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Regulation of troponin T expression during muscle development in sea bream *Sparus auratus* Linnaeus: the potential role of thyroid hormones

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Summary

In the sea bream *Sparus auratus* three stage-specific *fast troponin T* (*fTnT*) isoforms have been cloned and correspond to embryonic-, larval- and adult-specific isoforms. Characterisation, using database searches, of the putative genomic organisation of *Fugu rubripes* and *Tetraodon nigroviridis fTnT* indicates that alternative exon splicing in the 5′ region of the gene generates the different isoforms. Moreover, comparison of teleost *fTnTs* suggests that alternative splicing of *fTnT* appears to be common in teleosts. A different temporal expression pattern for each *fTnT* splice variant is found during sea bream development and probably relates to differing functional demands, as a highly acidic embryonic form (pI 5.16) is substituted by a basic larval form (pI 9.57). Thyroid hormones (THs), which play an important regulatory role in muscle development in flatfish and tetrapods, appear also to influence *TnT* gene expression in the sea bream. However, THs have a divergent action on different sea bream *TnT* genes and although the slow isoform (*sTnT1*) is TH-responsive, *fTnT*, *sTnT2* and the itronless isoform (*iTnT*) are unaffected. The present results taken together with those published for flatfish seem to suggest differences may exist in the regulation of larval muscle development in teleosts.

Key words: fast muscle, slow muscle, splice variants, teleost, temporal gene expression profile, thyroid hormones.

Introduction

Fish are an interesting alternative model in which to study muscle development and growth. In contrast to mammals and birds in which muscle hyperplasia stops at, or shortly after, birth, in fish it continues well into adulthood. Further differences also occur in the development and organisation of embryonic, larval and adult fish muscle. The structure, organisation and fibre type of fish muscle has been extensively studied using light and electron microscopy (Johnston, 1994; Koumans et al., 1991; Mascarello et al., 1995; Patruno et al., 1998; Rowlerson et al., 1995; Rowlerson et al., 1997; Veggetti et al., 1993; Watabe, 1999). However, with the exception of the zebrafish *Danio rerio* molecular studies of fish muscle and regulation of its development and turnover are far less numerous.

Thyroid hormones (THs), important regulatory factors during development, are known to be important in post-natal muscle development in vertebrates. It has been shown that in rats full differentiation of skeletal muscle phenotype is only achieved with appropriate thyroid hormone levels (Vadaszova et al., 2004). Moreover in rats, T3 treatment significantly changes the muscle contractile properties and myosin heavy chain (MHC) isoform expression in both fast and slow muscle types (Adams et al., 1999; Larsson et al., 1994; Larsson et al., 1995; Soukup and Jirmanova, 2000). In both thyroidectomised and thiourea-treated chick embryos prolonged expression of slow MHC and myosin light chains (MLC) and inhibition of neonatal fast MHC isoforms occurs in the fast *posterior latissimus dorsi* muscle, whereas in the slow *anterior latissimus dorsi* muscle slow muscle differentiation is delayed and expression of embryonic fast MHC isoforms persist and there is induction of fast MLCs (Gardahaut et al., 1992). During amphibian metamorphosis, larval muscle fibres die and give place to newly formed adult fibres and associated MHC isoform switching, which seems to be under the control of THs (Chanoine and Hardy, 2003). In zebrafish (Liu and Chan, 2002), TH treatment makes muscle tissue less compact and in developing larvae of *Epinephelus coioides* differences in locomotion between control and TH-treated fish are observed (de Jesus et al., 1998). TH-induced and spontaneous metamorphosis of the flounder *Paralichthys olivaceus*, causes biochemical changes in muscle proteins. In particular, different protein isoforms of fast troponin T (fTnT), the tropomyosin-binding subunit of the striated muscle troponin complex, are present in muscle from pre- and post-metamorphic larvae (Yamano et al., 1991).

Fast TnT has been extensively studied in mammals and birds and diverse fTnT protein isoforms have been identified, which arise as a consequence of alternative splicing of the 5′ region of the gene (Perry, 1998). In rat (Bucher et al., 1999) and mouse

(Jin et al., 1998a; Wang and Jin, 1997) the *fast skeletal TnT* (*fTnT*) gene is composed of 19 exons and in quail it is composed of 25 exons (Bucher et al., 1999). Isoform complexity of mammalian and avian *fTnT* genes is increased by the mutually exclusive nature of exons 16 and 17 during splicing. In these organisms the latter process appears to account for developmentally specific isoform expression and an isoform containing exon 17 is the major expressed exon in neonatal fast muscle, whereas exon 16 is present in the majority of postnatal *fTnT* isoforms, which are the predominant isoforms in adult white muscle (Bucher et al., 1999; Jin et al., 1998b; Jozaki et al., 2002; Wang and Jin, 1997).

The evidence from mammals and birds provides a possible explanation for the switch in expression of *fTnT* isoforms in embryonic/larval and juvenile fish muscle. However, in the only molecular study of *TnTs* in fish (although two *fTnT* genes with a similar organisation to higher vertebrate *fTnT* genes were identified in zebrafish) no evidence of alternative splice variants was reported (Hsiao et al., 2003). The present study reports, for the first time in a teleost, the sea bream *Sparus auratus*, cloning and characterisation of three different cDNA encoding different *fTnT* isoforms, which are the product of a single gene. Alternative splicing of a single gene appears to give rise to the three isoforms identified, one of which is a larval-specific isoform and generates a putative protein with markedly different biochemical characteristics. In contrast to the flounder, the sea bream does not undergo such a radical metamorphosis, raising questions about the potential role of THs in isoform switching during the larval/juvenile transition of this species. In order to assess the involvement of THs in regulation of *TnT* gene transcription, the ontogeny of isoform switching was related to thyroid hormone levels in developing sea bream. In addition, to further assess the effect of THs on *TnT* expression, experiments were performed in which T3 was administered to sea bream larvae and juveniles and the expression of *slow TnT* (*sTnT*) and *fTnT* isoforms was analysed.

Materials and methods

fTnT cDNA library screen

A lambda phage cDNA library made from sea bream *Sparus auratus* L. larvae aged 20–100 days post-hatch [d.p.h. (Nowell et al., 2001)] plated at a density of 1000 plaque forming units (pfu) was screened at low stringency with a 891-bp partial clone of an putative skeletal muscle *sTnT* from *Hippoglossus hippoglossus* (G.E.S., unpublished results). Nitrocellulose membranes containing plaque DNA were prehybridised for 2 h at 50°C in hybridisation solution alone (6× SSC, 0.1% SDS, 100 μg ml^{-1} tRNA, 5× Denhardt's solution) and then overnight at low stringency (50°C) in hybridisation solution to which ^{32}P-labelled putative skeletal muscle *sTnT* probe, prepared by random priming (Megaprime, random labelling kit, Amersham Biosciences, UK), had been added. Low stringency washes were carried out, by washing membranes twice for 30 min at room temperature in 1× SSC/0.1% SDS solution, followed by two washes of 30 min at 50°C in 1× SSC, 0.1% SDS. The membranes were then exposed overnight at –80°C to Biomax MS film (Kodak, Palo Alto, CA, USA). Positive plaques were isolated, automatically excised into pBluescript SK(+/–) (Stratagene, La Jolla, CA, USA), DNA purified and cDNA clones sequenced to give threefold coverage using BigDye Version 3 (Perkin-Elmer, Berkshire, UK) chemistry and an ABI 3700 sequencer.

Animal and tissue sampling

Adult sea bream, maintained at the Marine research station of the Centre of Marine Sciences (CCMAR, University of the Algarve, Portugal) were anaesthetized in MS-222 (125 mg l^{-1}, Sigma-Aldrich, Madrid, Spain) and killed by decapitation in accordance with National legislation for the welfare of animals. White muscle, red muscle, heart and liver were collected immediately into RNAlater reagent (Sigma-Aldrich, Madrid, Spain) and stored at –20°C until RNA extraction.

Pools of sea bream eggs (*N*=3) were collected at 30% epiboly (12 h post fertilisation – h.p.f.), 90% epiboly (18 h.p.f.), 2-somite stage (24 h.p.f.) and when the most posterior somites had formed (36 h.p.f.). Larvae and juveniles (*N*=3) were collected at hatching (1 d.p.h.) and at 4, 15, 46, 64, 75 and 89 d.p.h. The small size of the sea bream larvae meant that several different pools composed of several larvae (50–100 mg) of the same age were collected for each sample point until 46 d.p.h. and thereafter individual fish were collected and analysed. Larvae and juveniles were anaesthetised in MS-222 (125 mg l^{-1}; Sigma-Aldrich, Madrid, Spain) before being snap frozen in liquid nitrogen and stored at –80°C until use. Classification of sea bream stages in the present study was in accordance with previous studies that used morphology (Loy et al., 1999; Mascarello et al., 1995; Parra and Yufera, 2001; Patruno et al., 1998; Polo et al., 1991; Rowlerson et al., 1995). In general, all stages prior to hatch in sea bream are considered embryos, after hatching until approximately 90 d.p.h. larvae, and thereafter juveniles.

