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Thyroid hormone receptor expression during metamorphosis of Atlantic halibut

(*Hippoglossus hippoglossus*)

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Abstract

Flatfish such as the Atlantic halibut (*Hippoglossus hippoglossus*) undergo a dramatic metamorphosis that transforms the pelagic, symmetric larva into a benthic, cranially asymmetric juvenile. In common with amphibian metamorphosis, flatfish metamorphosis is under endocrine control with thyroid hormones being particularly important. In this report we confirm that tri-iodothyronine (T_3) levels peak at metamorphic climax during halibut metamorphosis. Moreover we have isolated cDNA clones of TRα and TRβ genes and confirmed the presence in halibut of two TRα isoforms (representing the products of distinct genes) and two TRβ isoforms (generated from a single gene by alternative splicing). Real time PCR was used to assess expression of these genes during metamorphosis. TRβ shows the most dramatic expression profile, with a peak occurring during metamorphic climax.

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1. Introduction

The link between thyroid hormones (TH) and the onset and progression of amphibian

metamorphosis (reviewed by Brown and Cai, 2007; Tata, 2006; Sachs et al., 2000) has

been known for almost a century (Gudernatsch, 1912). More recent research, notably

some incisive and elegant transgenic animal studies, has emphasised the importance of

thyroid hormone receptors (Buchholz et al., 2006; Buchholz et al., 2004; Schreiber et al.,

2001) and iodothyronine deiodinases in the process (Brown, 2005; Cai and Brown,

2004). However, amphibia are not the only vertebrates that undergo metamorphosis.

The development of fish involves a metamorphosis from larval to juvenile form that is

manifest most dramatically in flatfish (pleuronectiformes) such as sole, halibut and

flounder. In these species, the pelagic larva undergoes extensive remodelling which leads

to a loss of external bilateral symmetry and generates a benthic juvenile with both eyes

on the same, pigmented, side of the body. These morphological changes are scarcely less

profound than those of amphibian metamorphosis but are clearly distinct from them. On

the other hand, some of the biochemical and physiological changes that accompany

metamorphosis, such as switching of globin (Wakahara and Yamaguchi, 2001; Miwa and

Inui, 1991) and keratin (Campinho et al., 2007a) types and the production of new

isoforms of muscle proteins (Yamano et al., 1991a; Campinho et al., 2007b) are similar in

flatfish and amphibia. Still more significantly, there is strong evidence that thyroid

hormones regulate metamorphosis in flatfish in much the same manner as in amphibia.

Hence, metamorphosis in Japanese flounder (*Paralichthys olivaceus*) and Atlantic halibut

(*Hippoglossus hippoglossus*) is induced precociously by TH treatment and Japanese

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flounder metamorphosis is delayed or abolished following exposure to agents that inhibit TH synthesis (DeJesus et al., 1993; Solbakken et al., 1999).

THs exert their genomic effects by binding to thyroid hormone receptors (TRs). THs also have non-genomic effects (Davis and Davis, 1986), some of which are also mediated via the TRs mentioned above (Hiroi et al., 2006). However the genomic actions alone of thyroid hormones may be sufficient for metamorphosis in amphibia (Buchholz et al., 2004). TRs are members of the nuclear receptor superfamily, and act as hormone-regulated transcription factors (reviewed in Zhang and Lazar, 2000; Oetting and Yen, 2007). They usually activate the transcription of target genes when complexed with hormone but act as repressors in the absence of ligand, although some genes are repressed rather than induced by THs. There are two principal isoforms of TRs in vertebrates, designated TRα and TRβ, which are the products of distinct genes. The mammalian TRα and TRβ genes produce a variety of additional receptor isoforms via alternative splicing and the use of alternative transcription start sites, (Lazar, 1993; Flamant and Samurat, 2003). The functions of TRα and TRβ have been extensively investigated in transgenic mice and it is clear that, *in vivo*, they have both common and isoform-specific roles (reviewed by Cheng, 2005; Flamant and Samurat, 2003). For example, TRβ isoforms are more important than those of TRα in the inner ear (Forrest et al., 1996a), liver, and in TH homeostasis (Forrest et al., 1996b), whereas TRα isoforms play a more significant role in the heart (Mai et al., 2004) and GI tract and also in temperature regulation. These differences in function *in vivo* between TRα and TRβ may arise as a result of slight biochemical differences between the isoforms (for example, see

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Guissouma et al., 2002). However, microarray analysis of TH-induced gene expression in liver from TRα and TRβ deficient mice indicates that the two isoforms regulate almost the same set of genes (Yen et al., 2003). Hence, some or all of the functional differences between the receptor types may simply be a reflection of differences in their expression profiles.

