Morphological and functional modifications of the gastrointestinal tract during metamorphosis in Atlantic halibut *(Hippoglossus hippoglossus)*

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Scientific environment

The work presented in this thesis was mainly conducted in the Marine Developmental Biology (MDB) group at the Department of Biology, University of Bergen. Three research stays were part of this study and were hosted by: the Institute of Marine Research, Austevoll Aquaculture Research Station, Storebø (Norway) for sampling; the Max Planck-Institute for Plant Breeding Research, Köln (Germany), a partner of the EU project, LIFECYCLE (project nº. 222719; http://www.lifecycle.gu.se/) for the 454 transcriptome analysis (paper I); and the Comparative and Molecular Endocrinology (CME) group, CCMAR, University of Algarve, Faro (Portugal), for meetings with Deborah Power, co-supervisor and project collaborator, and to work with Ricardo Alves on the data for paper I. The work was financed by the European Community FP7 (LIFECYCLE; project no. 222719) and Research Council of Norway (Gutfeeling; project no. 190019).

Most striking was the influence of the thyroid food. It caused a precocious differentiation of the body, but suppressed further growth.

J. F. Gudernatsch, 1912

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Abstract

Metamorphosis in flatfish is the transition from a symmetrical larva to an asymmetrical juvenile stage, allowing the organism to utilize new resources in a different habitat and thus providing an evolutionary advantage. In addition to the overt changes in external morphology, coordinated maturation of many tissues and organs occur, including the gastrointestinal (GI-tract). In flatfish species, the GI-tract is extensively remodelled during larval development and its appropriate development is crucial for adapting to the shift in habitat and diet that accompanies the transition into a juvenile. Thyroid hormones (THs) play a central role in this process, translating the environmental cues into a coordinated program that remodels the organism. This thesis uses Atlantic halibut (*Hippoglossus hippoglossus*), a valuable aquaculture species, to provide new insights into flatfish metamorphosis. The transition from larvae to juvenile is here explored based on transcriptional changes during metamorphosis, functional development of the GI-tract and the establishment of neuroendocrine pathways for appetite-regulation.

To study the main molecular mechanisms associated with Atlantic halibut metamorphosis, reference transcriptomes of the GI-tract, head and skin were generated using 454 pyrosequencing. The resulting large set of good quality reads has been assembled into a significant number of contigs and successfully annotated using a multi Blast step approach. Functional analyses revealed that the most prominent biological processes are equally common between the three regions analysed (GI-tract, head and skin) despite significant differences in tissue complexity. Additionally, unique sets of biological processes associated with tissue-specific morphology and function were identified for each region. For the GI-tract, the focal organ of the present thesis, a total of 206 GO-terms were found to be unique, including gastric acid secretion process. Using SOLiD sequencing technology, it was revealed that during Atlantic halibut development hundreds of genes are significantly ($p<0.05$) up- or down-regulated at the whole larvae level, indicating that many key transcriptional modifications underlie the significant changes in tissue that occur between premetamorphic and juvenile stages. It is well established that initiation of metamorphosis in Atlantic halibut is associated with a surge of THs, thus THs levels increase until the metamorphic climax and decrease in

the post-climax stage. The TH cycle during metamorphosis was accompanied by a change in the expression profile of key elements involved in the thyroid signalling pathway, and the change in their expression profile occurred in synchrony with increasing THs levels at metamorphic climax. The coordinated changes in gene expression of TH signalling pathway players, THs levels and tissue morphology confirm the importance of the TH system in orchestrating the Atlantic halibut postembryonic development. The list of candidate genes for future studies aimed at understanding GI-tract development during metamorphosis includes many TH responsive genes.

The functional ontogeny of the GI-tract was studied using an integrative approach to test whether the multiple functions of the Atlantic halibut stomach develop synchronously during metamorphosis. The onset of gastric function was determined using *in vivo* pH analysis in the GI-tract lumen, combined with quantitative PCR (qPCR) of the gastric proton pump α and β subunits and pepsinogen A2. The results indicate that gastric function is established during metamorphic climax. A 3D model series of the GI-tract development and *in vivo* observations imply that the stomach's short-term reservoir function is established before metamorphosis, although the midgut acts as the main storage compartment until this function shifts to the stomach as its volume increases at metamorphic climax. The motility function of the GI-tract was investigated using *in vivo* analysis and results show that phasic and propagating contractions are established well before metamorphosis, but the number of contractions registered in the midgut decreases synchronously with the stomach's increasing peristaltic activity at metamorphic climax.

The stomach's role in appetite control was studied via changes in ghrelin mRNA expression. Ghrelin is mainly produced in the stomach and known as an important orexigenic factor in mammals and some fish species. During metamorphosis climax of Atlantic halibut, ghrelin levels significantly ($p<0.05$) increased parallel to stomach development. However, ghrelin expression did not change in developing Atlantic halibut in response to food intake. To explore potential changes in appetite regulation in Atlantic halibut during metamorphosis, spatial and temporal gene expression patterns of neuropeptide Y (NPY), peptide YY (PYY), pro-opiomelanocortin (POMC-C) and cocaine- and amphetamine-related transcript (CART) in the brain were analysed. NPY,

PYY and POMC-C gene expression did not change during ontogeny, but CART was significantly ($p<0.05$) down-regulated when metamorphosis commenced. Only PYY gene expression responded significantly ($p<0.05$) to food intake in the premetamorphic stage. Results for the other appetite-regulating factors were inconclusive, however Atlantic halibut larvae fasted for 44 h fasting showed significant responses of PYY, NPY, POMC-C and ghrelin after refeeding ($p<0.05$).

In summary, the results in this thesis reveal that the Atlantic halibut transcriptome is highly dynamic through post-embryonic development and that the TH signalling pathway is critical for the radical remodelling of organs during flatfish metamorphosis. The remodelling of the GI-tract, specifically the stomach development and volume growth, is linked to the surge of TH levels during the climax of metamorphosis, and the morphological modifications are connected with a set of functional changes. Furthermore, the development of the stomach during metamorphosis and emergence of proteolytic activity are correlated with a significant rise in stomach ghrelin, crucial for the gastric involvement in appetite regulation. Functional specializations of the GI-tract, such as its putative osmoregulatory function, are already well established at the onset of exogenous feeding and are therefore independent of metamorphosis. For the first time analysed, the neuroendocrine control of appetite regulation during Atlantic halibut larval development revealed no link between the onset of appetite-regulation with THs and metamorphosis, with the exception of ghrelin. Therefore, more studies will be required to better understand feeding and the onset of appetite regulation in fish larvae. Overall, the results in this thesis have opened up several new avenues of research and new molecular resources have been developed which will contribute to future studies aimed at understanding the complex process of flatfish metamorphosis.

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List of publications

Paper I

Gomes, A.S., Alves R.N., Stueber K., Thorne, M.A.S., Sináradóttir, H., Reinhard, R., Clark, M.S., Rønnestad, I. and Power, D.M.

New insights about tissue-specific modifications associated with metamorphosis in Atlantic halibut (*Hippoglossus hippoglossus*).
Manuscript

Paper II

Gomes, A.S., Kamisaka, Y., Harboe, T., Power, D.M. and Rønnestad I.

Functional modifications associated with gastrointestinal tract organogenesis during metamorphosis in Atlantic halibut (*Hippoglossus hippoglossus*).
BMC Developmental Biology (*in press*)

Paper III

Gomes, A.S., Jordal A-E. O., Olsen, K., Harboe, T., Power, D.M. and Rønnestad I.

Neuroendocrine control of appetite in Atlantic halibut (*Hippoglossus hippoglossus*); changes during metamorphosis and effects of feeding.
Manuscript

Chapter 1

General Introduction

1. Metamorphosis

Metamorphosis means "changing form" (Greek; *meta* meaning change and *morphe* meaning form), as it occurs during the dramatic and abrupt transformation of a larva into a juvenile. The best-known and described examples of such transformations occur in amphibians and insects. Nonetheless, there is no clear consensus about the term metamorphosis (Bishop et al. 2006) and for clarity in the present thesis the definition of McMenamin and Parichy (2013) has been adopted. Namely, metamorphosis is *"an irreversible developmental and physiological change that affects multiple traits during postembryonic development and is brought about by one or more systematically acting endocrine mediators, but is independent of sexual maturation, sex-specific modifications, or senescence"*.

Among vertebrate metamorphoses there are diverse examples of morphogenesis, but all of them share common principles (Laudet 2011). These common patterns are related to the ecological role and the triggers of metamorphosis, as well as its extent beyond morphological changes alone. In most species, the environment plays an important role in the transformation of a larva into its adult form, i.e. the metamorphosis is triggered in close connection to the environmental conditions. The hormonal system has a key part in this process, translating the environmental cues into a coordinated program that remodels the organism. In addition, metamorphosis is an ecological transformation and the larva and adult frequently do not live in the same habitats, or at least do not compete for the same resources. Metamorphosis is also an extensive transformation of the animal and is never limited to just morphological changes: it includes biochemical, histological and physiological remodelling and affects a range of different tissues and systems. Metamorphosis is thus a change in form and function, allowing the organism to transit between life-stages in order to utilize new resources in a different habitat. This enables larvae and adult to occupy different ecological niches and therefore eliminates the

competition between them. The resulting increase in the overall chances of survival may explain why vertebrates undergo such an energy-intensive process like metamorphosis.

1.1. Flatfish metamorphosis

The best-studied cases of metamorphosis in vertebrates are found in amphibians, particularly in *Xenopus laevis*. The knowledge acquired from the work on *Xenopus* has been crucial to understand metamorphosis in other vertebrates, such as the teleosts, the most species rich (>26 000, Nelson (2006)) and diverse group of vertebrates. Among teleosts, several types of metamorphosis have been characterized and some of the most dramatic morphological transitions are described in marine teleosts (McMenamin and Parichy 2013). In the latter organisms, the larval stages are planktonic or pelagic, and metamorphosis coincides with the final recruitment to its adult habitat (benthic or pelagic). There are various metamorphic transformations found in marine teleost species, notably substantial morphological remodelling that allows juveniles and adults to exploit different ecological niches. Coral reef fishes provide a good example of a rapid and radical remodelling. The changes in pigmentation and body shape associated with the transition to their adult form can be as fast as a few hours (McCormick et al. 2002). Yet, one of the most remarkable examples of metamorphosis is provided by the Pleuronectiform (flatfish).

