VILO) to remove possible genomic DNA contamination and synthesise cDNA. However, challenges started to arise, since first, we did not obtain enough (at least not for all samples) total RNA from the brain tissue (~0.4 mg) that would be necessary for our downstream analysis (Table 5, Figure 15). In addition, qPCR with RNA isolated with TRI-reagent, and cDNA synthesis with VILO (which includes DNase treatment) showed amplification in the NRT (Figure 15, B), meaning that the DNase treatment was insufficient to completely remove all genomic DNA contamination. As no product was amplified on the NTC (Figure 15, B), this excluded completely the hypothesis of primer dimer. Therefore, we attempted to use MACHEREY-NAGEL NucleoSpin RNA XS to isolate total RNA, since it could isolate and concentrate total RNA of high integrity from samples smaller than 5 mg. In addition, NucleoSpin RNA XS included on column DNase treatment, which means we would not need to do an extra step for this procedure. However, the total RNA concentration obtained from NucleoSpin RNA XS was much lower than TRI-reagent (Figure 12) and the 260/230 ratio ranged from 0.06-0.46 showing that the RNA purity was low (Table 5). As a third alternative, we tested QIAGEN RNeasy Mini Kit to isolate total RNA, which also included DNase treatment. The total RNA concentration yield was higher when using RNeasy than obtained using Tri Reagent and NucleoSpin (Figure 12), and the 260/280 and 260/230 ratios was above 2.0 and 2.2 respectively for most samples (Table 5).

After we had decided for the best method to isolate the total RNA from both gut and brain tissues of halibut larvae, we wanted to test if using VILO was an efficient method for cDNA synthesis. Therefore, we tested our cDNA by qPCR using specific primers to amplify *ef1a* (Figure 16, A). However, our results were not satisfactory, since a product was also amplified in the NRT (Figure 16, B). It was, clear at this point that we had genomic contamination in our samples, so we decided to introduce an extra step to remove it by using Ambion DNA-free as DNase treatment method. We used DNase treated total RNA isolated with QIAGEN kit (selected method, which includes DNase treatment), and an extra DNase treatment with the Ambion kit and cDNA synthesis with SuperScript III. Using this method, we successfully removed any genomic contamination from our samples, as we could observe from our NRT melt curve analysis (Figure 17, B). This qPCR plate also included VILO treated RNA, where products were quantified on the NRT (Figure 17, A). As a result of this we decided to isolate

total RNA with RNeasy, treat with DNase with Ambion and synthesize cDNA with SuperScript III. In addition, primers were always designed that at least one of the primers is spanning an exon-exon junction to reduce as much as possible the amplification of genomic DNA.

Table 5. Total RNA concentration (ng/µL) and purity (A260/280 and A260/230) using Tri Reagent, MACHEREY-NAGEL NucleoSpin RNA XS with DNase treatment, and QIAGEN RNeasy Mini Kit with DNase treatment. RNA was isolated from gut and brain tissue from larvae at stage 5 (Sæle et al., 2004).

Method	Tissue	Sample	Concentration (ng/µL)	260/280	260/230
Tri Reagent	Gut	1	461.3	1.95	1.47
		2	425.3	1.92	1.38
		3	489.6	1.93	1.81
		4	504.9	1.94	1.61
		5	557.3	1.97	1.14
		6	564.6	1.95	1.37
		7	163.1	1.61	0.47
		8	405.2	1.64	1.41
		9	375.8	1.84	2.11
		10	545.7	1.91	1.10
		11	520.9	1.92	1.97
		12	456.9	1.92	1.97
	Brain	1	447.6	1.89	1.46
		2	217.1	1.90	0.98
		3	227.1	1.85	1.20
		4	159.6	1.85	1.01
		5	239.3	1.83	1.8
		6	288.0	1.88	1.55
		7	186.9	1.51	1.67
		8	281.1	1.63	0.66
		9	222.3	1.74	1.68
		10	194.9	1.72	1.47
NucleoSpin	Gut	13	406.0	2.16	0.46
		14	193.0	2.16	0.18
	Brain	13	116.0	2.15	0.16
		14	103.6	2.34	0.23
QIAGEN	Gut	15	344.2	2.11	2.29
		16	695.8	2.11	2.31
		17	769.9	2.10	2.21
		18	284.8	2.13	0.41
	Brain	15	293.9	2.08	2.28
		16	372.9	2.09	2.32
		17	483.4	2.17	2.11
		18	252.4	2.11	2.33

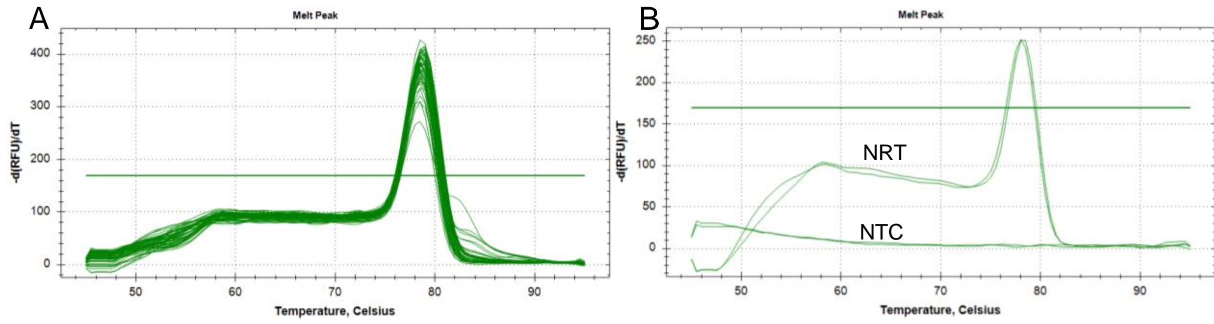


Figure 15. qPCR melt curve using tissue from gut and brain using VILO as DNase treatment and cDNA synthesis. samples of gut and brain (A), NTC and NRT (B). Primer: ef1a.