Total RNA extraction

Total RNA was extracted from 100 mg of adult sea bream striated white muscle, red muscle, heart and liver from three different individuals using Tri reagent (Sigma-Aldrich, Madrid, Spain) and following the manufacturer's instructions. In all developmental samples and treated samples, Tri was also used to extract total RNA from triplicate samples of 50–100 mg of pooled sea bream embryos and larvae up until 46 d.p.h. and from triplicate samples of individual fish of 64, 75 and 89 d.p.h.

Northern blot analysis

Total RNA (3 μg) from white muscle, red muscle, heart and liver was fractionated on a 1.5% agarose/5.5% formaldehyde gel run in 1× Mops. RNA was transferred to nylon Hybond-N membranes (Amersham Biosciences, Buckinghamshire, UK) with 10× SSC and cross-linked using UV light (Stratalinker; Stratagene, La Jolla, CA, USA). A 3′ UTR DNA probe was prepared for northern blotting by digesting the putative sea bream larval *fTnT* cDNA isolated in the library screening with *Xho*I and *Sac*I (0.1 i.u μl^{-1}; Promega, Madison, WI, USA). A

probe corresponding to the 3′ UTR of sea bream *fTnT* that would hybridise with all the isoforms arising from the sea bream *fTnT* gene was used.

The membrane was hybridised overnight under high stringency conditions (65°C in 6× SSC, 0.1% SDS, 100 μg ml^{-1} tRNA, 5× Denhardt's solution) with the [^{32}P]dCTP-labelled *fTnTsb* 3′ UTR DNA probe. The membrane was subsequently washed using high stringency conditions (65°C in 1× SSC, 0.1% SDS for 30 min) and exposed for several hours or overnight at –80°C to Biomax MS film (Kodak, Palo Alto, CA, USA).

Identification of fTnT variants in other teleosts

Sea bream *fTnT* cDNA sequences were used to identify and retrieve presumptive homologues of sea bream *fTnTs* in other teleosts using tBLASTX (Altschul et al., 1990) and a number of databases: GenBank (www.ncbi.nlm.nih.gov), zebrafish (www.ncbi.nlm.nih.gov/genome/seq/DrBlast.html), Medaka (*Oryzia latipis*; Medaka_EST_database), *Fugu* (*Fugu rubripes*; http://Fugu.hgmp.mrc.ac.uk) and *Tetratodon nigroviridis* (www.genoscope.cns.fr/externe/tetranew/).

Full-length cDNA were retrieved, translated using BioEdit and protein multiple alignments performed in Clustal X (Thompson et al., 1997). A Pearson multiple comparison analysis was performed to establish identity between the sea bream fTnT isoforms and the fTnT sequences retrieved from the databases.

Putative genomic organisation of sea bream fTnT gene

In order to establish the putative genomic organisation of sea bream *fTnT* a computer-based analysis was carried out using the puffer fish *Fugu rubripes* and *Tetraodon nigroviridis* genomes (Aparicio et al., 2002; Jaillon et al., 2004). The *Fugu* and *Tetraodon* scaffolds giving the most significant hit by tBLASTx analysis (Altschul et al., 1990) with the sea bream *fTnT* sequences were retrieved. Pairwise alignment of sea bream *fTnT* cDNA sequences with the selected *Fugu* and *Tetraodon* scaffolds using Spidey mRNA-to-genome software (Wheelan et al., 2001) permitted identification of the putative exon/intron boundaries of the sea bream gene.

Developmental expression of sea bream fTnT gene – semi-quantitative RT–PCR analysis

A semi-quantitative RT–PCR strategy was employed to analyse the developmental expression of the *fTnT* gene in sea bream. First strand cDNA (20 μl total reaction volume) was synthesised using 0.5 μg total RNA of the different sea bream embryonic, larval and juvenile stages and adult tissues. Before cDNA synthesis, all samples were treated with DNase using the DNA free kit (Ambion, Austin, TX, USA) according to the manufacturer instructions. cDNA synthesis was carried out in 0.05 mol l^{-1} Tris–HCl, pH 8.3, 0.075 mol l^{-1} KCl, 3 mmol l^{-1} $MgCl_2$, 0.01 mol l^{-1} DTT, 1 mmol l^{-1} dNTP, 5 pmol μl^{-1} random hexamer primers, 4 i.u. of RNAse inhibitor (Promega, Madison, WI, USA) and 10 i.u. of Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Synthesis reactions were carried out in an iCycler thermocycler (Perkin-Elmer) for 10 min at 25°C followed by 50 min at 42°C, and synthesis was terminated by heating for 2 min at 70°C. cDNA corresponding to three independent pools (50–100 mg/extract) of sea bream larvae were prepared for each developmental stage and for samples of adult sea bream white muscle, red muscle, heart and liver.

Initial RT–PCR experiments were conducted with *fTnT* to determine optimal cDNA concentration and PCR cycle number, and to ensure that amplification occurred in the logarithmic phase of the reaction. The internal standard selected to normalise the results was the expression of *18s ribosomal RNA* (rRNA).

Amplification of *fTnTsb* was carried out in a 25 μl reaction volume containing ~20 ng of cDNA for each of the samples described and 1.5 mmol l^{-1} $MgCl_2$, 0.1 mmol l^{-1} dNTPs, 1 pmol μl^{-1} of sea bream-specific *fTnT* forward and reverse primer (5′-ACAAGTCCACTCTCACCATG-3′ and 5′-TCTCAATCCTGTCCTTGAGG-3′, respectively) and 0.6 i.u. Taq polymerase (Sigma-Aldrich, Madrid, Spain). Primers were selected to amplify the entire N-terminal region of the fTnTsb protein, which in terrestrial vertebrates suffers alternative splicing (Perry, 1998). The forward primer was located in the 5′ UTR region of the isolated sea bream *fTnT* cDNAs (forward pointing arrow in Fig. A1D). The reverse primer was designed in a constitutively expressed region of the sea bream *fTnT* cDNAs (backwards pointing arrow in Fig. A1D).

The PCR reactions were performed in an iCycler (Perkin-Elmer) thermocycler, using the following cycle; 1 min at 95°C followed by 28 cycles of 30 s at 95°C, 1 min at 56°C and 30 s at 72°C, followed by a final step of 1 min at 72°C. Negative reactions without sample cDNA were also performed.

The housekeeping gene *18s*, was amplified in each sample in a 25 μl reaction containing ~20 ng of cDNA, 1 pmol μl^{-1} of forward and reverse primer (5′-TCAAGAACGAAAGTCG-GAGG-3′ and 5′-GGACATCTAAGGGCATCACA-3′ respectively), 1.5 mmol l^{-1} $MgCl_2$, 0.1 mmol l^{-1} dNTPs and 0.6 i.u. of Taq polymerase (Sigma-Aldrich). The thermocycle utilised was; 1 min at 95°C followed by 16 cycles of 30 s at 95°C, 1 min at 56°C and 30 s at 72°C, followed by a final step of 1 min at 72°C. RT–PCR reaction products (equal volume) from amplified target genes and 18s rRNA were mixed and fractionated on the same agarose gel (2.5%) and analysed by densitometry using LabWorks version 4.5 software (Ultra-Violet Products, Cambridge, UK). Results are expressed as the mean and standard error of three independent samples.

Experiments – T3 treatment

The experiments described comply with the Guidelines of the European Union Council (86/609/EU) and national legislation. Larvae were acclimated to seawater in three 125 l aquaria (100–150 larvae/tank) for at least 2 weeks in an open system (38 p.p.t. salinity, 1162 mOsm kg^{-1} H_2O) with a water temperature of 19±1°C and under natural photoperiod for February in the Algarve. Larvae were fed twice daily on dry food (grade 10 fish pellets, Anivite, Alverca, Portugal).

The objective of the experiments was to alter TH balance in the sea bream larvae and to this end the diet was supplemented with T3 (Sigma-Aldrich). Based upon previous morphological studies (Loy et al., 1999; Mascarello et al., 1995; Parra and Yufera, 2001; Patruno et al., 1998; Polo et al., 1991; Rowlerson et al., 1995) the timing of TH treatment was initiated well before the larvae/juvenile transition, when fish still had a clear larval morphology and before the peak of THs that accompany the larvae/juvenile transition in sea bream (D.M.P., unpublished results). Experiments lasted for 31 days and started when larvae were 57 d.p.h., tank conditions were maintained and each vessel contained approximately 100 larvae and represented a different experimental group (T3 treatment and control). Animals were fed as previously with the exception that food was pre-treated with either T3 dissolved in ethanol (10 mg g^{-1} dry food) or with the vehicle, ethanol, alone (control). No mortality was detected in any of the experimental groups during the experiment. Preliminary trials were conducted to optimise the experimental circuit, hormone dose, duration and route of hormone administration.