Flatfish metamorphoses includes migration of one eye so that it joins the other eye on the right or left hand side of the head, pigmentation of the ocular side of the body and extensive craniofacial remodelling (Table 1) (Sæle et al. 2004, Schreiber 2006, 2013). The external morphological modifications are accompanied by internal modifications as well as biochemical and physiological changes that affect a wide range of tissues and physiological processes (Schreiber and Specker 1998, Power et al. 2008, Laudet 2011, Schreiber 2013).

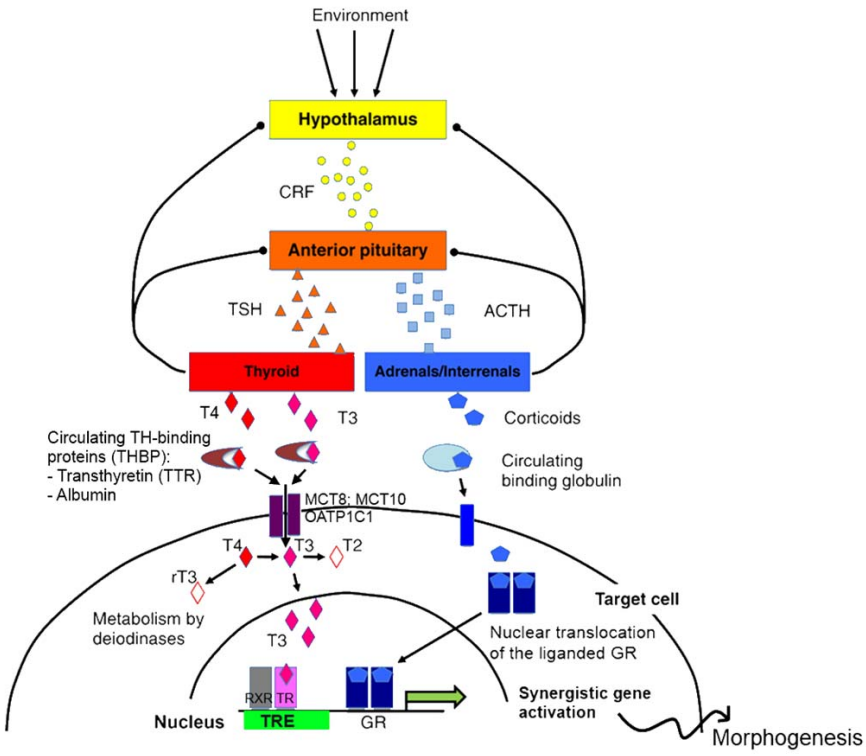
Table 1: Lists of major ecological, external and internal changes during flatfish metamorphosis. In addition, examples of flatfish species where one or more of the external and internal changes have been identified are listed (grey column). Modified from McMenamin and Parichy (2013).

Niche shift	External changes	Internal changes	Species and References
Pelagic to Benthic Plankton feeders to preying on invertebrates and fish	Body flattening/ asymmetry Eye migration Asymmetrical pigmentation and intensification Cranial asymmetry	Stomach and gastric digestion development Loss of swim bladder Erythrocyte development Haemoglobin appears Muscle development	Atlantic halibut *Hippoglossus hippoglossus* (Solbakken et al. 1999, Sæle et al. 2004, Murray et al. 2006, Campinho et al. 2012) Japanese flounder *Paralichthys olivaceus* (Inui and Miwa 1985, Inui et al. 1989, Miwa and Inui 1991, Yamano et al. 1991, Miwa et al. 1992, Tanaka et al. 1996, Yoo et al. 2000) Summer flounder *Paralichthys dentatus* (Keefe and Able 1993, Schreiber and Specker 1998, Martinez and Bolker 2003) Senegalese sole *Solea senegalensis* (Ribeiro et al. 1999, Fernández-Díaz et al. 2001) Spotted halibut *Verasper variegatus* (Tagawa and Aritaki 2005) Various species (Schreiber 2001, Power et al. 2008, McMenamin and Parichy 2013, Schreiber 2013)

1.2. Endocrine control of flatfish metamorphosis

The morphological and physiological changes that occur during flatfish metamorphosis are mediated by hormones and also involve environmental cues. The thyroid hormones (THs) are generally considered as the key regulators of flatfish metamorphosis (Miwa et al. 1992, Inui et al. 1995, Schreiber and Specker 1998, Power et al. 2008), in common to what has been observed in *Xenopus* (Box 1) (Brown et al. 2005, Brown and Cai 2007, Denver 2009a, Ishizuya-Oka 2011, Laudet 2011).

Box1. The hypothalamo-pituitary-thyroid axis and the thyroid hormone signalling pathway controlling *Xenopus* metamorphosis.



The environment controls the production of corticotropin releasing factor (CRF), which acts on the anterior pituitary and triggers the production of both thyrotropin (TSH) and corticotropin (ACTH). TSH stimulates the production of thyroxine (T4) and triiodothyronine (T3) from the thyroid gland. ACTH controls the production of corticoids. The THs circulating in the plasma mainly bind to specific TH binding proteins (THBP), such as Transthyretin (TTR) and albumin. Cellular uptake of T3 and T4 is achieved by organic anion (OATP1C1), monocarboxylate (MCT8 and MCT10), and amino acid transporters. In the target cells thyroid hormones (THs) are metabolized by deiodinases. Outer ring deiodinases (mainly deiodinase 2, DIO2) convert T4 into T3 (the active hormone). The inner ring deiodinases (mainly DIO3) transform T4 and T3 into the inactive compounds reverse T3 (rT3) and diiodothyronine (T2), respectively. In the nucleus, T3 binds to the thyroid hormone receptors (TR), which forms a heterodimer with the retinoid X receptors (RXR) (RXR-TR). The heterodimer RXR-TR bind to the thyroid hormone responsive elements (TRE) and activate the transcription of the specific target genes. Corticoids, once entering the cells, bind to the glucocorticoid receptor (GR). This receptor is located in the cytoplasm without a ligand, after ligand binding the receptor translocates into the nucleus where it binds DNA to activate or repress target genes. Some cases of synergistic gene activation between TR-RXR and GR have been demonstrated. Adapted from Denver (2009a) and Laudet (2011).

Thyroid hormones

The two thyroid hormones (THs), 3,5,3'5'-tetraiodothyronine (thyroxine; T4) and 3,5,3'-triiodothyronine (T3), are produced by the thyroid follicles (reviewed in: Yamano (2005)), which, and unlike tetrapods, are found scattered throughout the connective tissue of the lower jaw (pharyngeal region) and usually aggregate around the ventral aorta (Khanna 2004, Norris and Lopez 2010). T4 is the major hormone synthesized in the thyroid gland, and it is metabolized into a more biologically potent T3 by outer-ring deiodination in peripheral tissues, particularly in the liver (Visser et al. 1988, Bianco et al. 2002). The role of THs in flatfish metamorphosis has been studied for several years and experimentally verified (Inui and Miwa 1985, Miwa et al. 1992, Yamano 2005, Schreiber et al. 2010). In addition, TH levels peak at metamorphic climax during the larvae-to-juvenile transition in various flatfish species such as the Japanese flounder (*Paralichthys olivaceus;* Miwa et al. (1988) and Yamano (2005)), Summer flounder (*Paralichthys dentatus*, Schreiber and Specker (1998)), Senegalese sole (*Solea senegalensis*; Klaren et al. (2008)) and Atlantic halibut (*Hippoglossus hippoglossus*; Einarsdóttir et al. (2006) and Galay-Burgos et al. (2008)), highlighting a central role for THs in flatfish metamorphosis.

Thyroid hormone (TH) biosynthesis

The level of TH production is regulated by the hypothalamo-pituitary-thyroid axis (Box1) through the stimulatory action of thyrotropin-stimulating hormone (TSH) on the thyroid tissue, which produces THs (reviewed in: Szkudlinski et al. (2002)). In teleosts as well as in mammals, TSH is produced by the pituitary thyrotrophs and this hormone is composed of two non-covalently linked subunits: a hormone-specific β subunit (TSHβ) coupled to a glycoprotein α subunit (GSUα) (reviewed in: MacKenzie et al. (2009)). TSH regulates the iodide uptake by the sodium/iodide symporter (NIS) into the follicular cells, essential for the iodination of tyrosine residues in the glycoprotein thyroglobulin (Tg) (Eales et al. 1999, Blanton and Specker 2007). Fish take up iodine from the water via the gills and from their diet (Hunn and Fromm 1966, Eales and Brown 1993). In the follicular cells, iodide is oxidized by thyroid peroxidase (TPO) and afterwards incorporated into tyrosyl groups of Tg (Degroot and Niepomniszcze 1977). Iodination occurs at specific Tg tyrosine sites, giving rise to diiodotyrosine (DIT) and monoiodeotyrosine (MIT) (Degroot and Niepomniszcze 1977, Boas et al. 2006). The intramolecular coupling of either two DIT residues, gives rise to T_4, while a MIT with a

DIT residue results in the formation of T_3. Enzymatic degradation of the Tg is stimulated by TSH and the THs are released into the circulation (Eales and Brown 1993).