The teleosts also possess TRα and TRβ but, in contrast to mammals, Japanese flounder, conger eel (*Conger myriaster*) and Atlantic salmon (*Salmo salar*) have two distinct TRα genes (Yamano et al., 1994; Marchand et al., 2001; Jones et al., 2002; Kawakami et al., 2003a). Conger eel also has two TRβ genes, and whilst only a single TRβ gene has been found in other teleosts examined to date, further TRβ isoforms have been described that result from alternative splicing (Yamano and Inui, 1995; Marchand et al., 2001; Kawakami et al., 2003b). We have previously described (Llewellyn et al., 1999) a full length TRα (henceforth designated as TRαA) from a commercially important flatfish species, the Atlantic halibut (*Hippoglossus hippoglossus*). Here we describe partial clones of a second TRα gene (designated TRαB) and the TRβ gene from this key species. Real time PCR was subsequently used to assess the expression of all three TR genes during metamorphosis and has been related to changes in whole body TH concentration.

Materials and Methods.

2.1 Sampling

Halibut larvae were cultured using standard commercial procedures. Broodstock fish were stripped for eggs and milt, and eggs were fertilised *in vitro* and then placed in open system egg incubators at 5.0-5.5 °C for 14 days and then transferred to open system silos. For the next 45-50 days the larvae were kept in the dark at a temperature of 5-6°C, after which they were transferred to the first feeding tanks at 11°C and fed live feed (*Artemia*) for approximately 60 days. After metamorphosis the bottom dwelling juveniles were transferred to weaning tanks and gradually weaned for 10 days on dry feeds. Samples were taken from a standard commercial production cycle (Fiskeldi Eyjafjarðar Ltd, Iceland) at regular intervals and larvae were staged according to the criteria of Sæle et al (2004). Larvae were stored at -20°C in RNAlater prior to RNA extraction using Tri-reagent.

2.2. *TH extraction and Radioimmunoassay*

The T_4 and T_3 content of halibut larvae was assessed by specific radioimmunoassay (RIA). Individual larvae of stages 5 (n = 10), 6 (n = 10), 7 (n = 8), 8 (n = 7), 9 (n = 6) and 10 (n = 6) were extracted in methanol, re-extracted in 50μl methanol, 200μl chloroform and 100μl barbital buffer, and centrifuged (3,000 rpm for 30 min at 4°C). Then, the upper phase was removed, lyophilised, reconstituted in assay buffer, heat treated (65°C, 2h) and assayed. Assays for both T_3 and T_4 were highly specific and reproducible and were performed under equilibrium conditions using anti-T_3 and anti-T_4 sera (Sigma-Aldrich) as previously described (Einarsdottir et al., 2006). Statistically significant differences in the concentration of T_4 or T_3 detected for different stages were compared by ANOVA on log

transformed data, followed by All Pairwise Multiple Comparison Procedures (Holm-Sidak method). Results were considered to be significantly different at p<0.05.

2.3. Cloning of TRs

PCR using degenerate primers was used in order to isolate partial clones of halibut TRs. A multispecies alignment was performed using TR sequences from a variety of vertebrate species and degenerate PCR primers were designed that corresponded to particularly well conserved motifs. One primer pair (forward primer: AATGYCGCTTCAARAARTGYAT, reverse primer: GTAAACTGRCTRAAGGCYTC) was used in RT-PCR reactions in which the substrate was RNA isolated from metamorphosing halibut larvae. The PCR products generated were gel purified, subcloned into pGEM-T (Promega, UK) and sequenced.

2.4 Sequence analysis

Complete or partial nucleotide sequences of TRs from *Fugu rubripes* were obtained by BlastN searching of the *Fugu* genome (http://fugu.biology.qmul.ac.uk) with the halibut TRαA, TRαB and TRβ sequences. The intron-exon structure of the *Fugu* TRβ gene was deduced using Spidey (http://www.ncbi.nlm.nih.gov/spidey/) to identify sequences within the individual *Fugu* scaffold M001021 that showed strong similarity with known teleost TR mRNAs, followed by manual comparison of the putative splice sites in the *Fugu* sequence with the consensus sequences for vertebrate 5′ and 3′ splice junctions.

2.5 Real-time RT-PCR analysis of gene expression

RNA was prepared from five replicate individuals from stages 5 (larvae prior to onset on metamorphosis) through to stage 10 (fully metamorphosed juveniles) using Tri-reagent (Sigma-Aldrich) and subsequently treated with DNAse (Ambion) according to the manufacturer's instructions. cDNA was prepared from 0.5µg aliquots of each RNA using random hexamer primers and superscript II reverse transcriptase (Invitrogen).