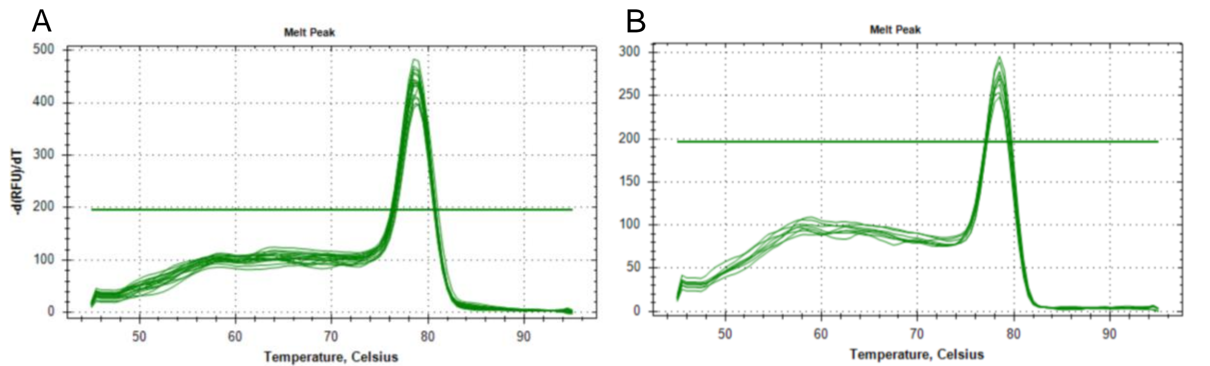


Figure 16. qPCR melt curve using tissue from gut and brain showing samples (A) and NRT (B). RNA was isolated, and DNase treated with QIAGEN followed by DNase and cDNA synthesis with VILO. Primer: ef1a.

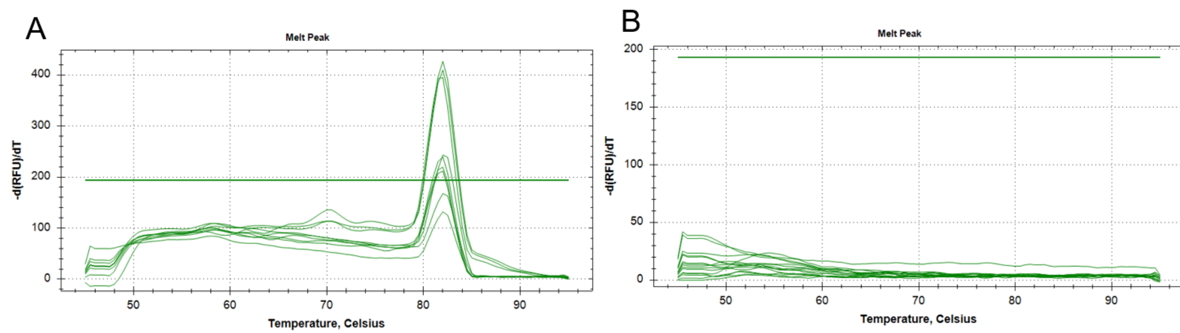


Figure 17. qPCR melt curve using gut tissue, showing NRT VILO (A) and NRT Ambion DNA-free (B). RNA was isolated, and DNase treated with QIAGEN. Primer: ef1a.