In teleosts, the stimulatory role of TSH on TH biosynthesis (Ng et al. 1982, Inui et al. 1989, Leatherland and Farbridge 1992) and the negative feedback of T3 and T4 on TSH β transcription (Larsen et al. 1997, Pradet-Balade et al. 1997, Pradet-Balade et al. 1999, Manchado et al. 2008) have been established. However, the factors controlling the secretion of TSH by the pituitary gland are still unclear. In teleosts, the hypothalamic thyrotropin releasing hormone (TRH) does not always act as a thyrotropin-releasing factor (reviewed in: De Groef et al. (2006)), as it does in mammals (Van Heuverswyn et al. 1984). Therefore, in teleosts it has been hypothesized that the secretion of TSH may be under the control of the corticotropin-releasing factor (CRF) (MacKenzie et al. 2009), such as occurs in tadpoles (Manzon and Denver 2004, Denver 2009b, Denver 2009a).

Deiodination

The conversion of T4 to T3 depends on the expression and activity of selenocysteine deiodinases. In flatfish, three deiodinase genes have been isolated (Itoh et al. 2010, Campinho et al. 2012). T4 is converted by the deiodinase enzymes DIO1 and DIO2 into the active form T3 (reviewed in: Gereben et al. (2008)). Both T3 and T4 are inactivated by a third deiodinase, DIO3 (reviewed in: Gereben et al. (2008)). Recent studies have shown that TH availability during flatfish metamorphosis is regulated by the coordinated action of DIO2 and DIO3 (Isorna et al. 2009, Itoh et al. 2010, Campinho et al. 2012), as occurs in other vertebrates (Brown 2005, Galton 2005). However, the action of deiodinases and the mechanisms regulated by THs during flatfish metamorphosis remain largely unexplored.

Thyroid Hormone (TH) transport

Once THs are released into the circulation, less than 1% is estimated to be present in the free form (Eales and Shostak 1985, Weirich et al. 1987). The majority of the THs are bound to plasma proteins, namely thyroid hormone binding proteins (THBP) (Power et al. 2009). In teleosts, two out of three THBP present in mammals have been identified, albumin (Richardson et al. 1994) and transthyretin (TTR) (Santos and Power 1999, Yamauchi et al. 1999). The T4-binding globulin (TBG) present in mammals remains to

be identified in fishes (Norris and Lopez 2010). In teleosts, TTR has a higher affinity for T3 than T4 (Yamauchi et al. 1999, Kawakami et al. 2006) or similar affinity (Morgado et al. 2008), as in birds and amphibians (Yamauchi et al. 1993, Prapunpoj et al. 2000) but in contrast to what is observed for mammals (Chang et al. 1999, Richardson 2009). So far, it has been possible to identify TTR genes in various fish orders, including the Pleunonectifome European flounder (*Platichthys flesus*), nevertheless TTR has not been identified, to date, in Atlantic halibut. In addition, relatively few physiological studies of TTR have been carried out in teleosts.

The THs are hydrophobic and for a long time it was thought that they access the cytoplasm of the target cells by passive diffusion. Recent studies have shown that the majority of THs enter the target cell through proteins present in the plasma membrane of the cell. In mammals, several TH membrane transporters have been identified (Visser et al. 2011). All of them belong to the solute carrier (SLC) superfamily: Slco1c1 (Organic anion-transporting polypeptide 1c1; Oatp1c1), Slc16a2 (Monocarboxylate transporter; MCT8) and Slc16a10 (Monocarboxylate transporter 10; MCT 10). In fish it has also been demonstrated that less than 10% of the total TH transport is through passive diffusion (Riley and Eales 1993, 1994). MCT8 has been identified as specific thyroid hormone membrane transporters in mammals (Friesema et al. 2003). Orthologs of mammalian thyroid hormone membrane transporters have also been described in non-mammalian species such as *Xenopus tropicalis* (Connors et al. 2010), zebrafish (*Danio rerio*) (Arjona et al. 2011) and fathead minnow (*Pimephales promelas*, Muzzio et al. (2014)), where a role in TH transport has been proposed.

Thyroid hormone receptors

The THs activate a downstream signalling pathway when they bind specifically to thyroid hormone receptors (TRs). These TRs are members of the nuclear receptor superfamily and activate expression of TH-target genes in the presence of the hormone or repress expression in its absence (Buchholz et al. 2006, Oetting and Yen 2007). The TRs bind to specific regulatory DNA sequences of the target genes known as TH response elements (TREs). Typically, TREs are composed by repeats of the half-site sequence AGGTCA (Velasco et al. 2007). The number of half-sites, spacing between and orientation are variable between TREs (Paquette et al. 2011). Although TRs can bind to TREs as monomers or homodimers, they preferentially bind as heterodimers with the retinoid X receptor (RXR) (Lazar et al. 1991, Paquette et al. 2011). The

different forms of TR to bind to TREs and preferences for different TREs need to be considered in the regulation of T3 responsiveness (Velasco et al. 2007, Brent 2012).

In contrast to anurans and most vertebrates, in which two TRs (TRα and TRβ) encoded by two distinct genes exist, most teleost fish have three TR genes: TRαA, TRαB and TRβ (Yamano and Inui 1995, Yamano and Miwa 1998, Kawakami et al. 2003, Galay-Burgos et al. 2008, Kawakami et al. 2008, Takayama et al. 2008). The additional copy of the TRs in fish is probably the result of the teleost-specific whole genome duplication event (Nakatani et al. 2007). In flatfish, TRα and TRβ expression increases during metamorphosis up to climax, but the pattern of expression prior, during, and after metamorphosis seems to be species-specific (Figure 1) (Yamano and Miwa 1998, Marchand et al. 2004, Galay-Burgos et al. 2008, Manchado et al. 2009).

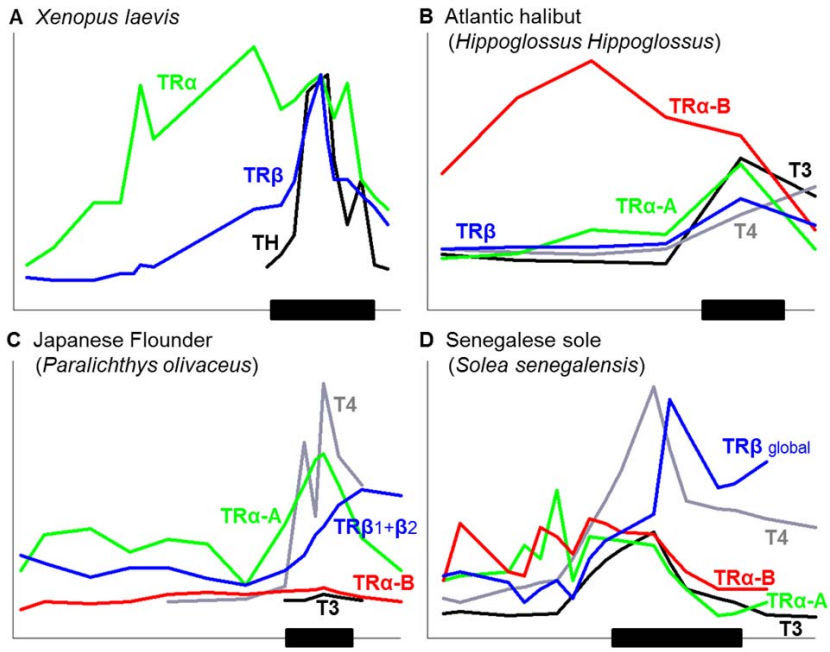


Figure 1: A comparison of the developmental profiles of thyroid hormones (T3 and T4) and its receptors (TRαB, TRαB, TRβ) between (A) *Xenopus laevis* and the flatfishes: (B) Atlantic halibut (*Hippoglossus hippoglossus*), (C) Japanese flounder (*Paralichthys olivaceus*) and (D) Senegalese sole (*Solea senegalensis*). Developmental stage is indicated by the x-axis and the horizontal black bar specifies the approximate metamorphic period. The relative values used are based on: (A) *Xenopus* (Yaoita and Brown 1990); (B) Halibut (Galay-Burgos et al. 2008); (C) Flounder (Yamano and Miwa 1998); and (D) Sole (Klaren et al. 2008, Manchado et al. 2009).

Studies so far indicate that TRs play a critical role in flatfish metamorphosis by mediating TH signalling. However, it is still not known which TR isoform is involved in tissue specific TH-driven maturation during flatfish metamorphosis and which molecular pathways are influenced by the different TRs.

Growth hormone/ Insulin-like growth factor-1 system

In addition to the TH system, the growth hormone (GH)/Insulin-like growth factor I (IGF-I) axis also plays an important role in promoting growth and regulating metabolism in larval stages of teleosts (Björnsson et al. 2002, Yousefian and Shirzad 2011). In teleosts and specifically in Atlantic halibut and common sole (*Solea solea)*, GH receptor (GHR) mRNA expression and IGF-I body content peak prior to the metamorphic climax, and then decrease during metamorphic climax (Hildahl et al. 2007, Ferraresso et al. 2013). Furthermore, in Atlantic halibut, when comparing normally and abnormally metamorphosed larvae, the IGF-I content and GHR mRNA levels were reduced in the abnormal fish, suggesting that the GH/IGF-I system has a regulatory role in metamorphosis. In addition, modification in the distribution and abundance of cells expressing IGF-I has been observed in the remodelling craniofacial region of the Atlantic halibut, suggesting its involvement in the morphological transformations during metamorphosis (Hildahl et al. 2008). However, the potential influence of GH/IGF-I axis on flatfish metamorphic events remains to be established (Power et al. 2008).

1.3. Local Mechanisms of morphogenetic changes

Flatfish metamorphoses include the remodelling of many existing tissues as well as the formation of entirely new tissues and organs that are crucial for the adult form (McMenamin and Parichy 2013, Schreiber 2013). Thus, metamorphosis requires cellular migration, proliferation, growth and death. In flatfish, both external and internal morphological changes are crucial for development and therefore also for commercial production. However, in mass-cultured commercial flatfish species, abnormal metamorphosis (arrested development) is still a common phenomenon (Power et al. 2008). The numbers of studies describing the tissue-specific morphological changes during metamorphosis of flatfish have recently increased, yet the underlying mechanisms of this extensive remodelling remain largely unexplored. The following examples demonstrate some of the tissue-specific remodelling/maturation during flatfish metamorphosis.