Larval samples were collected from each experimental group before feeding at 64, 75 and 89 d.p.h. Larvae were killed with an overdose of MS-222 (Sigma-Aldrich) and larvae (*N*=12) were snap frozen in liquid nitrogen or fixed (*N*=3) in 4% paraformaldehyde (PFA; Sigma-Aldrich) at 4°C overnight. Frozen samples were used for either RT–PCR or to determine TH content by radioimmunoassay (RIA). Fixed samples were washed twice for 5 min with phosphate buffer with 5% Tween 20 (Sigma-Aldrich) and stored in 100% methanol at 4°C. The heads of fixed sea bream were removed and embedded in paraffin and serial 8 μm longitudinal sections were cut and mounted on 3-aminopropyltriethoxysilane (APES)-coated slides.

Sea bream TnT genes in T3-treated animals – semi-quantitative RT–PCR analysis

Expression of the sea bream *fTnT* gene was determined by semi-quantitative RT–PCR as described above or for *sTnT1*, *sTnT2* and *iTnT* genes using a previously established RT–PCR (Campinho et al., 2005). The amount of cDNA included in RT–PCR reactions was assessed using amplification of *18s rRNA*. Reaction products (equal volume) from amplified target genes and *18s rRNA* were mixed and fractionated on the same agarose gel (2.5%) and analysed by densiometry using LabWorks version 4.5 software (Ultra-Violet Products, Cambridge, UK). The results are presented as mean ± standard error (s.e.m.) of three individual samples and statistical differences were assessed by two-way analysis of variance (ANOVA) as described below.

TH extraction and radioimmunoassay

The T4 and T3 content in T3-treated and control animals was extracted and assessed by RIA. Five frozen individual animals per sampling time of each experimental group were extracted in methanol and centrifuged at 1430 ***g*** for 30 min at 4°C. Then, the upper phase was removed, lyophilised, reconstituted in assay buffer (0.01 mol l^{-1} PBS, pH 7.6) and assayed.

Assays for both T3 and T4 were highly specific and reproducible and were performed as previously described (Einarsdóttir et al., 2006) under equilibrium conditions, using T2777 anti-T3 (<0.01% cross reactivity with T4; Sigma-Aldrich) and T2652 anti-T4 polyclonal sera (~3% cross reactivity with T3; Sigma-Aldrich). Results are presented as means ± s.e.m. of five individuals and the existence of significant statistical differences are assessed by two-way ANOVA as described below.

Thyroid follicular activity analysis

In order to analyse thyroid follicle activity the sectioned animal heads were dewaxed in xylene (2× 10 min), rehydrated through an alcohol series (90%, 70%, 50%), washed in phosphate-buffered saline (PBS; KH_2PO_4 1.7 mmol l^{-1}, Na_2HPO_4 5.2 mmol l^{-1}, NaCl 150 mmol l^{-1}) and then stained with Haematoxylin and Eosin for 5 min. Slides were rinsed in deionised water and mounted in PBX. Follicle number and thyrocyte cell height were determined at the junctions of the hypohyal bones in all animals so that comparative analysis could be performed. Cell height of four different thyrocytes per follicle lying 90° from one another were measured and a total of four different follicles per animal where analysed. Mean thyrocyte cell height was measured using a direct method (Kalisnik et al., 1977) without applying any correction factor for shrinkage. The results are expressed as mean of 16 measurements per animal, with three individual animals at each time point for each treatment analysed. Results are reported as mean ± s.e.m. and a two-way ANOVA was used to test for statistical differences as described below.

Statistical analysis

The data arising from semi-quantitative RT–PCR of *fTnT* developmental ontogeny, *TnT* genes, thyroid hormone concentrations and follicle parameters in control and T3-treated fish were each assessed by two-way ANOVA. If statistically significant differences were detected between treatments, a Tukey's HSD multiple comparison test was applied. All the statistical analysis was performed using Sigma Stat software version 3 (SPSS, Chicago, IL, USA). Differences were considered statistically significant at $P<0.05$.

Results

Characterisation of sea bream fast TnT isoforms

Three different full-length cDNAs for the sea bream *fast TnT* gene (*fTnTsb*) were isolated from a sea bream larval library and their identity assigned after tBLASTx analysis (Altschul et al., 1990), as sea bream embryonic (*efTnTsb*; DQ473445), larval (*LfTnTsb*; DQ473444) and adult (*afTnTsb*; DQ473443) *fast TnT*, respectively (Fig. A1). Clustal X nucleotide (nt) sequence analysis (Thompson et al., 1997) indicated that all sea bream *fTnT* clones isolated most probably result from alternative splicing of the same gene (Fig. A1D). An additional tBLASTx search (Altschul et al., 1990) performed against the *Fugu* database (http://*Fugu*.hgmp.mrc.ac.uk) using all sea bream *fTnT* sequences gave a highly significant match with a single

mayfold, M0001617 (http://*Fugu*.hgmp.mrc.ac.uk). In the Unigene database, mayfold M0001617 gave the most significant hit with the human *fTnT* locus.

The *efTnTsb* cDNA is 1107 base pairs (bp) and contains a 39 bp 5′ untranslated region (UTR) and a 193 bp 3′ UTR. The ATG translation start site (bold in Fig. A1A) is located at nucleotide (nt) 40 and spans a coding region of 860 nt, terminating in a TAG termination codon at nt 900 (bold in Fig. A1A). The cDNA encodes a putative protein of 287 amino acids (aa) (Fig. A1A) with a predicted molecular mass of 33.8 kDa and a pI of 5.16 (Wilkins et al., 1998). The cDNA contains a well-conserved Kozak sequence (underlined in Fig. A1A) as well as a consensus polyadenylation signal just before the beginning of the poly(A) tail (double underlined in Fig. A1A).

The presumptive adult *afTnTsb* cDNA is 973 bp and contains a coding region that spans 695 bp from the ATG translation start site at nt 68 until the TGA termination codon at nt 763 (bold in Fig. A1B). The *afTnTsb* encodes a putative 232 aa protein (Fig. A1B) with a predicted molecular mass of 27.8 kDa and a pI of 9.39 (Wilkins et al., 1998). The 5′ UTR of *afTnTsb* has 67 nt and the 3′ UTR of 193 nt is identical in sequence and length to the 3′ UTR of *efTnTsb* (Fig. A1B,D). The sea bream cDNA *LfTnTsb* is 1006 bp and includes a 5′ UTR region of 108 nt, and a 3′ UTR region of 193 nt identical in size and sequence to the other two sea bream *fTnT* cDNAs isolated (Fig. A1C,D). The *LfTnTsb* cDNA has a presumptive coding region of 686 nt starting at an ATG translation start site at nt 109 until the TAG termination codon at nt 795 (bold in Fig. A1C) and encodes a predicted protein of 229 aa with a molecular mass of 27.2 kDa and pI of 9.57 (Wilkins et al., 1998).

Clustal X multiple sequence alignment (Thompson et al., 1997) of the isolated sea bream *fTnTs* revealed that *efTnTsb* cDNA shares 78% and 82% nt sequence identity with *LfTnTsb* and *afTnTsb*, respectively. The isoforms *afTnTsb* and *LfTnTsb* share 95% sequence identity. Sequence comparison of the deduced sea bream fTnT proteins revealed that efTnTsb shares 80 and 81% sequence identity with LfTnTsb and afTnTsb, respectively (Table 1), and that the latter two deduced proteins share 99% sequence identity. The differences in sequence conservation between the sea bream *fTnT* cDNA arise as a consequence of localised insertions or deletions, as the remainder of the sequence is 100% conserved, indicating the different forms are probably generated by alternative splicing (Fig. A1D). The *efTnTsb* cDNA contains an insertion of 146 bp in the first third of the 5′ region that corresponds to a putative embryonic exon encoding 55 amino acids, of which 27 residues are glutamic acid and this accounts for the significantly lower pI of the deduced protein (Fig. A1A and Fig. 1). The *efTnTsb* cDNA shares an additional 9-nt putative exon with *afTnTsb* but not with *LfTnTsb* (Fig. A1D). This putative exon is located just before the putative embryonic exon in *efTnT* and is in-between the identical regions in all *fTnTsb* isoforms (Fig. A1D) and encodes three amino acids, EYD (Fig. 1). The differences observed between the three different *fTnTsb* cDNAs are not solely located in the coding region. Although the nt sequences of the 5′ UTR of *afTnTsb* and *LfTnTsb* are identical (Fig. A1D), in *efTnTsb* only nt 23–49, which precede the ATG translation start site, are identical.