Expression of the genes encoding TRα1, TRα2 and TRβ was quantified using real-time RT-PCR using fluorogenic 5′ nuclease assays performed on an ABI PRISM® 7700 Sequence Detection System (Perkin-Elmer Biosystems, UK). The sequences of the primers and TaqMan probes used in each assay were as follows: TRαA, primers TTGTTGGACATTGGCTCCATT and CCGCCTCATTGTCCTGTGAT, probe TGCCTCAGTACCGTCCAGCGG; TRαB, primers TGCGTCGCTCCCTGTTCT and TTGGTGCTGGACGACTCAAA, probe TCGATCAGACGCCGCTTCGCT; TRβ, primers TGGTGACTGACGCCCATATG and CAGGAATTTCCGCTTCTGCTT, probe CCACGAATGCCCAGGGCAACC. RT-PCR assays, using 5% of the cDNA generated in the reactions described above, were normalised with respect to expression of 18s rRNA, for which the primers were GCATGCCGGAGTCTCGTT and TGCATGGCCGTTCTTAGTTG, and the probe sequence was CCACGAATGCCCAGGGCAACC. Isoform specificity of the probes and primers was confirmed by experiments in which each primer/probe combination was tested for its ability to amplify DNA from the plasmid clones of TRαA, TRαB and TRβ. Each individual sample was analysed in triplicate. One way analysis of variance (ANOVA) was used to assess differences between means, after testing for homogeneity of group

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variances and normality of residuals. Tukey post hoc tests subsequently identified which stages were significantly different ($p<0.05$) from the others for each of the genes analysed.

2.6 PCR-analysis of TR-β alternative splicing products

To check for the presence of splice variants involving the hinge region of halibut TRβ, two primers were designed (AGCTGCAGAAGACGGTGTG and CAGGTGTATTTGCCATGGAC) that spanned the potential alternatively spliced exons. These were used in PCR reactions in which the substrate was cDNA from a variety of adult tissues and whole larvae prepared as described above. PCR reactions were performed for 40 cycles.

3. Results

3.1 Cloning of thyroid hormone receptors

We have previously described a full length halibut cDNA clone representing a thyroid receptor α gene (Llewellyn et al., 1999). However, three different thyroid hormone receptor genes, TRαA, TRαB and TRβ have previously been identified in Japanese flounder (Yamano et al., 1994; Yamano and Inui, 1995), conger eel (Kawakami et al., 2003a) and Atlantic salmon (Marchand et al., 2001; Jones et al., 2002). Moreover, our analyses of the publicly available sequence from the *Fugu* genome indicate the presence of two distinct TR α genes and a single TRβ gene in this species also (data not shown).

The Atlantic halibut TR gene that we had previously isolated shared highest similarity with Japanese flounder TRαA.

RT-PCR with degenerate primers was used to obtain partial clones of other halibut TRs. The degenerate primers utilised flank the sequences that encode the hinge (D) region and part of the ligand binding (E) domain of TR. When used with cDNA prepared from metamorphosing halibut, the primers amplified fragments of 358bp and 331bp. Both fragments were subcloned and sequenced, and used in blast searches and phylogenetic analyses. The 358bp PCR fragment was unambiguously identified as TRβ. The 331bp fragment was found to encode a TRα different from the one previously isolated (Llewellyn et al., 1999) and showed highest similarity to Japanese flounder TRαB, suggesting the amplified 331bp fragment represents halibut TRαB. The larger size of the TRβ PCR product (358bp) is the result of a 27bp insertion present in most teleost TRβ genes described to date (see below), but absent in all TRαs, all tetrapod TRβs, and TRs from dogfish and lamprey (GenBank accession numbers ABC49722, ABC49723, AAO53268, AAO532689). The amino acid sequences encoded by the cloned halibut TRαB and TRβ, together with the corresponding region of halibut TRαA, are shown in Fig 1.

3.2 Expression of TRs during metamorphosis

Real time PCR was used to quantify expression of halibut TRαA, TRαB and TRβ during metamorphosis. The larvae used were staged as described previously (Sæle et al., 2004) and expression levels were normalised with respect to the expression of 18s rRNA. The

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results of these analyses are presented in Fig. 2. TRαA is expressed in all stages with an

apparent peak at stage 9 (metamorphic climax). However, there was considerable

variability between individual larvae at this stage and this peak was not statistically

significant. TRαB is expressed throughout metamorphosis with a peak at stage 7 and a

marked, statistically significant decline (p<0.05) in expression on the completion of

metamorphosis at stage 10. TRβ shows the most dramatic expression profile with a clear,

statistically significant peak (p<0.05) during stage 9.

3.3 Atlantic halibut whole body TH during metamorphosis

T_3 and T_4 contents were determined in larvae from stage 5-10 and the results are shown

in Fig. 2. T_4 levels are low and relatively constant from stage 5 to stage 8 larvae and low

levels of T_3 are also detected and decline significantly (p<0.05) from stage 5 to stage 8

larvae. Subsequently, T_3 levels peak dramatically at metamorphic climax (stage 9) and

are still significantly (p<0.05) elevated in post-metamorphic (stage 10) animals. Prior to

stage 9 T_4 is considerably more abundant that T_3, but this relationship is inverted in

stages 9 and 10.