Flatfish cranial asymmetry and eye migration

The cranial transformation of the flatfish is a unique event, and a result of an additional period of postembryonic asymmetric remodelling during metamorphosis (reviewed in: Schreiber (2013)). Recent studies have shown that during flatfish metamorphosis at least one gene pathway involved in vertebrate embryonic organ lateralization (nodal-lefty-pitx2) is re-expressed. Pitx2 was asymmetrically re-expressed in the left habenula of pre-metamorphic Japanese flounder and spotted halibut (*Verasper variegatus*) larvae, and this re-expression appears to initiate eye migration (Suzuki et al. 2009b). After metamorphosis, the adult flatfish species exhibit either both eyes on the right side (dextral) or both eyes on the left side (sinistral). One morphologically abnormal characteristic in flatfish is the failure of the eye to migrate (Okada et al. 2001, Okada et al. 2003, Saele et al. 2006a, Saele et al. 2006b). The incidence of eye migration failure or reversed eye migration is higher in aquaculture fish compared to wild populations. Nevertheless, new rearing protocols have successfully reduced incomplete eye migration for some aquaculture flatfish species (Sæle et al. 2003, Harboe et al. 2009).

Changes in skin and pigmentation

Teleosts skin becomes increasingly stratified and complex during metamorphosis when differentiating into its adult structure (Campinho et al. 2007, Rakers et al. 2010). Keratins are generally used as a marker for epidermal development and differentiation (Suzuki et al. 2009a), and in flatfish their expression was down-regulated at metamorphic climax (Campinho et al. 2007) by the THs (Infante et al. 2007). Therefore, TH regulation of skin development in flatfish, particularly in Atlantic halibut, seems to be reminiscent of what has been observed in mammals and anurans (Campinho et al. 2012).

Asymmetric pigmentation is another common feature of flatfish metamorphosis. In contrast to other teleosts, in flatfish just the upper (ocular) side of the body becomes pigmented, whereas the lower side that rests on the sea floor remains unpigmented (reviewed in: Power et al. (2008) and McMenamin and Parichy (2013)). The pigmentation of fish skin is mainly due to the presence of melanophores, and in adult flatfish the melanophores are only present on the ocular pigmented side (Watanabe et al. 2008, Nakamura et al. 2010, Yamada et al. 2010). Abnormal pigmentation is an important issue in flatfish aquaculture and covers a range of different conditions, like albinism, ambicoloration, and mosaicism (reviewed in: Power et al. (2008)). In addition,

it is important to remember that external factors such as light, behaviour and nutrition are also important for pigmentation (reviewed in: Pittman et al. (2013)).

Gastrointestinal tract remodelling

The maturation of the gastrointestinal (GI-) tract in flatfish seems to be TH-dependent and occurs in parallel with the change in diet that accompanies the shift from a pelagic to a demersal lifestyle. This event is often compared to what has been observed in anurans, where the intestine changes from a long coiled tube into a complex and differentiated organ (reviewed in: Laudet (2011)). In addition, the number of mucosal folds increase and the epithelium and mesenchyme proliferate (Shi and Ishizuya-Oka 2001, Schreiber et al. 2009). In most flatfish, the GI-tract gradually develops from a short coiled tube into a segmented and differentiated adult organ (Boulhic and Gabaudan 1992). During intestinal maturation, the epithelium changes structure and function while molecular changes lead to the production of enzymes necessary for the adult digestion mode (Zambonino et al. 2008, Pittman et al. 2013). One of the most striking transformations during flatfish metamorphosis is the development of a stomach, a key event in the life-history of altricial-gastric species. Stomach development in flatfish species has been shown to be a TH-driven event (Miwa et al. 1992). Moreover, in Japanese, winter (*Pseudopleuronectes americanus*) and summer flounders and Atlantic halibut, the gastric glands start to develop just prior to metamorphosis, while pepsinogen mRNA levels increase significantly in the post-metamorphic stage (Miwa et al. 1992, Huang et al. 1998, Douglas et al. 1999, Murray et al. 2006). This indicates that the morphological and functional changes linked to GI-tract maturation develop synchronously during metamorphosis. However, studies describing the stomach and intestinal development in flatfish have focused mainly on morphological aspects and only a few have targeted specific functional features such as acquisition of gastric proteolytic activity. Therefore, the underlying mechanisms of GI-tract remodelling and their functional implications for digestive capacity remain largely unexplored in teleosts, particularly in flatfish.

A more detailed description of the morphological and functional development of the GI-tract during flatfish larval stages will be given in the next sections.

2. Structural and functional characteristics of the piscine GI-tract

The vertebrate GI-tract is a highly specialized structure that digests the ingested food, absorbs nutrients and expels waste products from the organism. At the onset of first feeding, the GI-tract of fish larvae consists of a foregut (that develops into esophagus and stomach (Smith et al. 2000)), midgut and hindgut. Alternatively, bucco-pharynx, oesophagus, stomach anlage, intestine and anus are terms used for the different regions of the GI-tract. The piscine GI-tract is often categorized based on the presence or absence of a stomach: agastric species do not possess a stomach; precocial species have a functional stomach at the onset of exogenous feeding; and altricial species develop a functional stomach during metamorphosis (reviewed in: Rønnestad et al. (2013)). Altricial-gastric species undergo one of the most extraordinary post-embryonic developments, their GI-tract experiences rapid and extensive remodelling, which prepares them for the shift from a pelagic to a benthic habitat and a change in feeding behaviour (Pittman et al. 1990, Luizi et al. 1999).

2.1. Stomach

The morphology of the vertebrate stomach varies among species, probably in relation to species-specific diet and habitat. In addition, the primitive chordate Amphioxus does not have a stomach (Smith et al. 2000) and within fish, agastric species such as members of the family *Cyprinidae* are stomachless (Koelz 1992, Day et al. 2011). These observations raise intriguing questions about the evolutionary origin of the vertebrate stomach. Recent studies have shown that the loss of the stomach correlates with the absence of the genes involved in the gastric function: H^+/K^+-ATPase subunits α and β and pepsinogens (Ordoñez et al. 2008, Castro et al. 2013). The diet and/or environmental factors may have caused selective pressure that led to the loss of gastric acidification, and this phenomena has occurred multiple times during vertebrate evolution (Castro et al. 2013). Furthermore, Castro et al. (2013) suggests that the loss of the stomach in agastric species may be an irreversible event, in line with Dollo's law (complex characters lost in evolution cannot be regained (Marshall et al. 1994)).

Altricial-gastric fish larvae lack a "true" stomach and proteolytic capacity until after metamorphosis, which is similar to what occurs in most anuran larvae. During metamorphosis, extensive modifications occur in the GI-tract and stomach differentiation takes place. Yet, little is known of the molecular mechanisms regulating

stomach differentiation in altricial-gastric species. Structural differentiation of the stomach epithelium and gastric gland development marks the transition period from larva to juvenile in altricial-gastric species. The adult fish stomach has the same general organization typical of vertebrates, with mucosa, submucosa, muscularis externa and serosa (Murray et al. 1994). Within the inner layer, i.e. the gastric mucosa, the oxynticopeptic cells composing the gastric glands are found (Murray et al. 1994). It is the oxynticopeptic cells that are responsible for the secretion of hydrochloric acid (HCl) and pepsinogen in fish and other non-mammalian vertebrates (Koelz 1992, Sugiura et al. 2006). In contrast, the mammalian stomach contains two specialized cell types: parietal (oxyntic) cells that secrete HCl and the chief (peptic) cells that secrete pepsinogen (Koelz 1992, Rhoades and Bell 2009).

For altricial-gastric species it is hypothesized that the stomach becomes completely functional during/at the end of the metamorphic climax, when the gastric glands are developed. The timing of appearance of the first gastric glands and subsequent completion of stomach development have been identified in several flatfish species (Boulhic and Gabaudan 1992, Segner et al. 1994, Bisbal and Bengtson 1995, Baglole et al. 1997, Luizi et al. 1999, Ribeiro et al. 1999, Gisbert et al. 2004, Hachero-Cruzado et al. 2009). However, the stomach does not only act as a proteolytic chamber, it fulfils several other important functions in digestion, protection and storage (Smith et al. 2000, Stevens and Hume 2004). Stomach digestion corresponds to the first phase of mechanical and chemical breakdown of food. Peristaltic movements in the stomach mix the ingested food with the secreted gastric acid (HCl) and pepsin, initiating the proteolysis. In addition, production of gastric acid kills many of the bacteria and pathogens ingested with food and represents part of the first line of immune defence that protects the organism. The short-term reservoir function of the stomach requires the establishment of a functional esophagus and pylorus sphincters. The simple constriction between the stomach region and the intestine observed in pre-metamorphic stages develops and transforms into a muscular valve, the pyloric sphincter, during metamorphosis (Pedersen and Falk-Petersen 1992, Segner et al. 1994, Luizi et al. 1999, Kamisaka and Rønnestad 2011). In the case of Japanese flounder the pyloric sphincter seems to be functional before the onset of metamorphosis (Rønnestad et al. 2000a). This feature allows the stomach to increase digestion efficiency by retaining food during longer periods of time, and regulates the passage of food into the intestine to maximise

digestion and absorption. The stomach terminates in the pylorus, and in some fish species is followed by the pyloric caeca. This structure is unique in fish and its development in teleosts is considered as an adaptation for increasing the absorptive intestinal area (Buddington and Diamond 1987, Rønnestad et al. 2000b).

2.2. Intestine

Intestinal remodelling during metamorphosis in anurans is crucial for the transition from a herbivorous larva to a carnivorous adult (reviewed in: Laudet (2011)). The straightening and shortening of the larval intestine is accompanied by an increase in size of the connective tissue and muscle layers. Meanwhile, the epithelial cells undergo apoptosis or differentiate into adult progenitor/stem cells which rapidly proliferate to form a more folded epithelial surface (Shi and Ishizuya-Oka 2001, Schreiber et al. 2009, Hasebe et al. 2013). The molecular programs behind larval epithelium cell death (apoptosis) as well as *de novo* growth and proliferation of adult epithelium have been extensively studied in anurans over the last decade (Schreiber et al. 2005, Heimeier et al. 2010).