Northern blot analysis

The 3′ UTR probe of *efTnTsb* cDNA used for northern blotting hybridises with all *fTnT* isoforms identified in sea bream. In northern blots no *fTnT* transcripts were detected in adult heart or liver and in red muscle transcripts were present in low abundance and were only detected after overnight exposure of films (Fig. 2, lane 2). Comparison of the relative abundance of *fTnT* transcripts in sea bream white and red muscle reveals that transcripts are most abundant in the former tissue (Fig. 2). This agrees with results in tetrapods in which *fTnT* is exclusive to white muscle.

Identification of fTnT variants in other teleosts

Sequences homologous to the sea bream *fTnT* cDNAs exist in zebrafish, cod *Gadus morhua*, Atlantic salmon *Salmo salar*, medaka *Oryzias latipes* and *Tetraodon nigroviridis* (Fig. 1). Multiple sequence alignment of the predicted amino acid sequence of the retrieved *fTnT* cDNAs demonstrated that fTnT isoforms also occur in other teleosts (Fig. 1). In zebrafish a second *fTnT* gene was identified confirming previous reports (Hsiao et al., 2003).

Tetraodon is the only teleost in which three isoforms

Table 1. *Protein sequence similarity matrix after Clustal X multiple alignment*

	efTnTsb	afTnTsb	LfTnTsb	efTnTtn	LfTnTtn	afTnTtn	fTnTmd-2	fTnTmd-1	fTnTss	fTnTgm	fTnTazf	fTnTbzf-2	fTnTbzf-1
efTnTsb	ID	0.808	0.797	0.878	0.738	0.752	0.721	0.731	0.675	0.711	0.668	0.672	0.682
afTnTsb		ID	0.987	0.754	0.913	0.931	0.892	0.905	0.836	0.879	0.827	0.831	0.841
LfTnTsb			ID	0.762	0.925	0.918	0.903	0.892	0.836	0.891	0.830	0.842	0.828
efTnTtn				ID	0.823	0.811	0.726	0.718	0.654	0.702	0.659	0.676	0.669
LfTnTtn					ID	0.982	0.882	0.870	0.793	0.852	0.800	0.820	0.806
afTnTtn						ID	0.875	0.887	0.797	0.845	0.801	0.814	0.824
fTnTmd-2							ID	0.986	0.801	0.860	0.834	0.825	0.811
fTnTmd-1								ID	0.801	0.849	0.831	0.814	0.824
fTnTss									ID	0.857	0.784	0.787	0.784
fTnTgm										ID	0.809	0.813	0.799
fTnTazf											ID	0.834	0.828
fTnTbzf-2												ID	0.982
fTnTbzf-1													ID

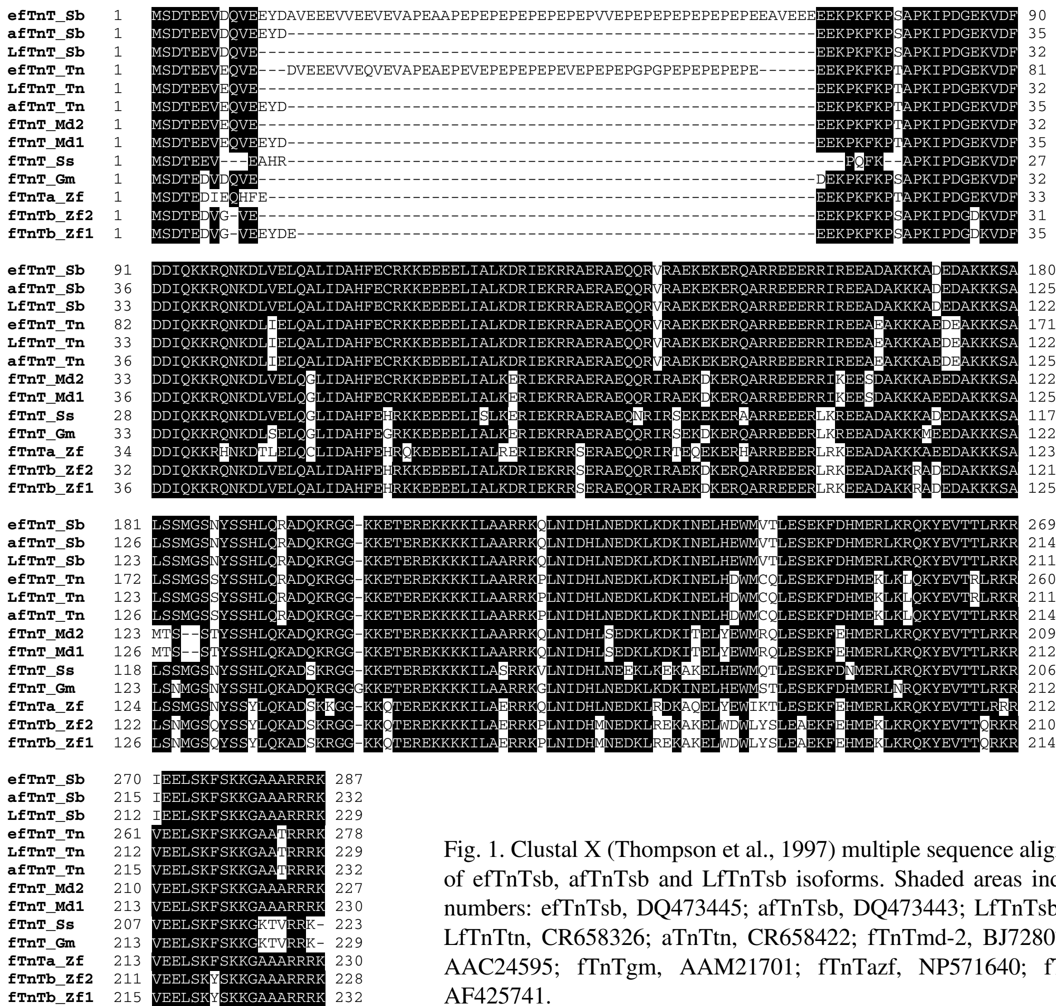

Fig. 1. Clustal X (Thompson et al., 1997) multiple sequence alignment of putative protein sequences of efTnTsb, afTnTsb and LfTnTsb isoforms. Shaded areas indicate identical residues. Accession numbers: efTnTsb, DQ473445; afTnTsb, DQ473443; LfTnTsb, DQ473444; efTnTtn, CR660426; LfTnTtn, CR658326; aTnTtn, CR658422; fTnTmd-2, BJ728074; fTnTmd-1, BJ729852; fTnTss, AAC24595; fTnTgm, AAM21701; fTnTazf, NP571640; fTnTbzf-2, BC065452; fTnTbzf-1, AF425741.

matching the three sea bream fTnT cDNAs were identified. In the medaka (*fTnTmd-1* and *-2*) and zebrafish (*fTnTbzf-1* and *-2*) two isoforms are found that seem to correspond to *afTnTsb* and *LfTnTsb* cDNAs, respectively (Fig. 1). The present results support the notion of alternative splicing of the *fTnT* gene in sea bream, medaka, *Tetraodon* and zebrafish. In each of these species the protein predicted isoforms differ only by the presence or not of the peptide EYD or EYDE (aa 11–14 in Fig. 1). Overall the level of conservation between these fTnT isoforms in the same species is approximately 98% and between the two forms in different species is always greater than 83% (Table 1). However, when the afTnTsb and LfTnTsb isoforms in sea bream are compared to the other teleost fTnT, two groups are evident on the basis of sequence similarity. The *Tetraodon* and medaka (advanced teleosts) share respectively, 92% and 90% identity with sea bream afTnT and LfTnT isoforms (Table 1), whereas the more ancient teleosts, zebrafish, *S. salar* and *G. morhua* share ~82%, ~83% and ~86.6% identity, respectively, to afTnTsb and LfTnTsb (Table 1).

Comparison of the embryonic fTnT isoforms identified in sea bream and *Tetraodon* revealed that they share 87.8% identity and are most dissimilar in the embryonic specific exon, which is 54 aa long in sea bream and 47 aa in *Tetraodon* (Fig. 1). The embryonic-specific exon in both species encodes a proline- and glutamic acid-rich sequence and this causes a change in the predicted pI of fTnT from basic to acid and presumably causes a change in protein function. The sea bream embryonic-specific exon (encodes amino acid 15–69; Fig. 1) is longer than the same region in *Tetraodon* (amino acid 12–59; Fig. 1) and encodes a unique C-terminal sequence (EAVEEE; aa 64-69; Fig. 1). An additional difference is that sea bream efTnT has the alternatively spliced peptide EYD whereas in *Tetraodon* all the clones identified coding efTnT did not posses this peptide (Fig. 1). Interspecies comparison of sea bream and *Tetraodon* efTnT isoforms to the presumed adult fTnT sequences revealed identity around 74% (Table 1). Likewise, the sea bream and *Tetraodon* efTnTsb share ~73% amino acid identity with the presumed adult and larval fTnT in medaka and ~70% when compared to zebrafish, *G. morhua* and *S. salar* fTnT proteins (Table 1).