3.4 TRβ splice variants

In teleosts, TRβ splice variants have been identified that involve the linker region of TRβ

(Yamano and Inui, 1995; Marchand et al., 2001; Kawakami et al., 2003b), which includes

the 27 nucleotide fish TRβ-specific insert described above (Fig. 1). To determine

whether any such splice variants arise during Atlantic halibut metamorphosis, RT-PCR

was performed using RNA from all larval stages and a variety of adult tissues with

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primers that flank the TRβ linker region. Each sample generated two discrete PCR

products (Fig. 3a), the smaller of which was of the size predicted from the sequence of

the TRβ clone (including the 27bp insert) while the larger fragment contained an

additional 60bp. Sequence analysis of the two fragments revealed that the smaller

fragment corresponded exactly to that of the Atlantic halibut TRβ sequence described

above, and that the larger fragment was identical to the smaller with the exception of a 60

bp insert directly upstream of the 27bp fish TRβ-specific insert (see Fig. 3c). This

resembles the situation in Japanese flounder where two similar TRβ splice variants occur.

We therefore follow the flounder nomenclature in designating the smaller variant TRβA

and the larger TRβB.

The real-time PCR analysis presented in Fig. 2 utilised TRβ primers that recognise

sequences common to both splice variants. It should be noted that the PCRs shown in

Fig. 3a are not quantitative and do not allow comparison of expression levels between

samples but do allow estimation of the ratio of the two splice variants which, as in a

competitive PCR, should be independent of cycle number. Therefore, to determine

whether there was a change in the ratio of the TRβ isoforms during metamorphosis a

more extensive RT-PCR analysis using RNA from 5 replicate larvae from each stage of

metamorphosis was performed. Both splice variants were found in all developmental

stages, with the ratio between the forms essentially constant and the smaller isoform

predominating (Fig. 3b). In contrast, Fig. 3a indicates that the ratio of the TRβ splice

variant isoforms varies considerably between different adult tissues, with the larger form

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making a more significant contribution to total TRβ levels in brain, cardiac muscle, red muscle and white muscle.

3.5 Structure of teleost TRβ genes

Clearly there is some variability in the TRβ isoforms found in different fish species, and between fish and tetrapods. Teleost TRβ genes characterised to date contain a 27bp insert missing from the corresponding gene from tetrapods (as well as from TRα genes from both fish and tetrapods). However, in Atlantic salmon (Marchand et al., 2001) this insert is absent from some transcripts due to alternative splicing. Similarly the 27bp insert appears present in some but not all TRβ transcripts from zebrafish (*Danio rerio*) since two zebrafish TRβ cDNA sequences have been deposited in GenBank that either include or lack the insert sequence (accession numbers NM_131340 and AF302242). Two alternatively spliced forms of TRβ have also been found in Japanese flounder (Yamano and Inui, 1995) and Atlantic halibut (this study), but in contrast to Atlantic salmon and zebrafish, they both contain the 27bp fish-TRβ specific insert with the larger isoform having an additional insert of 60bp immediately prior to it.

In an attempt to determine the origin of the different TRβ splice variants, we analysed the sequence of *Fugu* Mayffold M001021, which includes the TRβ gene. Comparison of the genomic sequence of *Fugu* TRβ to the full length TRβ cDNA (Nowell et al., 2001) from sea bream (*Sparus auratus*) and the partial cDNA from Atlantic halibut using Spidey (http://www.ncbi.nlm.nih.gov/spidey/) indicated that the coding sequence of *Fugu* TRβ is split into 9 exons (not shown). Exon 6 encodes both the 27bp fish-specific insert and the

larger splice variant (which includes the extra 60bp) present in both Atlantic halibut and Japanese flounder. This larger variant arises because the upstream intron has two potential 3′ (acceptor) splice sites. When the more downstream site is used the resulting mRNA contains the 27 bp fish specific insert. However, both the 27bp insert and the additional 60 nucleotides immediately upstream of it are included in transcripts generated by use of the more upstream splice site. The sequences of the alternatively spliced region of the Atlantic halibut TRβs, and the proposed origin of the alternatively spliced variants, are shown in Fig. 3c and Fig. 3d. Although the gene structure shown in Fig. 3d is based on analysis of the *Fugu* genome, we have obtained strong evidence that this organisation is conserved in Atlantic halibut. PCR reactions were performed on Atlantic halibut genomic DNA with primers that recognise sequences in exon 7 and the 5′ region of exon 6 (GTATTTGCCATGGACGCTTG and CGAGGAAGCTATGCTCTCTAATG respectively). This led to the isolation and subsequent sequencing of a 448 bp fragment that, as predicted, includes a contiguous sequence corresponding to the 27bp and 60bp TRβ-specific inserts (i.e. the sequences designated exon 6a and exon 6b in Fig. 3D), followed by a 325bp intron and a short region of exon 7 (data not shown).