APPENDIX 3 – RIN VALUES

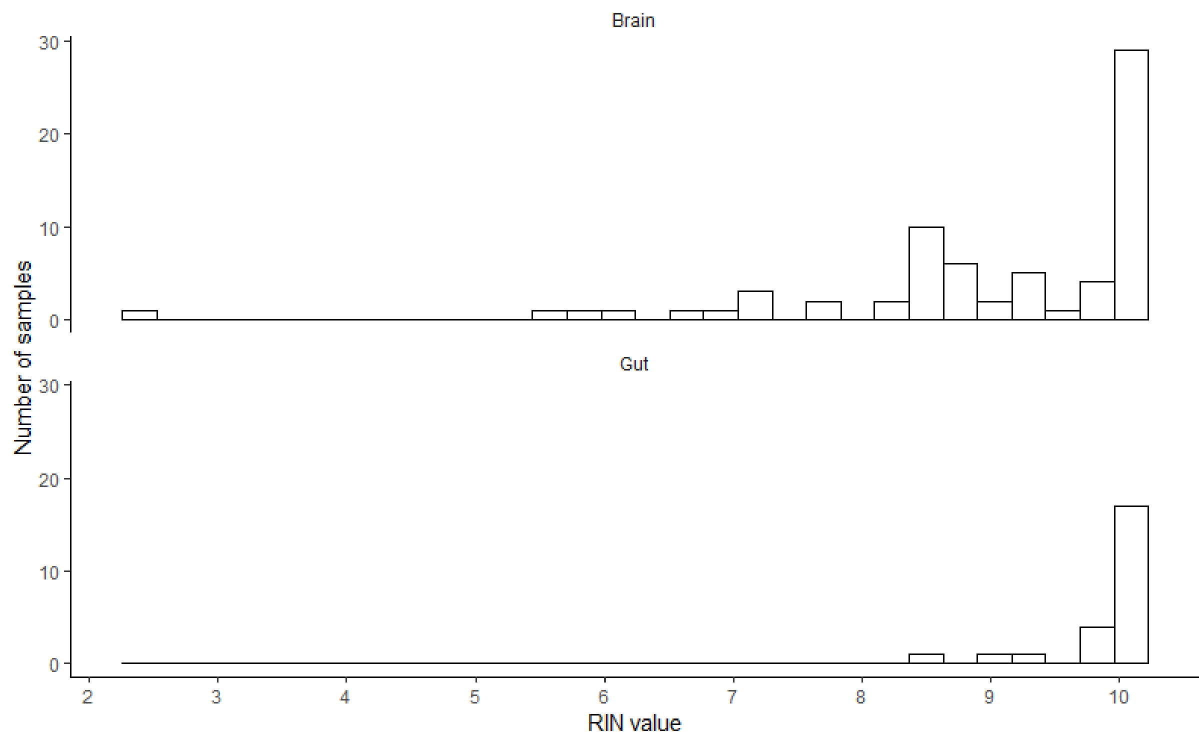
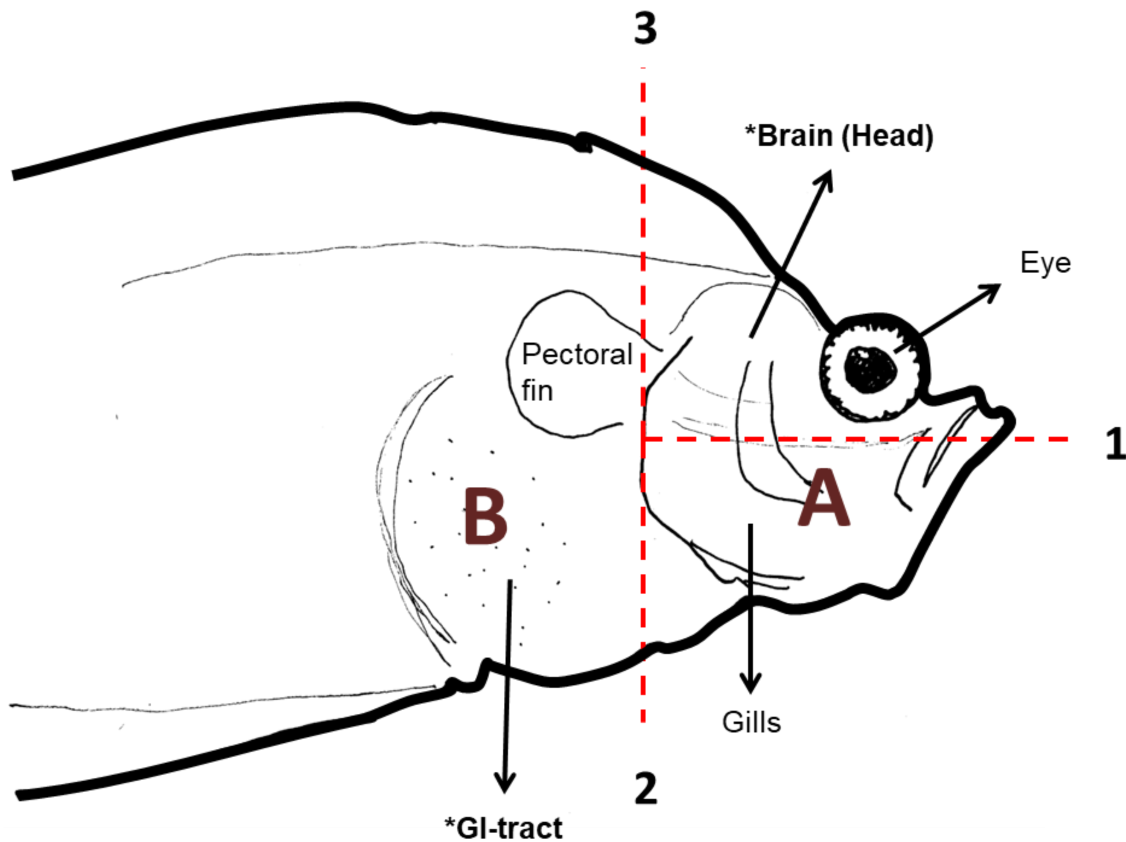


Figure 18. Histogram of RIN (RNA integrity) values in brain (n=72) and gut (n=24)

APPENDIX 4 – ATLANTIC HALIBUT LARVA DISSECTION

1. *Defrost the larvae on ice*
2. *Put the larvae in a petri-dish and keeping it submersed in RNAlater*
3. *Stabilize the larvae with a needle*
4. *Remove the eyes*
5. *Remove the side of the operculum to see the top of the gills*
6. *Make a horizontal cut below the eye socket: start from the pectoral fin start/edge of the operculum (cut 1)*
7. *Make a transversal cut (cut 2) to remove the gills and the lower jaw*
8. *Remove the pectoral fins*
9. *Peel off the skin of the abdominal wall (area B).*
10. *Remove the GI-tract by cutting through the oesophagus and at the anus*
11. *Make a transversal cut (cut 3) to free the area containing the brain*



Designed by Prof. Harald Kryvi and modified by Ana S. Gomes

APPENDIX 5 – STATISTICAL TESTS

SHAPIRO-WILK NORMALITY TEST

Table 6. Shapiro-Wilk normality test results for normal distribution analysis. Data are presented in p-values.

Dataset	W	P-value
Gut_10d_cck1	0.94128	0.055670
Gut_20d_cck1	0.91150	0.007147
Brain_10d_cck1	0.93748	0.051900
Brain_20d_cck1	0.93147	0.030940
Gut_10d_cck2	0.97648	0.625900
Gut_20d_cck2	0.92392	0.016430
Brain_10d_cck2	0.91596	0.012440
Brain_20d_cck2	0.96895	0.415100
Gut_10d_cck2	0.95937	0.205500
Gut_20d_cck2	0.93883	0.046730
Brain_10d_cck2	0.93969	0.060370
Brain_20d_cck2	0.58324	8.731e-09
Gut_10d_cckr2r2	0.98718	0.944100
Gut_20d_cckr2r2	0.98527	0.903100
Brain_10d_cckr2r2	0.87121	0.0008662
Brain_20d_cckr2r2	0.92700	0.022810
Gut_10d_cckr2r1	0.96137	0.236900
Gut_20d_cckr2r1	0.98385	0.866000
Brain_10d_cckr2r1	0.90069	0.004783
Brain_20d_cckr2r1	0.96070	0.240000
Gut_10d_pyya	0.93106	0.026960
Gut_20d_pyya	0.89053	0.001899
Brain_10d_pyya	0.93761	0.052350
Brain_20d_pyya	0.91435	0.009832
Gut_10d_npy	0.94485	0.072040
Gut_20d_npy	0.92052	0.013040
Brain_10d_npy	0.92699	0.025580
Brain_20d_npy	0.97691	0.656800
Gut_10d_pyyb	0.96584	0.322900
Gut_20d_pyyb	0.93732	0.041960
Brain_10d_pyyb	0.94081	0.065150
Brain_20d_pyyb	0.90722	0.006217