Comparison of the C-terminal region of the deduced teleost fTnT proteins with the same region of avian and mammalian

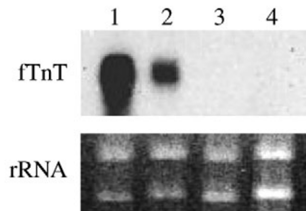

Fig. 2. Northern blot analysis of sea bream *fTnT* expression. Total RNA (3 μg) of sea bream adult white muscle (lane 1), adult red muscle (lane 2), adult heart (lane 3) and adult liver (lane 4). The probe utilised corresponded to the 3′ UTR of sea bream *fTnT* (top panel). The relative loading of total RNA was determined by staining with ethidium bromide (lower panel).

fTnT reveals that the teleost fTnTs are most like tetrapod fTnT isoforms bearing exon 17.

Putative genomic organisation of the sea bream fTnT gene

A tBLASTX search using the three fTnTsb against the *Fugu* and *Tetraodon* database (http://Fugu.hgmp.mrc.ac.uk; www.genoscope.cns.fr/externe/tetranew/) yielded a single scaffold, M001617 and SCA7217, respectively, further indicating they most probably result from alternative splicing of a single gene. Using Spidey mRNA to genomic sequence web interface (Wheelan et al., 2001) the entire *Fugu* scaffold M001617 aligned with the three cDNAs for the putative isoforms of the sea bream *afTnTsb*, *LfTnTsb* and *efTnTsb*. The same method was employed for *Tetraodon* scaffold SCAF7217, and *Tetraodon* full-length cDNA clones CR660426, CR658422 and CR657382, which matched *efTnTsb*, *afTnTsb* and *LfTnTsb* cDNAs, respectively, were also included in the analysis. The results of these alignments revealed that the *fTnT* gene in *Fugu* and *Tetraodon* is ~7.5 kb and ~5 kb, respectively. The putative *fTnT* gene in *Fugu*, *Tetraodon* and sea bream has 14 exons, all with perfectly conserved intron/exon boundaries.

A minimum coverage of 80% of the sea bream cDNA sequences occurs in the *Fugu* and *Tetraodon* alignment, and the sea bream *fTnT* cDNA coding region is totally mapped in the Tetraodontiform genomic sequence (Fig. 3). The lowest sequence identity (71.4%) between the sea bream sequences and the putative exons in *Tetraodon* occur in exon III (nt 70–83 in *efTnTsb*, nt 79–91 in *afTnTsb* and nt 128–141 in *LfTnTsb*). By contrast, the alternatively spliced exon IV shares 100% nucleotide sequence conservation between the three species and is flanked by conserved intron/exon boundaries. Overall, sequence identity between sea bream *fTnT* cDNA sequences and *Tetraodon* genomic sequence is 87.4% for *efTnTsb*, 87.8% for *afTnTsb* and 86.7% for *LfTnTsb*.

The embryonic isoform, *efTnTsb*, is composed of 13 exons and exon I of the adult and larval isoforms is missing, but all other exons are present (Fig. 3). The *afTnTsb* is composed of 13 exons, exons I–IV and VI–XIV, whereas *LfTnTsb* is composed of 12 exons, exons I–III and VI–XIV (Fig. 3). Comparison of

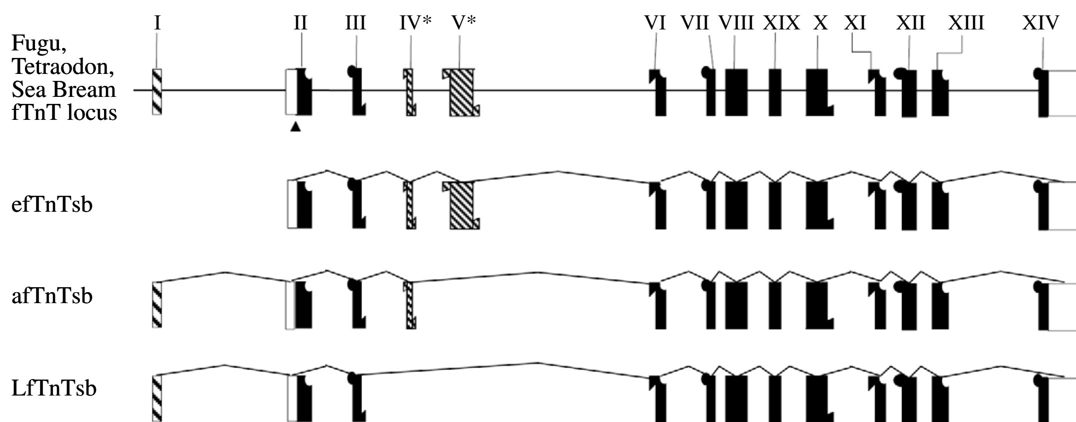

Fig. 3. Genomic organisation of the putative sea bream, *Fugu* and *Tetraodon fTnT* gene predicted after Spidey analysis using the sea bream *fTnT* isoform cDNA sequence isolated and the *Fugu* scaffold 1617. The same analysis was carried out using the sea bream *fTnT* isoforms isolated and *Tetraodon* scaffold SCAF7217, but including also the *Tetraodon* full-length cDNA clones CR660426, CR658422 and CR657382. Black boxes represent constitutive protein coding regions whereas the white boxes represent untranslated regions. Broad striped blocks represent alternatively spliced untranslated exons, while narrow striped blocks represent protein coding alternatively spliced exons. Exon II contains the ATG initiation codon (arrowhead) and is composed of part of the 5′ UTR and the start of the coding region. The sea bream, *Fugu* and *Tetraodon fTnT* locus has 14 exons from which exon I and XIV are untranslated exons. The exon numbers with an asterisk are alternatively spliced exons. Exon V is the larval-specific exon. Flush junction boundaries in exons indicate that they start or end in intact codons; saw tooth boundaries indicate that the upstream exon donates one nt to the codon while the other two are contributed by the downstream exon; concave/convex exon boundaries indicate that codon splitage takes place by the upstream exon donating two nt while the downstream exon contributes one. The *efTnTsb* isoform results form the incorporation of all exons except exon I. The *afTnTsb* isoform results from splicing of exons I–IV and VI-XIV whereas in the *LfTnTsb* isoform exons I–III and VI–XIV are spliced. Both *afTnTsb* and *LfTnTsb* have an extra 5′ UTR exon, exon I, which is absent from the *efTnTsb* (Fig. 4). Sequence conservation between this region (exon I) of the presumed adult sea bream *fTnT* cDNAs and the genomic sequence of *Tetraodon* was higher than 88%.

afTnTsb and LfTnTsb isoforms reveals that the three additional amino acids (EYD) in the isoform afTnTsb are the results of alternative splicing of exon IV (Fig. 3). Embryonic exon, V, is absent from adult and larval fTnT isoforms (Fig. 3).

The nucleotide sequence of the putative embryonic exon in the tetraodontiform genomic sequences and sea bream cDNA is highly conserved (84.2%). However 18 additional nucleotides encoding six amino acid residues (EAVEEE) are present in the sea bream embryonic exon (exon V). The putative *Tetraodon* embryonic exon is flanked by consensus intron/exon boundaries and an in frame TGA stop codon is located five nucleotides upstream of the 3′ exon/intron boundary. This suggests that the truncation in the 3′ end of the tetraodontiform embryonic exon is authentic and specific to *Fugu* and *Tetraodon* and that the differences between the sea bream and tetraondotiforms are species specific.

Developmental analysis of sea bream fTnT – semi-quantitative RT–PCR

RT–PCR co-amplified the three forms of *fTnTsb* (*efTnTsb*–411bp; *afTnTsb*–245 bp; *LfTnTsb*–236 bp) as primers that are localised in the common 5′ region of the cDNAs (arrows in Fig. A1D). Expression of the *fTnT* gene in sea bream was found to commence at 36 h.p.f. (Fig. 4), and was detected in all subsequent stages and in adult white and red muscle but was absent from heart and liver, in agreement with the results from northern blotting (Fig. 2 and Fig. 4A). The expression of *LfTnTsb* in adult white muscle is very low, although overall *fTnT* is highly expressed as a result of the high *afTnTsb* transcript abundance. The overall expression of *fTnT* in sea bream adult red muscle is extremely low and only *efTnTsb* and *afTnTsb* transcripts were expressed (Fig. 4A,B). Noticeable, is the fact that *efTnT* is highly expressed in relation to other isoforms in adult red muscle but has a residual expression in adult white muscle (Fig. 4C). Furthermore, *efTnTsb* and *afTnTsb* have an overlapping expression in adult red muscle and are both present in adult red muscle and in embryos before hatch (Fig. 4B).