In marine fish the intestinal remodelling is not as drastic as in anurans, however structural and functional changes occur during intestinal epithelium maturation until the adult mode of digestion is achieved (Zambonino et al. 2008). In altricial marine fish larvae the intestine region arises immediately after the pyloric constriction and has a bulb-like shape before initiation of metamorphosis. It has been hypothesized that this section may act as a storage compartment before stomach development (Luizi et al. 1999, Kamisaka and Rønnestad 2011) or may be the main region responsible for proteolytic digestion through trypsin-type activity with an alkaline pH (Walford and Lam 1993). The intestinal epithelium in fish also changes during development, becoming progressively more complex with an increase in the number of folds and absorptive activity (Luizi et al. 1999, Ribeiro et al. 1999).

The adult intestine is a complex multifunctional organ and the longest part of the GI-tract, situated between the pyloric sphincter (if stomach is present) and the ileorectal valve. The acidic chyme (if stomach is present) enters the intestine (midgut) and is neutralised by bicarbonate and digested by enzymes secreted from pancreas and mucosa. The chyme is progressively digested and absorbed and transported along the intestine through motility patterns and ultimately arrives to the rectum, where the

defecation reflex eliminates the faeces from the organism. The motility patterns commonly comprises a variety of phasic and propagating contractions (Holmberg et al. 2003). The propagating contractions are classified as anterograde (propagating in the anal direction) or retrograde (propagating in the oral direction). The smooth muscle contraction is the result of slow wave potentials (cycles of depolarization and repolarization). These slow waves can vary along the GI-tract and need to reach a "threshold" (mV) to cause a contraction in the muscle fibre and propagate. The GI-tract smooth muscle motility can be initiated in the smooth muscle itself, or by the action of the interstitial cells of Cajal (ICC) on the smooth muscle, neurotransmitters and/or hormones. The contractions are classified as either spontaneous or triggered by the presence of food. In adult vertebrates, motility is controlled by the autonomic nervous system (ANS), and in particular the enteric nervous system (ENS) located within the GI-tract wall. However, peristaltic contraction activity in the GI-tract smooth muscle is also possible in the absence of enteric neurons, due to the presence of ICCs (Huizinga et al. 2001, Holmberg et al. 2003). Nevertheless, in early developmental stages the ENS may play a particularly important role in the control of spontaneous activity, as has been suggested for mouse and zebrafish, where the ENS and GI-tract are developed before the ICCs (Wu et al. 2000, Holmberg et al. 2004).

The intestinal epithelium is the most vigorously self-renewing tissue of adult mammals (van der Flier and Clevers 2009, Barker et al. 2010). The rapid self-renewal of the epithelium is orchestrated by the proliferative compartment, i.e. the intestinal crypts (crypts of Lieberkühn). Furthermore, a number of signal transduction pathways, such as Shh (Sonic Hedgehog), Wnt, BMP (Bone Morphogenic Protein), and Notch, seems to play an important role in intestinal development in mammals (van der Flier and Clevers 2009, Kim et al. 2011). In marine fish, enterocytes are also capable of proliferation (Rombout et al. 1984). The continuous self-renewal capacity of the vertebrate intestinal epithelium provides the plasticity needed for this organ to adapt to different feeding conditions (Henning 1979). The processes behind self-renewal may have been preserved during evolution and therefore the same genetic program underlying this process may be conserved in teleosts and other vertebrates (Schreiber et al. 2009, Heimeier et al. 2010). Inadequate nutrition and other environmental factors during development may influence the capacity for self-renewal (Buddington 1994). However,

the molecular pathways regulating and controlling intestinal self-renewal in fish is still largely unstudied (reviewed in: Rønnestad et al. (2013).

3. Appetite and food intake control: Gut-brain axis

Control of food intake is essential for the development and survival of an organism. Food intake is affected by external factors, such as temperature and photoperiod, stress, predators and availability of food, and by internal factors, most of all appetite (Schwartz et al. 2000, Volkoff et al. 2010, Cardoso et al. 2012, Hoskins and Volkoff 2012). In vertebrates, appetite is physiologically controlled by the integration of peripheral and central signals in the hypothalamus (Demski and Northcutt 1983, Valassi et al. 2008). Appetite-stimulating (orexigenic) or appetite-inhibiting (anorexigenic) endocrine factors are the key drivers of feeding (Seim et al. 2012). These factors originate from the central nervous system (CNS) as well as from body energy storage (adipocytes), liver, and pancreas. In addition, both hunger (food desire) and satiety (sense of *fullness*) signals from the GI-tract regulate food intake on a meal-to-meal basis and are important for short-term appetite control (Sam et al. 2012). After a meal, the distension of the stomach and interactions between nutrients and the intestine wall trigger the secretion of several peptide hormones. The peripheral peptides convey their signals via sensory axons in the vagus nerve or via the blood and influence the central neuropeptide action. Gut hormones can reduce food intake either by decreasing hypothalamic orexigenic signalling or increasing anorexigenic signalling (Sam et al. 2012). When fasting, the stomach secretes ghrelin, the only orexigenic gut hormone, causing an increase in appetite (Higgins et al. 2007). Part of the orexigenic effect of ghrelin is mediated by upregulating the genes encoding for the potent appetite stimulants Neuropeptide Y (NPY) and Agouti-related peptide (AgRP) (Kojima and Kangawa 2005, Chen et al. 2009). The interaction and balance between anorexigenic and orexigenic factors originating from the GI-tract are important in the system controlling food intake, and an impairment of this balance can result in feeding behaviour disorders (Konturek et al. 2004).

The main gut hormones and hypothalamic factors involved in mammalian appetite control have also been identified in fish (Figure 2). Several of these factors have similar effects in goldfish (*Carassius auratus*) to those already described in mammals (Volkoff and Peter 2000, Volkoff et al. 2009, Volkoff et al. 2010). However, recent studies have

demonstrated that the function of some appetite-related hormones exhibit major differences between fish and mammals and also between fish species (Matsuda 2009, Hoskins and Volkoff 2012, Jonsson 2013).

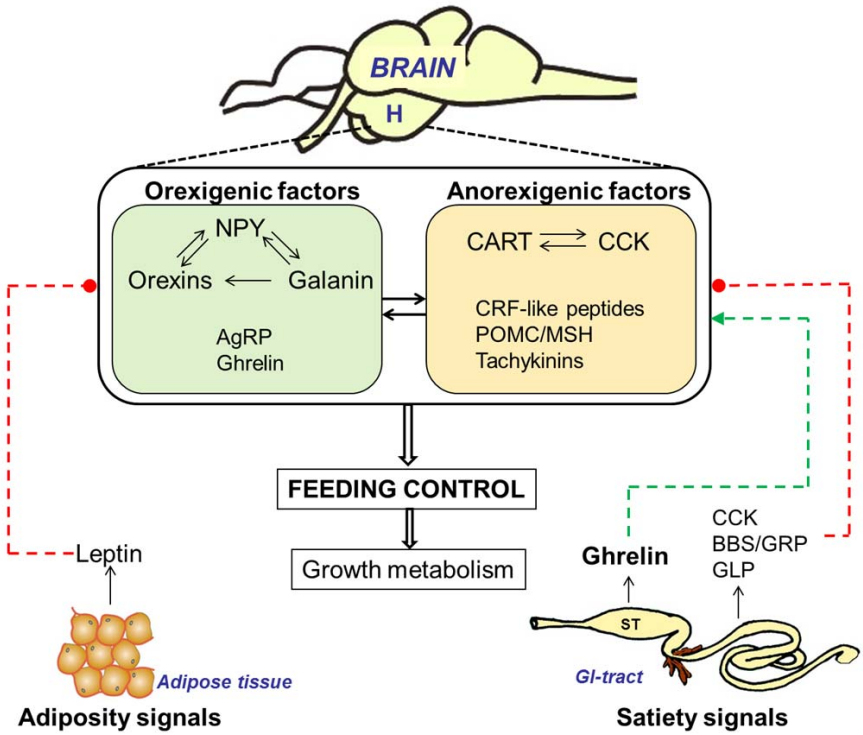


Figure 2: Schematic model of the central and peripheral peptides known to control food intake in fish. Peripheral hormonal signals from the adipose tissue and GI-tract reach the brain and influence the central neuropeptide signals. Peripheral signals are conveyed via sensory axons in the vagus nerve or reach the brain directly via the blood. Within the brain, central neuropeptide systems receive signals and integrate information from the periphery to adjust food intake. These central systems consist of both feeding stimulators (orexigenic factors) and feeding inhibitors (anorexigenic factors). Some of these neuropeptide systems interact with each other (indicated by arrows). Lines and arrows in green indicate an orexigenic pathway while lines and circles indicate an anorexigenic pathway. AgRP, agouti-related protein; BBS/GRP, bombesin/gastrin-releasing peptide; CART, cocaine- and amphetamine-regulated transcript; CCK, cholecystokinin; CRF, corticotropin-releasing factor; GLP, glucagon-like peptide; MSH, melanocyte-stimulating hormone; NPY, neuropeptide Y; and POMC, proopiomelanocortin. H, hypothalamus; ST, stomach. Adapted from Volkoff et al.(2005).