4. Discussion

4.1 The TR family in fish

A confusing collection of TR sequences have been described in fish including some isoforms that represent the products of distinct genes and others that arise through

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alternative splicing. Two TRα genes have been described in Japanese flounder, conger eel and Atlantic salmon and searches of the genome sequence of *Fugu rubripes* indicate two TRα genes in this species also. However, only one TRβ gene appears to be present in the *Fugu* genome and only one TRβ gene has been found in most teleost species, although multiple isoforms may be produced through alternative splicing. An exception is the conger eel which has at least two TRβ genes. In an attempt to determine relationships between the different receptor isoforms in teleosts, phylogenetic analyses were performed using teleost TR protein sequences (the regions corresponding to the amino acid sequences shown in Fig. 1). Unfortunately parsimony, maximum likelihood and distance methods, each gave trees with differing topologies and with branches often having very weak bootstrap support (not shown). However, all trees showed TRβs forming a separate branch, with the two conger eel TRβ genes grouped together. It therefore seems most likely that, following the whole genome duplication believed to have occurred during teleost evolution (Jaillon et al., 2004), duplicated TRα genes were retained whereas one of the duplicated TRβ genes was lost, with a subsequent TRβ gene duplication occurring in the anguilliform lineage.

Alternative splicing of TRβ transcripts is commonly seen in teleosts, with the TRβ genes of Atlantic salmon, zebrafish, Japanese flounder, Atlantic halibut and conger eel each producing two or more isoforms through alternative splicing. However, the alternative splicing products from the Atlantic salmon and zebrafish gene differ somewhat from those of the other species. Atlantic salmon and zebrafish produce alternatively spliced TRβ transcripts that either lack or possess the 27bp teleost-specific insert. In contrast, all

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TRβ mRNAs in Japanese flounder, Atlantic halibut and conger eel possess the 27bp

insert, but each of these species also produces an additional TRβ splice variant that

includes an additional 60 nucleotides immediately upstream of the 27 nucleotide insert

referred to above. Our analysis of the TRβ gene from *Fugu* shows the presence of a fish-

specific TRβ exon and indicates that the splice variants in Atlantic halibut, Japanese

flounder and Conger eel most likely arise due to the use of alternative 3′ splice sites (Fig.

3). There is as yet no evidence as to whether there are functional differences between the

TRβ splice variants or between TRαA and TRαB.

4.2 Whole body TH levels in metamorphosing larvae

The variations during Atlantic halibut ontogeny of T_4 and T_3 in the present study are

similar, but not identical, to those found in a previous study of the ontogeny of the

pituitary thyroid axis in the Atlantic halibut (Einarsdottir et al., 2006). In the present

study, much lower variability in TH levels between individuals of the same stage was

obtained and a significantly higher concentration of both T_4 and T_3 was detected at

metamorphosis (stage 9). The reason for the difference between the two studies may be a

consequence of the careful collection of individual samples instead of pools, use of a

validated staging scheme (Sæle et al., 2004) and the introduction of a heat treatment step

prior to RIA of samples. Although previous studies of THs during ontogeny of salmonids

(de Jesus and Hirano, 1992; Tagawa and Hirano, 1987; Leatherland et al., 1989), striped

bass (*Morone saxatilis*) and conger eel (Brown et al., 1987; Yamano et al., 1991b) have

not reported problems of interassay variability of T_3 in the same samples or between

different samples, this was found to be a difficulty with the Atlantic halibut larval

extracts. The use of a heat treatment is an indispensable step in sample preparation for the analysis of THs in human plasma samples (Neeley and Alexander, 1983) in order to overcome the "matrix" effect caused by complexity of biological samples (Selby, 1999). In the present study, after extensive validation sample heat treatment was introduced prior to assaying larval extracts.

T_4 levels were low and showed relatively little variation up until metamorphosis (stage 9) and immediately after metamorphosis (stage 10) when it was significantly higher than all previous developmental stages. A dramatic increase in T_3 occurred at metamorphic climax (Fig, 2). This is not surprising given that T_3 is the more potent form of thyroid hormone and that thyroid hormone treatment is known to drive metamorphosis. These observations provide further confirmation of the importance of thyroid hormones in flatfish metamorphosis and are remarkably similar to observations in the amphibian *Xenopus laevis* in which both T_4 and T_3 are elevated during metamorphosis. However, the results contrast somewhat to those in Japanese flounder (De Jesus et al., 1991) in which T_4 peaks but T_3 does not during metamorphosis. It would be of interest to reassesses the concentration of THs previously reported in a number of teleosts after the introduction of a heat treatment step in sample preparation.