The expression pattern of sea bream *fTnT* isoforms changed with hatching and from larval stages to adulthood (Fig. 4C). *efTnTsb* and *afTnTsb* are the only isoforms detected in embryonic stages at 36 h.p.f. (Fig. 4A and B) and *LfTnTsb* only after hatching, although in all subsequent stages analysed *LfTnTsb* is abundant (Fig. 4). *efTnTsb* is the most abundant isoform before hatching but is strongly downregulated after hatching and by 64 d.p.h. onwards is undetectable (Fig. 4C). Immediately after hatching, and in all larval and juvenile stages analysed, *LfTnTsb* is the most abundant isoform of *fTnT* and the ratio *afTnTsb:LfTnTsb* is always lower than 1 (Fig. 4C). The ratio of the two isoforms starts to change at 89 d.p.h. juveniles and in adult white muscle there is an ~tenfold inversion in the relative abundance of the two isoforms (Fig. 4C).

T3 treatment – sea bream TnT expression

The *efTnTsb* isoform was not detected in any of the samples collected from control or T3 experiments (Fig. 5A,B). No significant difference was observed in the expression of *afTnTsb* and *LfTnTsb* isoforms in control fish or those treated with T3 up until 64 and 75 d.p.h. (HSD, $P>0.05$; Fig. 5). However, at 89 d.p.h. an increase in expression of *afTnTsb* was noted in both control and T3-treated groups (HSD, $P<0.05$; Fig. 5A,B). At the end of the experiment (89 d.p.h.), *afTnTsb* expression in control and T3-treated juveniles was approximately three- and twofold higher, respectively, than

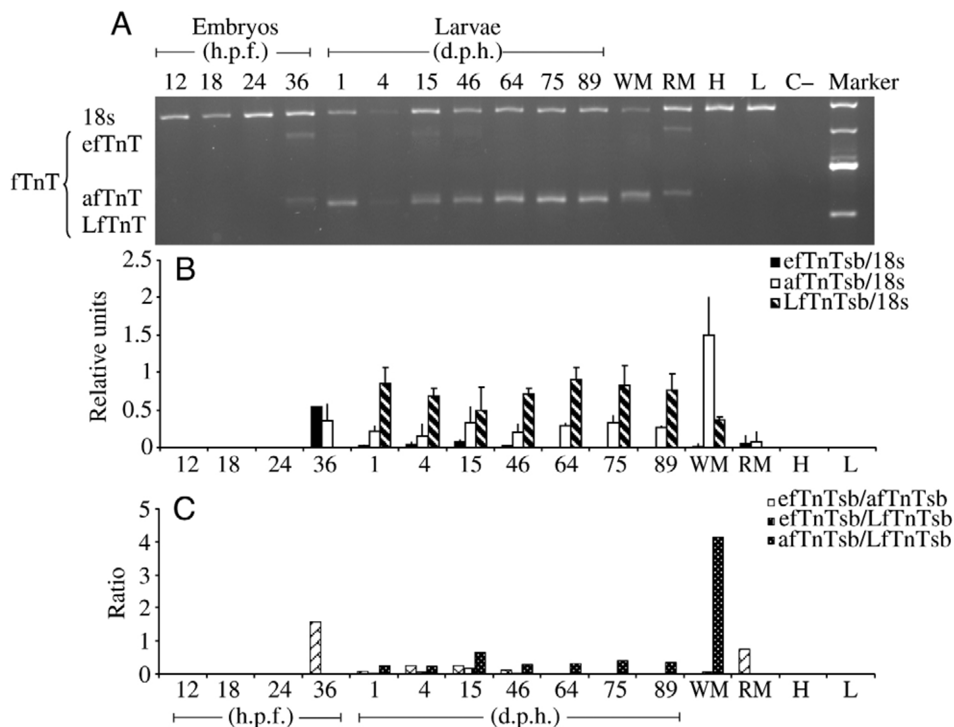

Fig. 4. Developmental expression of sea bream *fTnT* isoforms and *18s ribosomal RNA* (*18s rRNA*) assessed by RT–PCR. (A) Reaction products were fractionated on a 2.5% agarose gel. Embryos from 12, 18 and 36 h.p.f., larvae from 1 to 75 d.p.h. and juveniles of 89 d.p.h. were analysed. Adult white (WM) and red (RM) muscle, heart (H) and liver (L) were also analysed. A no template, negative control was used (C–). Three bands are detected which are products of the sea bream *fTnTsb* gene. (B) Graph showing the average expression of each *fTnT* isoform in relation to 18s rRNA of three samples. Values are means ± s.e.m. (C) Graph showing the ratio between the different sea bream *fTnT* isoforms at various stages and in different tissues.

after 18 days of treatment (75 d.p.h.; HSD, *P*<0.001; Fig. 5B).

By contrast, *LfTnTsb* expression did not change significantly in any of the experimental groups during the entire experiment (HSD, *P*>0.05; Fig. 5C). However, comparison of *LfTnTsb* expression levels between treatment groups revealed it is significantly lower in the T3 group compared to control animals (HSD, *P*<0.05, Fig. 5C). In the first 7 days of the experiment the ratio of *afTnTsb* to *LfTnTsb* in T3-treated and control animals was always below 1 (Fig. 5D). At the end of the experiment the ratio of *afTnTsb* to *LfTnTsb* in control and T3-treated larvae increased to ~1 (HSD, *P*<0.05; Fig. 5D).

The expression of *sTnT2sb* and *iTnTsb* in sea bream treated for 7 (64 d.p.h.), 18 (75 d.p.h.) or 31 (89 d.p.h.) days with T3 were not significantly different from control fish (one-way ANOVA, *P*>0.05; Fig. 6), suggesting that T3 does not induce or repress expression of *sTnT2* or *iTnT* (Fig. 6B) in sea bream. By contrast, *sTnT1sb* was significantly downregulated at the end of the first 7 days of treatment with T3 (HSD, *P*<0.05; Fig. 7). T3 treatment had a greater effect on expression of *sTnT1sb-1* isoform than on *sTnT1sb-2*. Hormone treatment decreased *sTnT1sb-1* isoform expression from the first 7 days of treatment to a level only attained in 89 d.p.h. control fish (31 days of treatment; Fig. 7A,B). In the case of *sTnT1sb-2*, after 18 days (75 d.p.h.) of treatment no significant differences in isoform expression were observed between control and T3-treated fish (HSD, *P*>0.05). In control fish after the first 18 days of experiments the ratio between *sTnT1sb-1* and *sTnT1sb-2* was ~1 and decreased to ~0.5 at 89 d.p.h. (Fig. 7D) and *sTnT1sb-2* became the most abundant sea bream *sTnT1* gene isoform; a feature characteristic of adult sea bream red muscle (Campinho et al., 2005). The change to an adult expression profile of the ratio of *sTnT1sb* isoforms occurred after only 7 days of treatment with T3, in comparison to the control group, and the effect persisted throughout the entire treatment time (HSD, *P*<0.05; Fig. 7D).

Radioimmunoassays for T4 revealed very low levels of this hormone in control and T3-treated sea bream after 7 days of treatment (64 d.p.h.; Fig. 8A). In control sea bream there was a significant increase in T4 levels, occurring after 18 and 31 days of treatment (HSD, *P*<0.001; Fig. 8A). From 75 d.p.h. onwards T3-treatment resulted in an approx. fivefold increase in T4 levels compared with control animals (HSD, *P*<0.001; Fig. 8A). The high levels of T4 (Fig. 8A) in T3-treated sea bream (despite a histological index indicative of an inactive thyroid) is confusing. However, if the cross