Although the understanding of the endocrine control of appetite and digestion in fish has recently improved, few studies have focused on larval stages. Only a limited number of hormones and neuropeptides have been identified and their expression profiles determined during larval development stages and next to no studies describes how they affect appetite and ingestion in larvae (Kortner et al. 2011a, Kortner et al. 2011b, Rønnestad et al. 2013). Furthermore, whether altricial fish larvae possess a satiety system that regulates appetite is still unknown. Basically, fish larvae behave as "feeding machines" and eat continuously despite having a full gut and this is particularly noticeable at the onset of exogenous feeding. This suggests that the feedback signalling system from the GI-tract to the CNS is still not fully established and raises questions about how metamorphosis may influence appetite control in fish larvae. The answer to these questions could contribute to improve larval feeding procedures, and consequently increase the production of high quality juveniles for aquaculture (Rønnestad et al. 2013).

4. Atlantic halibut: aquaculture production

Atlantic halibut (*Hippoglossus hippoglossus*) is the largest extant flatfish of the family Pleuronectidae and is distributed throughout the northern part of the North Atlantic Ocean and in parts of the Arctic Ocean. Atlantic halibut is traditionally a highly valued food fish, but due to overfishing and subsequent stock collapses it has become scarce. Nowadays it is listed as an endangered species (Sobel 1996) and most fisheries are closed, although there is commonly by-catch mortality through demersal fishing gear. The combination of high demand and low abundance has drawn the interest of the aquaculture industry and resulted in an emerging aquaculture production, initiated by Norway and Iceland in the mid 1980's. The production of Atlantic halibut has shown a positive trend during recent years but remains at a low level compared to major aquaculture species like Atlantic salmon (*Salmo salar*) (FAO 2012). This is partly due to persisting challenges in the production cycle, particularly at first feeding stages. Abnormal development during metamorphosis is still a frequent problem in Atlantic halibut production and abnormalities are commercially important, although their relative impact may depend on the end product, e.g., intact fish or fillets (Power et al. 2008). The identification of possible causes of abnormalities, such as lack of eye migration and bone deformities, has been a priority in the last two decades. Likewise, to obtain high quality juvenile/adult fish a good knowledge of the larval feeding behaviour and

digestion is essential to further improve feeding protocols, diets and ultimately larval quality.

Stage definition

The Atlantic halibut larvae has an inherent number of advantages for research: the relatively large size facilitates isolation and analysis of specific tissues from a single individual; the larvae are transparent until the pigmentation starts to intensify during metamorphosis; and their slow development allows for collection of well-defined developmental stages. Often, the sampling criteria are based only on age (days of hatching or days after first feeding) and do not consider the morphological criteria. The development of a well-define staging scheme for Atlantic halibut represented a fundamental step for comparisons between and within experiments. In addition, it facilitates studies of the ontogeny of organ and tissue systems during development and facilitates the identification of discrete changes in morphology or molecular patterns during key developmental stages in metamorphosis (Power et al. 2008).

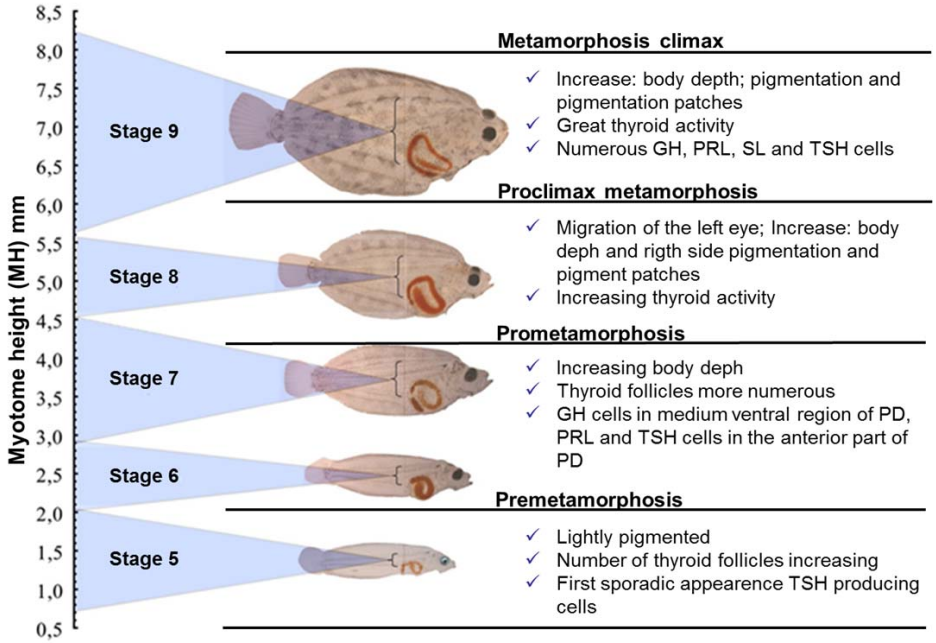


Figure 3*:* Myotome height (MH) size (in mm) ranges from stages 5 to 9. The Atlantic halibut larvae drawings are representative of the midrange of each stage. Major morphological changes are described for each developmental stage. A summary of the principal endocrine cells in the pituitary gland during ontogeny is presented. GH, growth hormone; PRL, prolactin; SL, somatolactin, TSH, thyroid stimulating hormone, PD pars distalis (adapted from Sæle et al. (2004), Einarsdóttir et al. (2006)).

Atlantic halibut can be grouped according to their external morphology and this permits identification of a sequence of distinct larval stages before, during and after metamorphosis. The first halibut larvae staging definition from Pittman et al. (1990) divided the yolk-sac larval phase of Atlantic halibut into four developmental stages. Later, Sæle et al. (2004) developed this approach further and established a detailed staging scheme from first feeding through metamorphosis to settlement (Figure 3). The stage definitions are based on external morphology, skeletal development, degree of ossification and asymmetry. These parameters are generally well-correlated with myotome height. For sample standardization, myotome height is used as benchmark parameter, so that individuals can be rapidly separated into the principal developmental

stages: 5: pre-metamorphosis; 6-7: prometamorphosis; 8: proclimax metamorphosis; 9: climax metamorphosis; and 10: post-metamorphosis. The Atlantic halibut larvae of stages 5–7 are bilaterally symmetrical and transparent, but with pigmented eyes. The transition to asymmetry starts during stage 8, in which larvae are still pelagic, but have a relatively large size and frequently have started to tilt to one side. At stage 9, the eye migration is advanced and reaches the midline, giving the larvae an asymmetric appearance. Distinctive skin pigment patterns emerge and the larvae rest occasionally on the bottom of the tank. In the post-metamorphic stage 10, the eye migration and pigmentation are complete. Thus, stage 10 corresponds to the juvenile individual. From this point on, the halibut adopts a demersal lifestyle and settles on the bottom of the tank.

Chapter 2

Thesis approach

Atlantic halibut metamorphosis is triggered by the action of THs and results in modifications that prepare the larva to its adult lifestyle, and involves the remodelling of body-shape (from symmetry to asymmetry) as well as the growth and differentiation of tissue/organs such as the GI-tract. Knowledge about the molecular basis behind these events in flatfish is still very rudimentary. This represents the central challenge of this thesis: To provide new insights into the drivers of Atlantic halibut metamorphosis, and determine to what extent they are coordinated by THs. The focus herein is on the remodelling of GI-tract morphology and function during larvae-to-juvenile transition. In detail, three major specific research questions were formulated and addressed:

Q1: Which molecular processes characterize GI-tract morphology and functional remodelling during metamorphosis? How does the Atlantic halibut GI-tract transcriptome change during development and which candidate genes are TH-dependent?

Incomplete or "arrested" metamorphosis is a common phenomenon in flatfish aquaculture production. Elucidation of the endocrine factors that control directly or indirectly the changes in gross external morphology and/or internal modifications during metamorphosis, will contribute to the understanding of why this process sometimes fails during aquaculture production. The study of flatfish metamorphosis, including Atlantic halibut, has been mostly based on a candidate-gene approach, by cloning homologues of genes identified as being differentially expressed during amphibian metamorphosis (reviewed in: Power et al. (2008)) and more recently using microarrays (Douglas et al. 2008). Nevertheless, in the past five years a shift towards next generation sequencing (NGS; also referred to as second-generation sequencing that uses a different chemistry to the conventional Sanger sequencing method) resulted in the generation of transcriptome resources for non-model teleost species. Transcriptome sequencing using NGS provides a massive output of sequence data that permits

characterization of expressed genes and generates resources for gene expression profiling studies. In this context, in **paper I**: 1) a reference transcriptome of three major tissues (GI-tract, head and skin) that are remodelled during Atlantic halibut metamorphosis are generated using 454 pyrosequencing technology; 2) the GI-tract transcriptome is compared to that of skin and head to establish enriched or tissue specific transcripts/networks; and 3) the differential expression profile of candidate transcripts in seven developmental stages is analysed using SOLiD sequencing technology.

To date, only two large scale transcriptome studies have been conducted in flatfish during the larval-to-juvenile transition. The first study used a microarray approach on the Atlantic halibut (Douglas et al. 2008) and the second approach in common sole used 454 pyrosequencing (Ferraresso et al. 2013). Despite the high costs compared to other NGS technologies, 454 is still widely used because of the long reads it produces, which facilitates read alignment during the *de novo* assembly (Mundry et al. 2012). The 454 approach is particularly advantageous when relatively few molecular resources (genome or expressed sequence tags, ESTs) are available to assist the assembly of sequencing data into contiguous sequences of sufficient length and sequencing depth for transcript identification. In this thesis, 454 pyrosequencing was used to produce a large set of good quality reads (head: 2,242,561; skin: 1,790,856; GI-tract: 2,070,247) that after trimming were then assembled into a significant number of contigs (head: 90,676; skin: 65,530; GI-tract: 38,426). A multi Blast step approach was used and allowed the successful annotation of 36% of the contigs in head (32,189), 66% in skin (43,295 contigs) and 58% in the GI-tract (22,237 contigs). Success of annotation of the halibut 454 sequencing output was similar to that achieved in previous 454 studies for other fish species (Coppe et al. 2010, Fu and He 2012, Pereiro et al. 2012, Yúfera et al. 2012). Our work improved the EST resources for Atlantic halibut by adding several thousands of new sequences to the public database. The new information provided is particularly useful for the progress of future more detailed development and morphogenesis studies.