CYST INTAKE

Table 7. Cyst intake. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship between cyst intake and attractant extracts. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

Dataset	Estimate	SD	t value	P-value
Control - Gemma	0.34356	0.40601	0.846	0.420
Control - Otohime	0.1845	0.42305	0.436	0.678
Control - Shrimp	0.07866	0.44038	0.179	0.865

MRNA EXPRESSION DURING ONTOGENY

Table 8. Expression during ontogeny. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between gut and brain tissue during development. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

Dataset	Estimate	SD	t value	P-value	
Gut_cck1	0.5066	0.2177	2.327	0.0296	*
Brain_cck1	0.23128	0.10686	2.164	0.0421	*
Gut_cck2	0.8211	0.1321	6.216	2.95E-06	***
Brain_cck2	0.1508	0.1417	1.064	0.299	
Gut_cckr1	0.22559	0.10488	2.151	0.042724	*
Brain_cckr1	0.3198	0.3464	0.923	0.366	
Gut_cckr2r2	0.16554	0.09248	-1.79	0.0872	.
Brain_cckr2r2	0.007032	0.176691	0.04	0.969	
Gut_cckr2r1	0.41418	0.15491	-2.674	0.0139	*
Brain_cckr2r1	0.07404	0.15651	0.473	0.641	
Gut_pyya	0.8356	0.1327	6.295	2.46E-06	***
Brain_pyya	0.04478	0.12571	0.356	0.725	
Gut_npy	0.1539	0.1824	0.843	0.408	
Brain_npy	0.22339	0.11472	1.947	0.065	.
Gut_pyyb	0.52702	0.07163	7.358	2.30E-07	***
Brain_pyyb	0.06548	0.08431	0.777	0.446	

RESPONSE TO FEEDING

Table 9. Atlantic halibut 10 dpff brain cck1. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value	
UNFED-ST1 Fed-Empty	-0.11011	0.13508	-0.815	0.42214	
UNFED-ST1 Fed-Full	-0.32148	0.13097	-2.455	0.02083	*
UNFED-ST2 Fed-Empty	-0.08227	0.11922	-0.69	0.49603	
UNFED-ST2 Fed-Full	-0.4166	0.15361	-2.712	0.01149	*
UNFED-ST3 Fed-Empty	-0.13253	0.12155	-1.09	0.28518	
UNFED-ST3 Fed-Full	-0.41816	0.13621	-3.07	0.00484	**
ST1 Full-Empty	0.211368	0.112744	1.875	0.4159	
ST2 Full-Empty	0.334331	0.117376	2.848	0.0497	*
ST3 Full-Empty	0.285622	0.108603	2.63	0.0895	.

Table 10. Atlantic halibut 20 dpff brain cck1. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value	
UNFED-ST1 Fed-Empty	-0.200569	0.115513	-1.736	0.0935	.
UNFED-ST1 Fed-Full	-0.403779	0.125141	-3.227	0.00318	**
UNFED-ST2 Fed-Empty	-0.193589	0.115203	-1.68	0.104	
UNFED-ST2 Fed-Full	-0.2499	0.117701	-2.116	0.0434	*
UNFED-ST3 Fed-Empty	0.001692	0.107052	0.016	0.9875	
UNFED-ST3 Fed-Full	-0.408721	0.125391	-3.26	0.00293	**
ST1 Full-Empty	0.203204	0.115256	1.763	0.48898	
ST2 Full-Empty	0.055421	0.110425	0.502	0.9961	
ST3 Full-Empty	0.410413	0.110576	3.712	0.00275	**

Table 11. Atlantic halibut 10 dpff gut cck1. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value	
UNFED-ST1 Fed-Empty	-0.2248	0.2277	-0.987	0.332	
UNFED-ST1 Fed-Full	0.2691	0.1894	1.42	0.166	
UNFED-ST2 Fed-Empty	-0.463	0.2507	-1.847	0.075	.
UNFED-ST2 Fed-Full	0.1341	0.1987	0.675	0.505	
UNFED-ST3 Fed-Empty	-0.6081	0.2664	-2.283	0.03	*
UNFED-ST3 Fed-Full	0.222	0.1926	1.153	0.258	
ST1 Full-Empty	-0.49385	0.18917	-2.611	0.09243	.
ST2 Full-Empty	-0.59712	0.2091	-2.856	0.04795	*
ST3 Full-Empty	-0.83011	0.21638	-3.836	0.00175	**

Table 12. Atlantic halibut 20 dpff gut cck1. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value	
UNFED-ST1 Fed-Empty	-0.36798	0.28436	-1.294	0.206	
UNFED-ST1 Fed-Full	0.31	0.22025	1.407	0.17	
UNFED-ST2 Fed-Empty	-0.02896	0.24894	-0.116	0.908	
UNFED-ST2 Fed-Full	0.01303	0.24504	0.053	0.958	
UNFED-ST3 Fed-Empty	-0.29595	0.27621	-1.071	0.293	
UNFED-ST3 Fed-Full	0.52963	0.20478	2.586	0.015	*
ST1 Full-Empty	-0.67798	0.25117	-2.699	0.07396	.
ST2 Full-Empty	-0.042	0.24166	-0.174	0.99998	
ST3 Full-Empty	-0.82558	0.23662	-3.489	0.00634	**

Table 13. Atlantic halibut 10 dpff brain cck2. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value	
UNFED-ST1 Fed-Empty	-0.07652	0.20353	-0.376	0.7099	
UNFED-ST1 Fed-Full	-0.37956	0.20481	-1.853	0.0748	.
UNFED-ST2 Fed-Empty	-0.3561	0.20286	-1.755	0.0905	.
UNFED-ST2 Fed-Full	-0.70665	0.26637	-2.653	0.0132	*
UNFED-ST3 Fed-Empty	-0.14758	0.18675	-0.79	0.4362	
UNFED-ST3 Fed-Full	-0.24913	0.19434	-1.282	0.2108	
ST1 Full-Empty	0.30304	0.19922	1.521	0.6477	
ST2 Full-Empty	0.35055	0.23772	1.475	0.6778	
ST3 Full-Empty	0.10155	0.18278	0.556	0.9936	