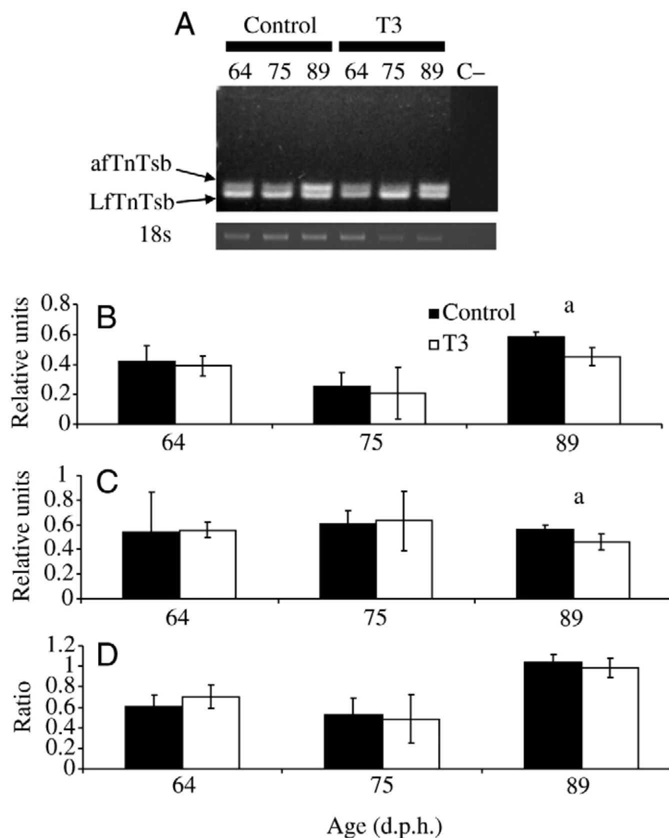

Fig. 5. Expression of sea bream *fTnT* gene isoforms *afTnTsb* (higher molecular mass), *LfTnTsb* (lower molecular mass) and *18s* rRNA assessed by RT–PCR after 7 (64 d.p.h.), 18 (75 d.p.h.) and 31 (89 d.p.h.) days of treatment with T3 and in control animals. Reaction products fractionated on a 2.5% agarose gel (A) and respective graphical representation of the relative expression of isoform *afTnTsb* (B) and *LfTnTsb* (C) against *18s*; (D) the *afTnTsb:LfTnTsb* ratio. The *efTnTsb* isoform is not represented graphically since no expression was detected in any treatment at any sampling point. [a]Significant statistical differences between the control and T3-treated groups.

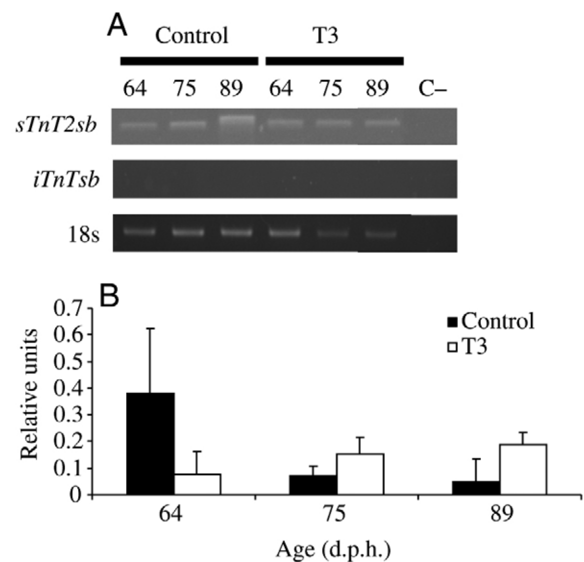

Fig. 6. Expression of sea bream *sTnT2*, *iTnT* genes and *18s rRNA* determined by RT–PCR after 7 (64 d.p.h.), 18 (75 d.p.h.) and 31 (89 d.p.h.) days of treatment with T3 in control animals. (A) Reaction products fractionated on a 2.5% agarose gel and (B) respective graphs of *sTnT2sb* expression relative to *18s rRNA*. The *iTnTsb* gene is not represented graphically since no expression was detected in any treatment group at any sampling time. No significant statistical differences were found in *sTnT2sb* expression between of the T3-treated and the control animals at any sampling time.

reactivity of the anti-T4 rabbit polyclonal serum (Sigma-Aldrich) with the high plasma T3 levels in T3-treated sea bream (Fig. 8B) is taken into account it readily explains the apparent increase in T4.

The level of T3 in control larvae (~10 pg T3 mg^{-1} animal) did not change significantly over the duration of the experiment (from 64 to 89 d.p.h.; HSD, $P>0.05$; Fig. 8B). In the T3-treated larvae after 7 days of treatment T3 levels were not significantly higher than control (HSD, $P>0.05$; Fig. 8B). However, after 18 and 31 days of T3 treatment there was, respectively, a 50-fold and ~100-fold higher concentration of T3 compared with control larvae (HSD, $P<0.001$; Fig. 8B).

To further analyse thyroid status after T3 treatment, thyroid follicle number (Fig. 9A) and thyrocyte cell height [index of thyroid activity (Cooley et al., 2001; Kalisnik et al., 1977); Fig. 9B], was assessed during normal sea bream ontogeny and during treatment with T3 (Fig. 9). In control larvae, follicle number was constant throughout the course of the experiment (HSD, $P>0.05$; Fig. 9B). However, control thyrocyte cell height was greater at 64 and 75 d.p.h. than at the end of the experiment (HSD, $P<0.001$; Fig. 9A,C). Moreover, at 64 and 75 d.p.h. colloid was absent or was vesicular, indicative of high follicle activity (Fig. 9 and Fig. 8A). At the end of the experiments 50% of the control follicles appeared inactive and had a squamous appearance (Fig. 9A). T3 treatment significantly increased follicle number in relation to control fish by the end of treatment (HSD, $P<0.001$; Fig. 9). However, there was a significant reduction in thyrocyte cell height in T3-treated fish compared with control animals (HSD, $P<0.001$; Fig. 9A,C). The effect of T3 on thyrocyte cell height was evident by the end of the first 7 days of the experiment and caused a 25% reduction in cell height compared with control animals (HSD, $P<0.001$; Fig. 9A,C). This difference in thyrocyte cell height between the T3-treated group and the control group is even more accentuated at 75 d.p.h. (Fig. 9A,C). At this time, thyrocyte cell height in

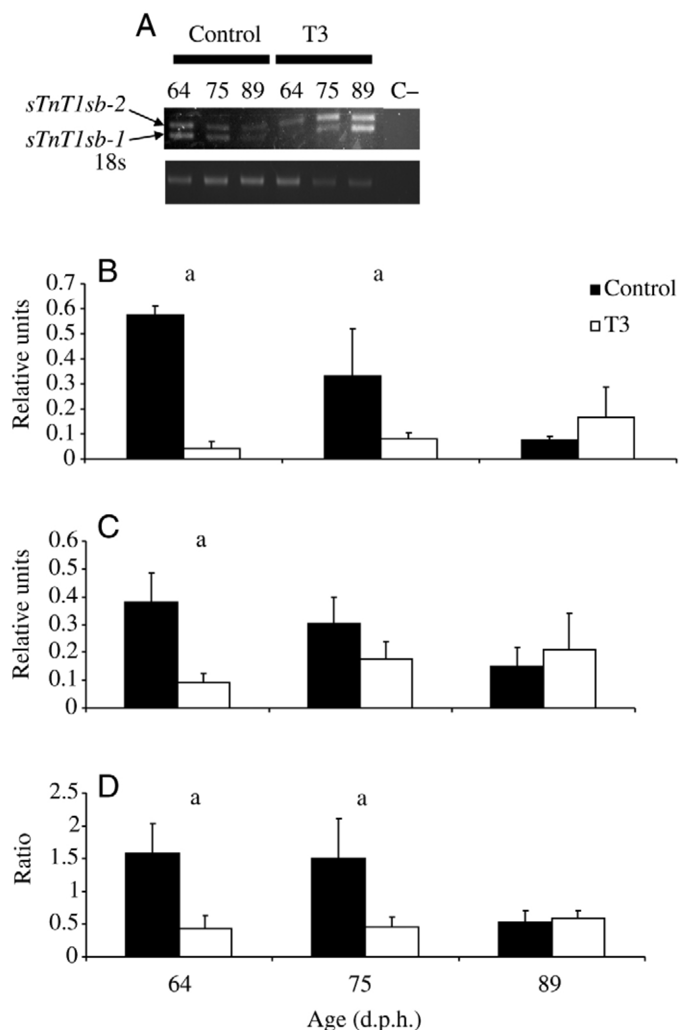

Fig. 7. Expression of sea bream *sTnT1sb* gene isoforms *sTnT1sb-1* (lower molecular mass), *sTnT1sb-2* (higher molecular mass) and *18s rRNA* determined by RT–PCR after 7 (64 d.p.h.), 18 (75 d.p.h.) and 31 (89 d.p.h.) days of treatment with T3 or in control animals. (A) Reaction products fractionated on a 2.5% agarose gel and (B,C) respective graphs of relative expression of isoform *sTnT1sb-1* (B) and *sTnT1sb-2* (C) against *18s rRNA*. (D) The ratio *sTnT1sb-1*:*sTnT1sb-2*. [a]Significant statistical differences between control and T3-treated animals.