Functional analyses revealed that the most prominent biological processes - biological regulation, cellular and metabolic processes - were equally common between the three tissues analysed (GI-tract, head, skin), which is in agreement with what has been observed in other teleost species (Pereiro et al. 2012, Yúfera et al. 2012). However, it

was possible to identify overrepresented and unique GO terms specific for each tissue. In the GI-tract, categories associated with cell proliferation and gene expression/transcription were overrepresented. Also, a total of 206 GO-terms were found to be unique in the GI-tract. Gastric acid secretion process was a specific-GO term associated with this tissue and the genes involved in this process were further investigated in **paper II** as gene-specific candidates for analysis of the onset of the stomachs functional capacity.

The Atlantic halibut (whole-larvae) transcriptome dynamics during development were analysed using SOLiD sequencing technology. SOLiD reads were mapped to a reference transcriptome which was generated based on the three tissue libraries (37,073 contigs). A large number of genes that are differentially expressed during metamorphosis were identified, particularly in the juvenile stage (majority of genes were up-regulated). Considering the large number of differentially expressed transcripts (when all stages were compared with two reference stages: premetamorphic stage 5 and juvenile stage), it is likely that key modifications in the transcriptome underpin the morphological and functional changes that occur between premetamorphic and juvenile stages. To validate the performance of SOLiD data analyses, mRNA expression in a set of several genes was determined with quantitative RT-PCR (qPCR). The list of genes was selected from those that displayed a different expression pattern across development in the SOLiD dataset and that are involved in different tissue processes. From the set of genes analysed, alpha-globin 1 and type I keratin isoform 2 mRNA expression displayed a strongly differential pattern throughout metamorphosis, in agreement with previous studies in Atlantic halibut (Campinho et al. 2007, Power et al. 2008). In the present study, the SOLiD dataset and the qPCR gene expression results were significantly correlated ($p<0.05$), confirming the robustness of the results obtained with the two methods.

Initiation of metamorphosis in Atlantic halibut is associated with a surge of THs (T4 and T3), which is why TH levels increase until the metamorphic climax and decrease in the post-climax stage (Galay-Burgos et al. 2008). However, until now no detailed analysis is available of the activity and contribution of the multiple players within the thyroid axis during metamorphosis. To address this question, the expression pattern of genes involved in the TH biosynthesis (Tg), availability (DIO1, DIO2, DIO3), cellular

transport (MCT8, MCT10) and action (TRαA, TRαB) in whole larvae were studied. Tg and TRs were not identified in the SOLiD sequencing dataset and this was probably due to their generally low tissue abundance further aggravated by the dilution effect caused by using mRNA from whole larvae rather than discrete tissue. As previously demonstrated for Senegalese sole (Manchado et al. 2008), Tg expression was differentially regulated during Atlantic halibut metamorphosis. The observed expression pattern (**paper I**) resembled the TH profile during larval development (Galay-Burgos et al. 2008): levels increased gradually and significantly ($p<0.05$) during development and reached a maximum at metamorphic climax, and afterwards decreased sharply.

The expression profiles of DIO1 and DIO2 transcripts during development were similar to those of TRαA and TRαB transcripts and were coincident with the pattern of T3 levels (Galay-Burgos et al. 2008, Campinho et al. 2012). The increase in T3 during metamorphic climax, which is mainly sustained by peripheral DIO1 and DIO2 activity, provides indirect evidence that the activity of DIO1 and DIO2 increases (Campinho et al. 2012).

The membrane transporter MCT10, responsible for transport of aromatic amino acids in addition to both T3 and T4, was not affected during development. On the other hand, the TH specific transporter MCT8 was significantly ($p<0.05$) increased during the metamorphic climax (**paper I**). The TH transporters have been poorly studied in teleosts and so far have only been described in two other species, zebrafish (Arjona et al. 2011) and fathead minnow (Muzzio et al. 2014). In fathead minnow, changes in the expression of TH transporter transcripts were tissue-specific. In Atlantic halibut, the transcriptional response of TH transporters to changes in the circulating concentration of THs differed from the strict TH-induced changes of deiodinases or TRs suggesting that their regulation may be less hormone dependent.

The coordinated gene expression profiles of key transcripts suggest a crucial role for the TH pathway in Atlantic halibut metamorphosis. However, since qPCR expression of candidate transcripts was assessed in the same whole-larval mRNA extracts used for SOLiD sequencing to permit robust validation, no data was obtained about tissue specific transcripts. Caution is therefore required in the interpretation of the results and the use of whole larval mRNA may explain the lack of significant changes in SOLiD analysis of low abundance transcripts such as Tg and TRs. Nonetheless the approach

taken and the results obtained have permitted the characterization of the transcriptome of the skin, head and GI-tract of Atlantic halibut and enriched understanding of the main molecular mechanisms associated with metamorphosis.

Q2: Does stomach organogenesis and multiple functions of the stomach develop synchronously and are they linked to TH-driven metamorphosis? What is the impact of the agastric-to-gastric transition during metamorphosis on the functional role of the GI-tract?

During metamorphosis the GI-tract of altricial-gastric species develops from a simple tubular form into a more complex folded structure, while the stomach becomes a distinct compartment and continues to acquire its multiple functions (see chapter 1, section 2.1). The establishment and maintenance of such a specialised and voluminous organ as the stomach requires a high energy investment, but it provides also an evolutionary advantage as it extends and facilitates the digestion of dietary proteins through the activity of pepsin (Castro et al. 2013) in combination with acid denaturation that eases proteolysis (Govoni et al. 1986, Rust 1995, Tonheim et al. 2005). The stomach is not present in all vertebrate species, and in particular some teleost lineages lack a stomach, and this has been correlated with the absence of the genes involved in gastric function: H^+/K^+-ATPase α and β subunits and pepsinogen (Castro et al. 2013). These are specific gene markers for stomach organogenesis and functional development that have been included in the consideration of **Q1**.

In **paper II**, we analysed the gastric function of the stomach through expression profiling of the gastric proton pump (H^+/K^+-ATPase) and pepsinogen A2 by qPCR combined with *in vivo* pH analyses. The gastric proton pump is a heterodimer composed of two subunits: α subunit, encoded by Atp4A, and β subunit, encoded by Atp4B, capable of pumping protons (H^+) against a gradient (Maeda 1994, Shin et al. 1997, Shin et al. 2009). Fundamental to gastric function is pepsinogen, the precursor of the enzyme pepsin, which is activated by acidic pH in the stomach. Pepsin digests proteins into smaller peptides, making them available for further digestion and absorption (Kageyama 2002). Pepsinogen A2 transcript expression was correlated with both proton pump subunits in Atlantic halibut and was in agreement with previous observations in other teleost species (Douglas et al. 1999, Gawlicka et al. 2001, Darias et al. 2005, Darias et al. 2007, Gao et al. 2013). In addition, we show that the increased mRNA

expression of gastric proton pump subunits occurred simultaneously with the increase in acidic capacity of the stomach. In summary, the gastric proteolytic capacity in Atlantic halibut larvae is established during the climax of metamorphosis, in synchrony with stomach development. It remains to be demonstrated that pepsinogen transcript abundance correlates with pepsin activity, however in winter flounder the increase in pepsin activity is simultaneous with the increase of pepsinogen IIb and proton pump gene expression (Douglas et al. 1999). The correlation between stomach development and TH-driven metamorphosis has been also documented for other pleuronectiformes species (Miwa et al. 1992, Huang et al. 1998). During metamorphic climax (stage 9), when T3 and T4 levels are high in Atlantic halibut (Galay-Burgos et al. 2008), the stomach volume grows more than 11-fold (**paper II -** 3D models). The series of 3D models representing the GI-tract development (**paper II**) also indicate that a functional pyloric sphincter is present prior to metamorphosis in Atlantic halibut, as previous observed for Japanese flounder by Rønnestad et al. (2000a). This observation was confirmed by *in vivo* visual studies of fed larvae and suggests that stomach reservoir function is established independent of metamorphosis.

The lack of a fully developed stomach to promote mixing and to mechanically degrade the ingested food at prometamorphosis (stage 6) was functionally compensated by the strong anterograde and retrograde contractile activity in the anterior midgut (midgut region 1). Similar observations were made in zebrafish, a stomachless species, where it has been suggested that the retrograde contractions in the anterior part of the midgut generate similar mechanical mixing to the stomach (Holmberg et al. 2003). The number of contractions in the midgut decreased at metamorphic climax synchronously with the stomach's increased peristaltic activity and the established proteolytic capacity.

Na^+/K^+-ATPase α subunit, the main enzyme involved in osmoregulation, was found to be expressed in the Atlantic halibut GI-tract from stage 5 to stage 10, with the highest mRNA expression levels at stage 5. This enzyme has previously been identified on the basolateral side of the epithelial cells of the Atlantic halibut intestine in yolk-sac larvae stages (Einarsdóttir et al. 2011). Our results suggest that during post-embryonic development the Atlantic halibut GI-tract is also osmotically functional, as previously shown for the teleosts Atlantic cod (*Gadus morhua*, Mangor-Jensen and Adoff (1987)) and European sea bass (*Dicentrarchus labrax*, Giffard-Mena et al. (2006)).

The transcript abundance of ghrelin, a putative orexigenic signalling gastric hormone, was significantly ($p<0.05$) correlated with the emergence of the gastric function (**paper II**). We suggest that the stomach's role in appetite control develops simultaneously with the establishment of the proteolytic function. The putative role of ghrelin in appetite control during stomach development was further investigated in **paper III**.

Q3: Does the development of a complete functional stomach affect appetite control in Atlantic halibut larvae? How does metamorphosis influence the gut-brain feedback signalling system?