Table 14. Atlantic halibut 20 dpff brain cck2. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value	
UNFED-ST1 Fed-Empty	-0.32916	0.16532	-1.991	0.0563	.
UNFED-ST1 Fed-Full	-0.34876	0.16661	-2.093	0.0455	*
UNFED-ST2 Fed-Empty	-0.23962	0.15961	-1.501	0.1445	
UNFED-ST2 Fed-Full	-0.18263	0.15613	-1.17	0.252	
UNFED-ST3 Fed-Empty	0.01989	0.14473	0.137	0.8917	
UNFED-ST3 Fed-Full	-0.26858	0.16142	-1.664	0.1073	
ST1 Full-Empty	0.0196	0.18914	0.104	1	
ST2 Full-Empty	-0.05699	0.17746	-0.321	1	
ST3 Full-Empty	0.28848	0.17077	1.689	0.538	

Table 15. Atlantic halibut 10 dpff gut cck2. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value	
UNFED-ST1 Fed-Empty	0.33319	0.143	2.33	0.02697	*
UNFED-ST1 Fed-Full	-0.02702	0.16279	-0.166	0.86934	
UNFED-ST2 Fed-Empty	0.44133	0.1379	3.2	0.00332	**
UNFED-ST2 Fed-Full	-0.11001	0.16802	-0.655	0.51778	
UNFED-ST3 Fed-Empty	0.2901	0.14513	1.999	0.05507	.
UNFED-ST3 Fed-Full	-0.23044	0.17611	-1.309	0.20096	
ST1 Full-Empty	0.3602	0.1458	2.471	0.1315	
ST2 Full-Empty	0.5513	0.1464	3.765	0.00219	**
ST3 Full-Empty	0.5205	0.1564	3.328	0.01112	*

Table 16. Atlantic halibut 20 dpff gut cck2. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value	
UNFED-ST1 Fed-Empty	0.16641	0.14288	1.165	0.2536	
UNFED-ST1 Fed-Full	-0.38165	0.17636	-2.164	0.0388	*
UNFED-ST2 Fed-Empty	0.01421	0.15107	0.094	0.9257	
UNFED-ST2 Fed-Full	-0.05808	0.15524	-0.374	0.7111	
UNFED-ST3 Fed-Empty	0.15619	0.1434	1.089	0.285	
UNFED-ST3 Fed-Full	-0.30939	0.17128	-1.806	0.0813	.
ST1 Full-Empty	0.54805	0.19685	2.784	0.0594	.
ST2 Full-Empty	0.07229	0.18519	0.39	0.9988	
ST3 Full-Empty	0.46558	0.19283	2.414	0.15	

Table 17. Atlantic halibut 10 dpff brain cckr1. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value	
UNFED-ST1 Fed-Empty	0.24708	0.26727	0.924	0.3634	
UNFED-ST1 Fed-Full	-0.23129	0.28775	-0.804	0.4285	
UNFED-ST2 Fed-Empty	0.45849	0.22399	2.047	0.0505	.
UNFED-ST2 Fed-Full	-0.04617	0.29979	-0.154	0.8787	
UNFED-ST3 Fed-Empty	0.25176	0.24024	1.048	0.3039	
UNFED-ST3 Fed-Full	-0.05529	0.26876	-0.206	0.8386	
ST1 Full-Empty	0.478372	0.329543	1.452	0.692	
ST2 Full-Empty	0.504661	0.309377	1.631	0.574	
ST3 Full-Empty	0.307049	0.294188	1.044	0.902	

Table 18. Atlantic halibut 20 dpff brain cckr1. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; $p<0.001$ (***), $p<0.01$ (**), $p<0.05$ (*), $p<0.1$ (.), $p<1$( ).

	Estimate	SD	t value	P-value
UNFED-ST1 Fed-Empty	-0.04602	0.39647	-0.116	0.908
UNFED-ST1 Fed-Full	-0.62849	0.4994	-1.258	0.219
UNFED-ST2 Fed-Empty	-0.11313	0.40655	-0.278	0.783
UNFED-ST2 Fed-Full	-0.68777	0.51201	-1.343	0.19
UNFED-ST3 Fed-Empty	-0.22308	0.42401	-0.526	0.603
UNFED-ST3 Fed-Full	-0.65629	0.50526	-1.299	0.205
ST1 Full-Empty	0.58247	0.29234	1.992	0.343
ST2 Full-Empty	0.57464	0.30156	1.906	0.395
ST3 Full-Empty	0.43321	0.30486	1.421	0.712

Table 19. Atlantic halibut 10 dpff gut cckr1. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; $p<0.001$ (***), $p<0.01$ (**), $p<0.05$ (*), $p<0.1$ (.), $p<1$( ).

	Estimate	SD	t value	P-value
UNFED-ST1 Fed-Empty	0.05569	0.18692	0.298	0.76788
UNFED-ST1 Fed-Full	-0.02415	0.19258	-0.125	0.90107
UNFED-ST2 Fed-Empty	0.07546	0.18557	0.407	0.68726
UNFED-ST2 Fed-Full	-0.03512	0.19338	-0.182	0.85716
UNFED-ST3 Fed-Empty	0.19318	0.17782	1.086	0.28626
UNFED-ST3 Fed-Full	0.0468	0.18754	0.25	0.80472
ST1 Full-Empty	0.07984	0.243844	0.327	1
ST2 Full-Empty	0.110575	0.243397	0.454	0.998
ST3 Full-Empty	0.146388	0.23168	0.632	0.989

Table 20. Atlantic halibut 20 dpff gut cckr1. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; $p<0.001$ (***), $p<0.01$ (**), $p<0.05$ (*), $p<0.1$ (.), $p<1$( ).