4.3 Expression of TRs during metamorphosis of Atlantic halibut

Using quantitative PCR, TRαB was found to be expressed at similar levels throughout most stages of metamorphosis with a clear decline in expression in metamorphosed juveniles (stage 10). TRαA appears to peak at stage 9 as does TRβ, with the latter but

not former peak being statistically significant. The TRβ measurements reflect the total amount of TRβ mRNA, i.e. both the TRβA and TRβB splice variants. RT-PCR analysis showed that the ratio of the small and large forms is essentially constant throughout metamorphosis, with the smaller (TRβA) form predominating.

The TR expression profiles we report for Atlantic halibut are similar, but not identical, to those described in two other flatfish, turbot (*Scophtalmus maximus*) and Japanese flounder (Marchand et al., 2004; Yamano and Miwa, 1998), with the changes in TR gene expression in all three flatfish species being markedly less dramatic than those that occur during amphibian metamorphosis (Yaoita and Brown, 1990). TRαB expression appears to contribute a greater proportion of total TR in Atlantic halibut than in Japanese flounder (no data on TRαB is available from turbot), and, more significantly, in Atlantic halibut it is TRβ that shows the most distinct peak during metamorphosis whereas in Japanese flounder and turbot it is TRαA. In turbot, TRβ expression has been reported as being low and relatively constant throughout metamorphosis, increasing thereafter. In Japanese flounder TRβ levels are elevated at metamorphic climax, but reach still higher level in post-climax individuals.

In summary, in the flatfish species analysed to date there is appreciable expression of TRs prior to metamorphosis with TRβ and/or TRαA peaking around metamorphic climax. TRα2 expression in Atlantic halibut and Japanese flounder is more constant although in both species there is a decline in transcript levels after the completion of metamorphosis. The expression profiles of TRs in flatfish are consistent with the model

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previously proposed for *Xenopus laevis* (Sachs et al., 2000) in which, prior to metamorphosis, TRs in the absence of ligand act as repressors of metamorphosis-associated genes. As THs accumulate the receptors become associated with ligand and activate rather than repress their target genes. The change in receptor expression detected during the present study may indicate that TRαA and TRβ are themselves the products of TH-target genes thus explaining their increased expression during metamorphic climax. Consistent with this, studies in zebrafish suggest that both TRαA and TRβ are TH-inducible (Liu et al., 2000). Further investigation is needed to fully understand the roles of THs in flatfish metamorphosis, especially the question of how THs can evoke a response that leads to body asymmetry. Important goals for future investigation will be to determine the expression profiles of the deiodinase enzymes that regulate TH levels in peripheral tissues and to identify genes that are induced or repressed at metamorphosis by THs.

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Figure Legends

Fig. 1. Partial amino acid sequences of TRαA, TRαB and TRβ from Atlantic halibut. An asterisk (*) indicates a residue identical to that of TRαA. Hyphens (-) represent spaces inserted to maximise similarity.

Fig. 2. The top three panels show the relative expression of TRαA, TRαB and TRβ. The bottom two panels show changes in whole-animal T_4 and T_3 content (pg/mg) during halibut metamorphosis. Bars indicate standard error. In these analyses, stages with the same letter are not significantly different, those with different letters are significantly different ($p<0.05$).

Fig. 3. A) Analysis by RT-PCR of expression of splice variants of TRβ during larval development and in adult tissues. The 197bp and 257bp PCR products correspond to TRβA and TRβB respectively. B) Ratio of TRβA:TRβB splice variants in different developmental stages as determined by RT-PCR analysis of RNA from five individual larvae of each stage. Error bars indicate standard error. C) cDNA sequences of the alternatively spliced region of the halibut TRβ isoforms TRβA and TRβB. The 27bp fish TRβ-specific insert is shown in bold and the additional nucleotides present in TRβB are underlined. D) Putative organisation of the Atlantic halibut TRβ gene, based on analysis of the TRβ sequence from *Fugu rubripes*. Rectangles represent exons with numbers corresponding to exon number in the *Fugu* gene. Exon 6 is alternatively spliced, with shading showing the 27bp fish-specific TRβ insert and the stippled box representing the additional 60 nucleotides present in TRβB. Dotted and dashed lines show the splicing patterns that generate TRβA and TRβB respectively. The arrows above exon 6 indicate alterative 3′ splice sites; the horizontal, double headed arrow indicates the region for which halibut genomic sequence has been obtained.