The reputation of fish larvae as "feeding machines" originates from the fact that they can ingest amounts of food above their own body weight. In the case of the Atlantic halibut larvae, the continuous ingestion of *Artemia* may result in incomplete digestion and food is defecated undigested or even alive, resulting in a loss of nutrients. The continuous feeding behaviour in fish larvae raises questions about the nature of appetite control mechanisms and the timing of their establishment during ontogeny. In mammals and adult teleosts, food intake is controlled by nutrient availability and signalling factors (see chapter 1, section 3). These include hormones and neuropeptides produced in the central nervous system (CNS), particularly in the hypothalamus (Demski and Northcutt 1983) and in peripheral organs, e.g. GI-tract. Hormones and neuropeptides can either stimulate (orexigenic) or inhibit (anorexigenic) appetite. In **paper III**, we explore the role in Atlantic halibut larvae undergoing metamorphosis of the hormone ghrelin and several well-known neuropeptides involved in appetite control in mammals: neuropeptide Y (NPY), peptide YY (PYY), pro-opiomelanocortin (POMC-C) and cocaine- and amphetamine-related transcript (CART).

The neuropeptides NPY, PYY, POMC-C and CART were present and predominantly expressed in the brain of Atlantic halibut at metamorphic climax, corroborating the results of studies in adults of other teleost species (reviewed in: Volkoff et al. 2005 (2005) and Volkoff et al. (2009)). However, the postprandial response of peptides is not conclusive. In common with what occurs in mammals, in the Atlantic halibut larvae there are indications that PYY acts as an important anorexigenic factor. This hypothesis is supported by the changes in mRNA expression observed both at premetamorphic stage 5 with a significant ($p<0.05$) increase 1 and 3 h after feeding, and also at 49 days post-first feeding (dpff; age groups comprising a mix of developmental stages 7, 8 and

9B) larvae with a significant ($p<0.05$) increase four hours after initiation of feeding (compared to a fasted control group).

POMC-C was significantly ($p<0.05$) down-regulated 30 min after feeding (compared to a fasted control group) at 49 dpff. This may imply that it acts only as a short-term response factor of appetite control in larval stages. NPY mRNA expression significantly ($p<0.05$) increased five hours after initiation of feeding (compared to a fasted control group). NPY is considered to be a potent orexigenic factor in mammals, but the late response to food intake in Atlantic halibut at 49 dpff suggests that this neuropeptide may play only a minor role in the appetite control system of Atlantic halibut larvae. For CART, no postprandial effect was observed. Furthermore there were no changes in the postpandrial response of the analysed neuropeptides, with the exception of PYY, during development from premetamorphosis to climax of metamorphosis.

Atlantic halibut larvae lack a stomach when exogenous feeding is initiated, and the stomach only becomes fully functional after the metamorphic climax (**paper II**). In this context, **paper III** evaluates how the development of a completely functional stomach influences the establishment of a ghrelin-dependent feedback mechanism. Ghrelin transcript abundance significantly ($p<0.05$) and sharply increased during metamorphosis climax, synchronously with stomach emergence and differentiation, as has previously been reported for Atlantic halibut larvae (Manning et al. 2008). However, no significant changes in mRNA expression levels of ghrelin were observed in relation to food intake during the development of Atlantic halibut larvae. Thus, a complete functional gut-brain feedback system may not be achieved until juvenile/adult stages.

The lack or inconclusiveness of a postprandial response of the peptides analysed in **paper III** may be explained by the big variety of developmental stages found in the refeeding experiment. The majority of the Atlantic halibut larvae in the refeeding experimental setup at 49 dpff were at the prometamorphic stage 7. At this age, and according to the standard rearing procedures applied, it would be expected that most larvae would be at the metamorphic climax stage. This shows that analysing fish larvae at a certain age does not guarantee that they are at the same developmental stage. In addition, individual variability in growth and development tends to cause problems in respect to sampling protocols and also raises questions in terms of result standardisation between studies.

Chapter 3

Conclusions and future perspectives

The purpose of this thesis was to improve the understanding of Atlantic halibut metamorphosis, focussing on the event cascade that results in the morphological and functional maturation of the GI-tract. In particular, the functional changes of the GI-tract that accompany the development of the stomach were established and if THs, through their regulation of stomach development, are involved in the establishment of a fundamental part of the appetite control gut-brain circuit was also revealed. **Paper I** reports for the first time the sequencing and characterisation of GI-tract, skin and head transcriptomes, major tissues remodelled during Atlantic halibut metamorphosis. This provided information about common and specific gene networks and tissue-specific candidate genes were identified that can be used as candidate markers in the future. The generation of large region/tissue specific transcriptomes in developing Atlantic halibut permitted transcriptome dynamics during metamorphosis to be analysed using SOLiD sequencing analyses. Hundreds of differentially expressed transcripts were identified between larvae-to-juvenile stages using SOLiD and these transcripts may have predetermined functions to ensure the correct course of metamorphosis. Extensive *in silico* analysis identified candidate networks of importance in skin, head and GI-tract and also putative TH responsive genes. Further studies are now required to determine the tissue and cell specific localization and function of candidate genes and to provide experimental evidence of the key networks/pathways involved in Atlantic halibut larvae-to-juvenile transition. It remains to be established if the general evolutionary conservation that occurs during early development of very divergent species also holds true for metamorphosis, a post-embryonic developmental event. However, taking into consideration that the role of THs during metamorphosis has been conserved in vertebrates it seems credible to assume they regulate similar cellular and molecular processes and that the outcome of the present study may be of much more general interest.

The coordinated gene expression profiles of key transcripts involved in the thyroid hormone axis suggest a crucial role of the TH pathway in Atlantic halibut metamorphosis (**paper I**). However, the potential regulatory effects of THs at the tissue level, particularly in the GI-tract, remains to be fully clarified. The highest mortality rates in Atlantic halibut aquaculture production occur during larval development, particularly during metamorphosis. The identification and characterisation of genes and gene networks controlling this development is crucial to optimize the industrial production of high quality larvae and juveniles. In this context, transcriptome analysis of Atlantic halibut larvae undergoing normal versus arrested (endpoint of a particular ontogenetic pathway) development will provide new insights into the molecular mechanisms that orchestrate metamorphosis.

The appropriate development of the GI-tract is crucial for survival and maturation as this organ controls the flux of nutrients into the organism. A key event of metamorphosis in Atlantic halibut is the functional maturation of the GI-tract and organogenesis of the stomach. The modifications in the GI-tract ensure that the larva is well adapted for the shift in habitat and diet that accompanies the transition into a demersal juvenile. Using an integrated approach with anatomical, biochemical, molecular and *in vivo* methodologies, we have described the development of the multifunctional stomach and GI-tract in developing Atlantic halibut (**paper II**). We have confirmed that organogenesis of the stomach and onset of gastric function is linked to TH action during metamorphosis. In **paper II**, we have also demonstrated that remodelling of the GI-tract, specifically the stomach development and volume growth, is linked to the surge of TH levels during the climax of metamorphosis, and the morphological modifications are connected with a set of functional changes. Nonetheless, the stomach's short-term reservoir function is established before metamorphosis, although the main storage function before this stage is assumed by the anterior part of the midgut. Moreover, a series of developmental events and functional specializations of the GI tract, such as its putative osmoregulatory function, are already well established at the onset of exogenous feeding and are independent of metamorphosis and presumably the action of THs. It was not possible in the present study to establish the molecular mechanisms that program cell and tissue maturation and understanding this process remains a priority. Additionally, phasic and propagating contractions, responsible for the mechanical breakdown of food and transportation of

chyme through the GI-tract, were observed before metamorphosis and are therefore independent of metamorphosis. However, the number of contractions in the midgut decreased at metamorphic climax synchronously with the stomach's increased peristaltic activity. It will be therefore interesting to investigate which systems regulate the motility patterns during Atlantic halibut development.

Feeding behaviour is closely associated with appetite control, and it changes during development parallel to stomach organogenesis and change in diet. In Atlantic halibut larvae, candidate genes involved in teleost adult appetite control system such as NPY, PYY, POMC-C and CART are present since first feeding (**paper III**). However, their postpandrial response is not affected during development with the exception of PYY, an important anorexigenic factor in mammals. The response of the neuropeptides NPY, POMC-C and CART to food intake was ambiguous and further more detailed studies are required. In relation to metamorphosis ghrelin mRNA levels sharply increased at the climax and were synchronous with stomach differentiation, its main site of production, although no response to feeding was observed. Intriguingly, CART was significantly down-regulated at metamorphic proclimax (stage 8) when ghrelin levels start to increase, which may indicate an as yet undescribed role for THs in the establishment of appetite control circuits during development. The results reported in **paper III** raise new questions about how appetite is actually controlled in fish larvae. Therefore, future studies are needed to investigate the endocrine regulatory factors involved in the feedback signalling system from the GI-tract to the CNS and to elucidate which factors determine satiety in fish larvae and how metamorphosis may influence it. Behavioural studies (for example using video-recording analysis) together with evaluation of ingestion rates, gut filling and transit time, digestion capacity and key genes involved in food intake control may help to better understand the physiological processes involved in appetite control of Atlantic halibut larvae. In addition, a comparative study between larval and juvenile Atlantic halibut stages will help to clarify which neuropeptides and hormones are involved in the appetite control of this species and how (if they do) change during ontogeny. To better elucidate the system controlling food intake in fish larvae, more mechanistic studies may be developed and applied to determine the sites of synthesis as well as the action of appetite control peptides.

The integration of morphological and physiological data, concerning both structure and function of the developing Atlantic halibut GI-tract into a mathematical model can provide a useful tool to predict and identify critical metamorphic changes and to determine the functional implications during normal or/and abnormal larval development. The findings presented in this thesis, *in silico* (**paper I**) and experimental data (**paper II** and **paper III**), together with previous studies in Atlantic halibut larvae, provides a starting point for numerical simulations, making use of existing models for larval stages of flatfish (Rønnestad and Conceição 2012) and recent advances in the area of biological systems mathematical modelling. These information can also be extrapolated to other teleost, particularly altricial-gastric species and provide new insights into how the functional remodelling of the digestive system affects feeding behaviour.

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