	Estimate	SD	t value	P-value	
UNFED-ST1 Fed-Empty	-0.20704	0.17928	-1.155	0.2576	
UNFED-ST1 Fed-Full	-0.0368	0.16788	-0.219	0.828	
UNFED-ST2 Fed-Empty	0.12815	0.15792	0.811	0.4237	
UNFED-ST2 Fed-Full	-0.21609	0.17992	-1.201	0.2395	
UNFED-ST3 Fed-Empty	0.3081	0.14819	2.079	0.0466	*
UNFED-ST3 Fed-Full	0.13968	0.15727	0.888	0.3818	
ST1 Full-Empty	-0.17024	0.24401	-0.698	0.982	
ST2 Full-Empty	0.34423	0.236	1.459	0.689	
ST3 Full-Empty	0.16842	0.20526	0.821	0.964	

Table 21. Atlantic halibut 10 dpff brain cck2r1. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value	
UNFED-ST1 Fed-Empty	-0.16334	0.20282	-0.805	0.4277	
UNFED-ST1 Fed-Full	-0.14439	0.17938	-0.805	0.4279	
UNFED-ST2 Fed-Empty	-0.12389	0.17796	-0.696	0.4923	
UNFED-ST2 Fed-Full	-0.52999	0.23699	-2.236	0.0338	*
UNFED-ST3 Fed-Empty	-0.27709	0.189	-1.466	0.1542	
UNFED-ST3 Fed-Full	-0.37975	0.19698	-1.928	0.0645	.
ST1 Full-Empty	-0.01895	0.19374	-0.098	1	
ST2 Full-Empty	0.4061	0.21631	1.877	0.414	
ST3 Full-Empty	0.10265	0.19572	0.525	0.995	

Table 22. Atlantic halibut 20 dpff brain cck2r1. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value	
UNFED-ST1 Fed-Empty	-0.27297	0.19088	-1.43	0.1638	
UNFED-ST1 Fed-Full	-0.46864	0.20641	-2.27	0.0311	*
UNFED-ST2 Fed-Empty	-0.16549	0.18311	-0.904	0.3738	
UNFED-ST2 Fed-Full	-0.51631	0.21047	-2.453	0.0206	*
UNFED-ST3 Fed-Empty	-0.31485	0.19405	-1.622	0.1159	
UNFED-ST3 Fed-Full	-0.40738	0.20135	-2.023	0.0527	.
ST1 Full-Empty	0.19567	0.19439	1.007	0.916	
ST2 Full-Empty	0.35082	0.19252	1.822	0.451	
ST3 Full-Empty	0.09253	0.1931	0.479	0.997	

Table 23. Atlantic halibut 10 dpff gut cck2rl. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value	
UNFED-ST1 Fed-Empty	-0.54014	0.22651	-2.385	0.0239	*
UNFED-ST1 Fed-Full	-0.17243	0.19517	-0.883	0.3842	
UNFED-ST2 Fed-Empty	-0.44519	0.21775	-2.044	0.0501	.
UNFED-ST2 Fed-Full	-0.07025	0.18762	-0.374	0.7108	
UNFED-ST3 Fed-Empty	-0.29547	0.2049	-1.442	0.16	
UNFED-ST3 Fed-Full	-0.49627	0.22241	-2.231	0.0335	*
ST1 Full-Empty	-0.36771	0.22304	-1.649	0.565	
ST2 Full-Empty	-0.37495	0.21238	-1.765	0.487	
ST3 Full-Empty	0.2008	0.22616	0.888	0.949	

Table 24. Atlantic halibut 10 dpff gut cck2rl. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value	
UNFED-ST1 Fed-Empty	-0.17992	0.17986	-1	0.3254	
UNFED-ST1 Fed-Full	-0.07785	0.1729	-0.45	0.6559	
UNFED-ST2 Fed-Empty	-0.25989	0.18562	-1.4	0.1721	
UNFED-ST2 Fed-Full	-0.22269	0.1829	-1.218	0.2332	
UNFED-ST3 Fed-Empty	-0.36883	0.19393	-1.902	0.0672	.
UNFED-ST3 Fed-Full	-0.11229	0.1752	-0.641	0.5266	
ST1 Full-Empty	-0.10207	0.19204	-0.532	0.995	
ST2 Full-Empty	-0.0372	0.20303	-0.183	1	
ST3 Full-Empty	-0.25654	0.20377	-1.259	0.807	

Table 25. Atlantic halibut 10 dpff brain cck2r2. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value	
UNFED-ST1 Fed-Empty	-0.19841	0.21302	-0.931	0.3599	
UNFED-ST1 Fed-Full	-0.0297	0.1777	-0.167	0.8685	
UNFED-ST2 Fed-Empty	-0.31042	0.19827	-1.566	0.1291	
UNFED-ST2 Fed-Full	-0.72332	0.26716	-2.707	0.0116	*
UNFED-ST3 Fed-Empty	-0.35588	0.20194	-1.762	0.0893	.
UNFED-ST3 Fed-Full	-0.39005	0.20477	-1.905	0.0675	.
ST1 Full-Empty	-0.16871	0.18139	-0.93	0.9379	
ST2 Full-Empty	0.4129	0.22571	1.829	0.443	
ST3 Full-Empty	0.03417	0.18968	0.18	1	

Table 26. Atlantic halibut 20 dpff brain cck2r2. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value
UNFED-ST1 Fed-Empty	-0.24058	0.22078	-1.09	0.285
UNFED-ST1 Fed-Full	-0.14817	0.21307	-0.695	0.493
UNFED-ST2 Fed-Empty	-0.19138	0.21662	-0.883	0.385
UNFED-ST2 Fed-Full	-0.14606	0.2129	-0.686	0.498
UNFED-ST3 Fed-Empty	-0.32145	0.2279	-1.41	0.169
UNFED-ST3 Fed-Full	-0.06505	0.2065	-0.315	0.755
ST1 Full-Empty	-0.092407	0.208166	-0.444	0.998
ST2 Full-Empty	-0.045315	0.205429	-0.221	1
ST3 Full-Empty	-0.2564	0.20879	-1.228	0.823