28

Figure 1

Figure 1

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                10          20          30          40          50          60
                |     |     |     |     |     |     |     |     |     |     |
TRαA   SVGMAMDLVL DDSKRVAKRR LIEENREKRK REEMVRTLQV RPEPDTAEWE LIRMATDAHR
TRαB   A********* ********** *******R** K****K***N ****TG**** ****V*E***
TRβ    V****T**** N****L***K *******R*R K**LQK*VWD *L**TQE**D ****V****M


                70          80          90          100
                |     |     |     |     |     |     |     |     |
TRαA   HTNAQGSSWK QKRKFL---- -----SDDIG QGPMVPTSDG DKVDL 96
TRαB   ******AQ** ******---- -----P*K** *S*VA***** ***** 96
TRβ    A*****NH** ******SAAG VKEDKP*E** *AS*AN*PE* N***I 105
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Figure 2

Figure 2

Figure 3

A

B
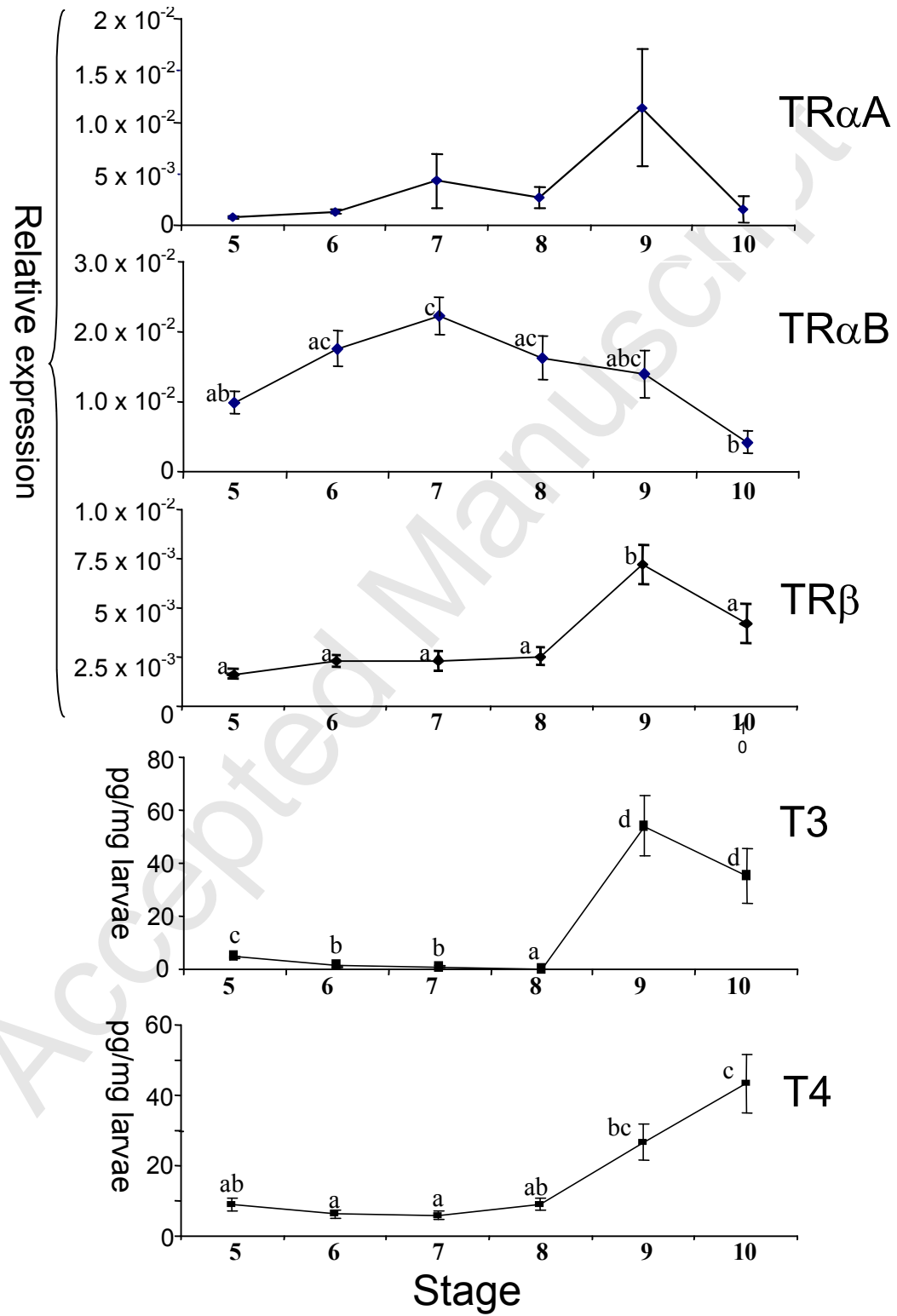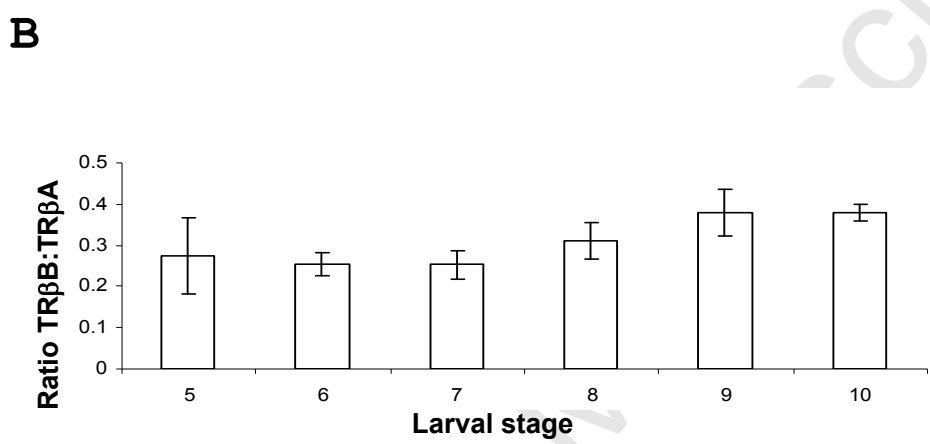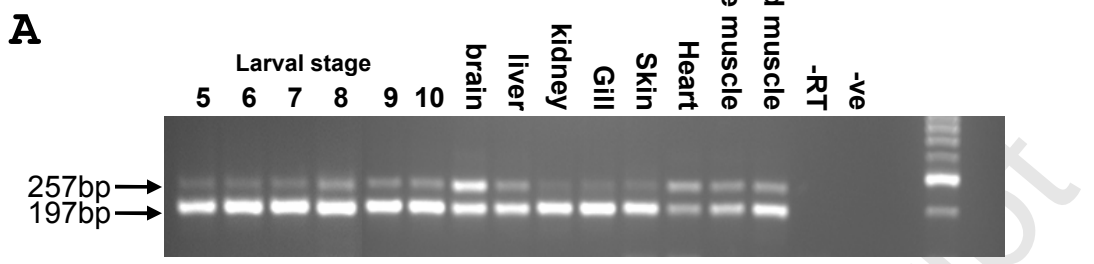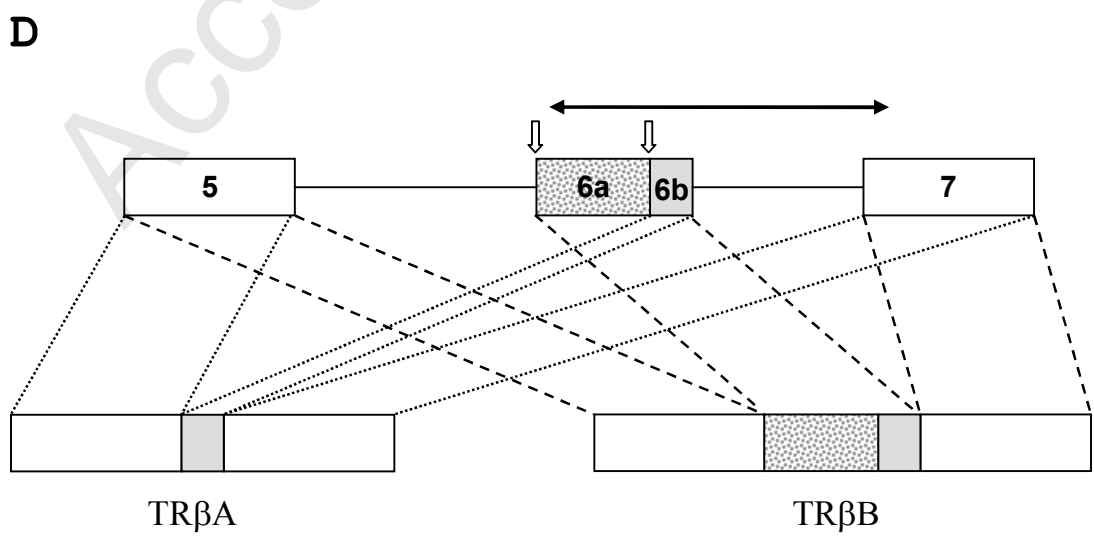

C

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TRβA    GGCCACGAATGCCCAGGGCAACCACTGGAAGCAGAAGCGGAAATTCCTG----------- 228
TRβB    GGCCACGAATGCCCAGGGCAACCACTGGAAGCAGAAGCGGAAATTCCTGGTCGAGGAAGC 240

TRβA    ------------------------------------------------AGTGCAGCGGG 240
TRβB    TATGCTTCTTAATGAAATAACATGTAATTTATTCTATACTTCTGACCAGAGTGCAGCGGG 300

TRβA    GGTGAAGGAAGATAAGCCTGAGGAAATTGGTCAAGCGTCCATGGCAAATACACCTGAAGG 300
TRβB    GGTGAAGGAAGATAAGCCTGAGGAAATTGGTCAAGCGTCCATGGCAAATACACCTGAAGG 360
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D