Table 27. Atlantic halibut 10 dpff gut cck2r2. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value	
UNFED-ST1 Fed-Empty	-0.003796	0.132268	-0.029	0.9773	
UNFED-ST1 Fed-Full	0.109243	0.126851	0.861	0.3962	
UNFED-ST2 Fed-Empty	0.256968	0.120334	2.135	0.0413	*
UNFED-ST2 Fed-Full	-0.001264	0.132142	-0.01	0.9924	
UNFED-ST3 Fed-Empty	0.08425	0.128015	0.658	0.5156	
UNFED-ST3 Fed-Full	-0.0363	0.133883	-0.269	0.7899	
ST1 Full-Empty	-0.113039	0.156121	-0.724	0.979	
ST2 Full-Empty	0.258231	0.150871	1.712	0.523	
ST3 Full-Empty	0.120253	0.158386	0.759	0.974	

Table 28. Atlantic halibut 20 dpff gut cck2r2. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value	
UNFED-ST1 Fed-Empty	0.10354	0.14167	0.731	0.4707	
UNFED-ST1 Fed-Full	0.21434	0.13614	1.574	0.1262	
UNFED-ST2 Fed-Empty	0.26041	0.13395	1.944	0.0616	.
UNFED-ST2 Fed-Full	0.05028	0.14447	0.348	0.7303	
UNFED-ST3 Fed-Empty	0.11901	0.14087	0.845	0.4051	
UNFED-ST3 Fed-Full	0.20443	0.13661	1.496	0.1454	
ST1 Full-Empty	-0.110801	0.179932	-0.616	0.99	
ST2 Full-Empty	0.210134	0.180614	1.163	0.854	
ST3 Full-Empty	-0.085422	0.179627	-0.476	0.997	

Table 29. Atlantic halibut 10 dpff brain pyya. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value	
UNFED-ST1 Fed-Empty	-0.20043	0.16442	-1.219	0.2334	
UNFED-ST1 Fed-Full	-0.24462	0.14895	-1.642	0.1121	
UNFED-ST2 Fed-Empty	-0.2823	0.1512	-1.867	0.0728	.
UNFED-ST2 Fed-Full	-0.50149	0.18687	-2.684	0.0123	*
UNFED-ST3 Fed-Empty	-0.25436	0.14952	-1.701	0.1004	
UNFED-ST3 Fed-Full	-0.37414	0.1569	-2.385	0.0244	*
ST1 Full-Empty	0.0442	0.16281	0.271	1	
ST2 Full-Empty	0.21919	0.17938	1.222	0.825	
ST3 Full-Empty	0.11979	0.15818	0.757	0.974	

Table 30. Atlantic halibut 20 dpff brain pyya. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	**SD**	**t value**	**P-value**	
UNFED-ST1 Fed-Empty	-0.35441	0.15867	-2.234	0.0337	*
UNFED-ST1 Fed-Full	-0.23168	0.1512	-1.532	0.1367	
UNFED-ST2 Fed-Empty	-0.24675	0.15208	-1.622	0.1159	
UNFED-ST2 Fed-Full	-0.27152	0.15356	-1.768	0.0879	.
UNFED-ST3 Fed-Empty	-0.06767	0.14204	-0.476	0.6375	
UNFED-ST3 Fed-Full	-0.20059	0.14939	-1.343	0.1901	
ST1 Full-Empty	-0.12273	0.14672	-0.836	0.961	
ST2 Full-Empty	0.02477	0.14412	0.172	1	
ST3 Full-Empty	0.13293	0.13554	0.981	0.924	

Table 31. Atlantic halibut 10 dpff gut pyya. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	**SD**	**t value**	**P-value**
UNFED-ST1 Fed-Empty	0.31015	0.22248	1.394	0.173877
UNFED-ST1 Fed-Full	0.36069	0.21868	1.649	0.10985
UNFED-ST2 Fed-Empty	0.18736	0.23225	0.807	0.426392
UNFED-ST2 Fed-Full	0.33785	0.22038	1.533	0.136106
UNFED-ST3 Fed-Empty	-0.02848	0.25142	-0.113	0.910592
UNFED-ST3 Fed-Full	0.35966	0.21875	1.644	0.11094
ST1 Full-Empty	-0.050537	0.269534	-0.187	1
ST2 Full-Empty	-0.150481	0.279878	-0.538	0.995
ST3 Full-Empty	-0.388144	0.296133	-1.311	0.779

Table 32. Atlantic halibut 20 dpff gut pyya. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	**Std.Error**	**t value**	**P-value**	
UNFED-ST1 Fed-Empty	-0.373417	0.177373	-2.105	0.044	*
UNFED-ST1 Fed-Full	0.008267	0.152781	0.054	0.957	
UNFED-ST2 Fed-Empty	-0.193882	0.165093	-1.174	0.25	
UNFED-ST2 Fed-Full	0.22493	0.141205	1.593	30.122	
UNFED-ST3 Fed-Empty	-0.05042	0.156198	-0.323	30.749	
UNFED-ST3 Fed-Full	0.170438	0.143967	1.184	0.246	
ST1 Full-Empty	-0.38168	0.21683	-1.76	0.4899	
ST2 Full-Empty	-0.41881	0.19675	-2.129	0.2707	
ST3 Full-Empty	-0.22086	0.19091	-1.157	0.8562	

Table 33. Atlantic halibut 10 dpff brain pyyb. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value	
UNFED-ST1 Fed-Empty	0.0267	0.13011	0.205	0.83897	
UNFED-ST1 Fed-Full	-0.1368	0.12392	-1.104	0.27937	
UNFED-ST2 Fed-Empty	0.0074	0.11729	0.063	0.95016	
UNFED-ST2 Fed-Full	-0.21627	0.14364	-1.506	0.14378	
UNFED-ST3 Fed-Empty	-0.18349	0.12619	-1.454	0.15746	
UNFED-ST3 Fed-Full	-0.38488	0.13677	-2.814	0.00901	**
ST1 Full-Empty	0.16349	0.1651	0.99	0.921	
ST2 Full-Empty	0.22367	0.17182	1.302	0.783	
ST3 Full-Empty	0.20139	0.17257	1.167	0.852	

Table 34. Atlantic halibut 20 dpff brain pyyb. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value
UNFED-ST1 Fed-Empty	-0.13732	0.13744	-0.999	0.326
UNFED-ST1 Fed-Full	-0.23728	0.14282	-1.661	0.108
UNFED-ST2 Fed-Empty	-0.21428	0.14156	-1.514	0.141
UNFED-ST2 Fed-Full	-0.10793	0.13592	-0.794	0.434
UNFED-ST3 Fed-Empty	0.11707	0.12518	0.935	0.358
UNFED-ST3 Fed-Full	-0.22912	0.14237	-1.609	0.119
ST1 Full-Empty	0.09996	0.178639	0.56	0.994
ST2 Full-Empty	-0.10635	0.176329	-0.603	0.991
ST3 Full-Empty	0.346193	0.168437	2.055	0.311

Table 35. Atlantic halibut 10 dpff gut pyyb. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value	
UNFED-ST1 Fed-Empty	0.08718	0.14267	0.611	0.5459	
UNFED-ST1 Fed-Full	0.4279	0.12667	3.378	0.0021	**
UNFED-ST2 Fed-Empty	0.11473	0.14124	0.812	0.4232	
UNFED-ST2 Fed-Full	0.21136	0.13642	1.549	0.1322	
UNFED-ST3 Fed-Empty	-0.16933	0.15722	-1.077	0.2903	
UNFED-ST3 Fed-Full	0.35226	0.12992	2.711	0.0111	*
ST1 Full-Empty	-0.34072	0.17027	-2.001	0.3399	
ST2 Full-Empty	-0.09663	0.17733	-0.545	0.9942	
ST3 Full-Empty	-0.5216	0.1868	-2.792	0.0582	.

Table 36. Atlantic halibut 20 dpff gut pyyb. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value	
UNFED-ST1 Fed-Empty	-0.25581	0.13424	-1.906	0.0667	.
UNFED-ST1 Fed-Full	0.06314	0.11878	0.532	0.5991	
UNFED-ST2 Fed-Empty	-0.26985	0.13499	-1.999	0.0551	.
UNFED-ST2 Fed-Full	0.20613	0.11279	1.828	0.0779	.
UNFED-ST3 Fed-Empty	-0.15285	0.12891	-1.186	0.2454	
UNFED-ST3 Fed-Full	0.07989	0.11805	0.677	0.5039	
ST1 Full-Empty	-0.31895	0.1857	-1.718	0.5185	
ST2 Full-Empty	-0.47598	0.1812	-2.627	0.0903	.
ST3 Full-Empty	-0.23274	0.1797	-1.295	0.7869	

Table 37. Atlantic halibut 10 dpff brain npy. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value	
UNFED-ST1 Fed-Empty	-0.13607	0.17612	-0.773	0.4465	
UNFED-ST1 Fed-Full	-0.23214	0.16303	-1.424	0.1659	
UNFED-ST2 Fed-Empty	0.01369	0.14831	0.092	0.9271	
UNFED-ST2 Fed-Full	-0.33734	0.19156	-1.761	0.0896	.
UNFED-ST3 Fed-Empty	-0.0563	0.15228	-0.37	0.7145	
UNFED-ST3 Fed-Full	-0.25323	0.1644	-1.54	0.1351	
ST1 Full-Empty	0.09608	0.15987	0.601	0.991	
ST2 Full-Empty	0.35103	0.16174	2.17	0.25	
ST3 Full-Empty	0.19693	0.14663	1.343	0.759	

Table 38. Atlantic halibut 20 dpff brain npy. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value	
UNFED-ST1 Fed-Empty	0.04148	0.10834	0.383	0.7047	
UNFED-ST1 Fed-Full	-0.20854	0.11897	-1.753	0.0906	.
UNFED-ST2 Fed-Empty	0.04691	0.10813	0.434	0.6678	
UNFED-ST2 Fed-Full	-0.2465	0.12074	-2.042	0.0507	.
UNFED-ST3 Fed-Empty	0.21804	0.10178	2.142	0.041	*
UNFED-ST3 Fed-Full	-0.20621	0.11887	-1.735	0.0938	.
ST1 Full-Empty	0.25002	0.129118	1.936	0.37846	
ST2 Full-Empty	0.293409	0.130363	2.251	0.21381	
ST3 Full-Empty	0.424258	0.124371	3.411	0.00841	**

Table 39. Atlantic halibut 10 dpff gut npy. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value
UNFED-ST1 Fed-Empty	-0.465487	0.307802	-1.512	0.141
UNFED-ST1 Fed-Full	0.354113	0.225645	1.569	0.127
UNFED-ST2 Fed-Empty	-0.020078	0.258034	-0.078	0.939
UNFED-ST2 Fed-Full	-0.146179	0.270788	-0.54	0.593
UNFED-ST3 Fed-Empty	-0.28395	0.285894	-0.993	0.329
UNFED-ST3 Fed-Full	-0.006912	0.256759	-0.027	0.979
ST1 Full-Empty	-0.8196	0.32823	-2.497	0.123
ST2 Full-Empty	0.1261	0.31976	0.394	0.999
ST3 Full-Empty	-0.27704	0.33113	-0.837	0.96

Table 40. Atlantic halibut 20 dpff gut npy. Estimate, standard error (SD), t-value and p-value indicating significance from a generalised linear model testing the relationship of mRNA expression between unfed, fed-empty and fed-full larvae. Significant codes; p<0.001 (***), p<0.01 (**), p<0.05 (*), p<0.1 (.), p<1( ).

	Estimate	SD	t value	P-value
UNFED-ST1 Fed-Empty	-0.04515	0.24457	-0.185	0.855
UNFED-ST1 Fed-Full	0.26426	0.2185	1.209	0.236
UNFED-ST2 Fed-Empty	-0.15201	0.25481	-0.597	0.555
UNFED-ST2 Fed-Full	-0.15228	0.25484	-0.598	0.555
UNFED-ST3 Fed-Empty	-0.42081	0.28372	-1.483	0.149
UNFED-ST3 Fed-Full	-0.11734	0.25142	-0.467	0.644
ST1 Full-Empty	-0.3094182	0.2753956	-1.124	0.871
ST2 Full-Empty	0.0002673	0.3120295	0.001	1
ST3 Full-Empty	-0.3034659	0.3327187	-0.912	0.943