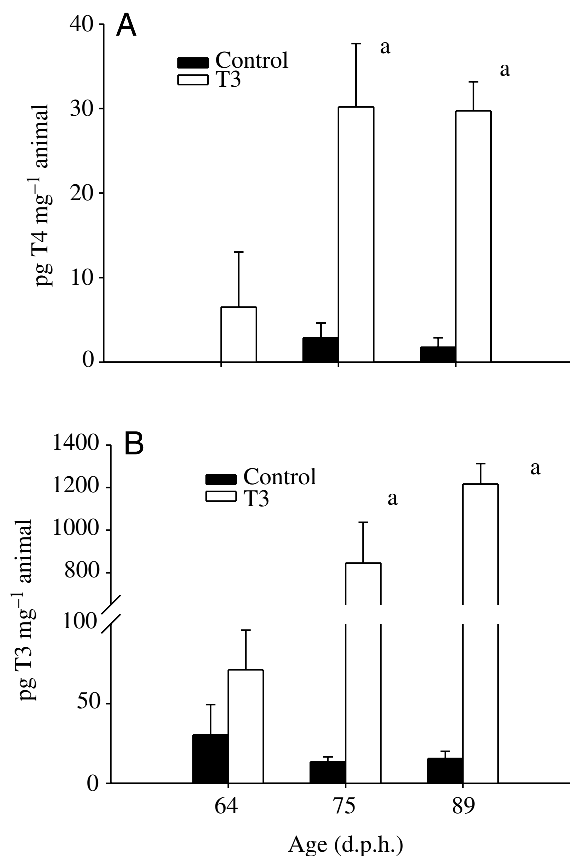

Fig. 8. Sea bream whole body thyroid hormone levels: (A) T4 and (B) T3 levels in control sea bream and in fish treated with T3 (N=5 per sampling time and treatment). Levels were measured after 7 (64 d.p.h.), 18 (75 d.p.h.) and 31 (89 d.p.h.) days of treatment. [a]Significant difference (Tukey's HSD, $P<0.001$) between T3 and the control group.

T3-treated animals was almost half that of the control (HSD, $P<0.001$; Fig. 9). Nonetheless at the end of the experiment thyrocyte cell height in the T3-treated and control groups were not significantly different (HSD, $P>0.05$; Fig. 9). The follicle lumen of T3-treated fish contained abundant colloid and few vesicles for the duration of the experiment (Fig. 9A), indicative of low activity.

Discussion

Molecular characterisation of the sea bream fTnT gene – fTnTsb

The deduced protein sequence of the cDNA clones for *fTnT* splice variants in sea bream differ in the N-terminal region but are identical in the mid- and C-terminal region (Fig. 1). A similar situation is found for *fTnT* genes in terrestrial vertebrates (Bastide et al., 2002; Breitbart et al., 1985; Briggs et al., 1988; Briggs et al., 1984; Briggs et al., 1987; Briggs and Schachat, 1989; Briggs and Schachat, 1993; Bucher et al., 1999; Gahlmann et al., 1987; Hastings et al., 1985; Jin, 1996; Jin et al., 1998a; Jin et al., 1996; Jozaki et al., 2002; Morgan et al., 1993; Ogut and Jin, 1998; Perry, 1998; Wang and Jin, 1997; Wang and Jin, 1998; Wu et al., 1994). The embryonic fTnT isoform in sea bream is larger (33.8 kDa) than afTnTsb and LfTnTsb, with a predicted molecular mass of ~28 kDa, which is similar to terrestrial vertebrate fTnT proteins (Jin et al., 2000; Perry, 1998).

In tetrapods, *TnT* genes are initially expressed in different skeletal muscle types, but as animals reach adulthood expression becomes restricted to specific white skeletal muscle (Wang et al., 2001). In general, sea bream *fTnT* has a similar expression pattern to that observed in tetrapods and it is principally expressed in fast muscle and is absent from cardiac muscle and non-muscle tissue. However, in contrast to tetrapods, in sea bream *fTnT* is also present in adult red muscle (Fig. 2), and this observation may explain previous biochemical data demonstrating fast tissue ATPase activity during the development of red (slow) muscle in sea bream (Mascarello et al., 1995). In fact, in *Clupea harengus* yolk-sac larvae, slow muscle adult fast myosin light chain isoforms were detected, and in *Oncorhynchus mykiss* larvae several fast-muscle-specific genes have been identified by *in situ* hybridisation in newly formed slow muscle fibres (Chauvigne et al., 2006). The hyperplastic capacity retained by adult fish muscle (Koumans and Akster, 1995; Mascarello et al., 1995; Mommsen, 2001) may explain the persistent expression of *fTnT* in both adult white and red muscle. Interestingly, in addition to expression of *sTnT2sb* and *iTnTsb* in adult sea bream red muscle, *efTnTsb* and *afTnTsb* are also present (Fig. 4) (Campinho et al., 2005). The co-expression of sea bream TnT genes (*sTnT2sb*, *iTnTsb*, *efTnTsb* and *afTnTsb*) in adult red muscle is similar to what occurs during early embryonic muscle development (Fig. 4) (Campinho et al., 2005) and may suggest that *de novo* formation of new red muscle fibres in adults recapitulates embryonic muscle development.

Various fTnT protein isoforms found in sea bream arise from alternative splicing of two exons (exon IV and V in Fig. 3) located in the 5′ region of the gene. Alternative splicing is responsible for the differing sequence of the N-terminal region of the sea bream fTnT proteins (Fig. 1) and also for their divergent pI and size. The occurrence of *fTnT* splice variants in teleosts is not restricted to the sea bream and database searches revealed the existence of homologous cDNA sequences in *Tetraodon*, medaka and zebrafish (Fig. 1). A similar situation also occurs in tetrapods: in chicken wing muscle about 79 different TnT proteins have been identified by two-dimensional SDS–PAGE (Yao et al., 1992), and in mouse the mRNA of at least 10 different isoforms of the *fTnT* gene have been identified (Breitbart et al., 1985). Alternative 5′ exon splicing of the mouse *fTnT* gene can generate 64 different isoforms (Perry, 1998). This huge genomic heterogeneity in tetrapod *fTnT* isoforms is further increased by the presence in avian and mammalian *fTnT* genes of two mutually exclusive alternatively spliced exons in the 3′ region (exon 16 and 17), which give rise to different C-terminal protein domains (Bucher et al., 1999; Jin et al., 1998b; Jozaki et al., 2002; Perry, 1998; Wang and Jin, 1997). In the present study no teleost C-terminal *fTnT* variants have been found (Fig. 1) and the predicted C-terminal fTnT protein is most similar to the protein encoded by tetrapod exon 17. This seems to indicate that the alternatively spliced C-terminal exon 17 in tetrapods may be the exon present in *fTnT* of the common ancestors of fish and land vertebrates and that exon 16 may have arisen in the terrestrial vertebrate lineage. The greater heterogeneity of tetrapod *fTnT* transcripts compared to teleosts is probably a consequence of the greater number of specialized muscles in tetrapods, which express alternatively spliced *fTnT* isoforms (Jin et al., 2000; Perry, 1998).

In *Fugu* and *Tetraodon* the putative *fTnT* genes are composed of 14 putative exons, have a well-conserved organisation (Fig. 3) and are ~7.5 kb and ~5 kb, respectively. This is in contrast to the ~16 kb *fTnT* gene in rat and more than 33 kb gene in quail (Bucher et al., 1999), which are composed of 19 and 25 exons, respectively. Despite the greater number of exons in terrestrial vertebrate *fTnT* there is conservation of the overall gene organisation in teleosts. In sea bream, *Fugu* and *Tetraodon* only two alternatively spliced exons (exon IV and V; Fig. 3) have been identified. The zebrafish differs from the other teleosts as it has two *fTnT* genes, which are each composed of 12 exons and are 12 and 15 kb, respectively (Hsiao et al., 2003). Database searches reveal alternative splicing of zebrafish *fTnTb* also occurs and two different cDNAs coding for different isoforms (Fig. 1) of one gene have been identified in the present study. One of the predicted zebrafish proteins contains four additional amino acids (EYDE), homologous to the sea bream EYD peptide found in the *efTnTsb* and *afTnTsb* isoforms (Fig. 1). Mouse, rat and chicken contain a significantly greater number of *fTnT* isoforms (13, 10 and 25, respectively) than fish (Breitbart et al., 1985; Perry, 1998; Wang and Jin, 1997). This has been related to their differential expression in specialised muscles of terrestrial vertebrates (Jin et al., 2000; Perry, 1998).

Interestingly the biochemical characteristics of the embryonic form of *fTnT* in sea bream (54 aa), *Tetraodon* (48 aa), human (8 aa) (Perry, 1998), rat (13 aa) and rabbit (12 aa) (Briggs and

Schachat, 1993) are similar and the high glutamic acid content encoded by the embryonic exon confers an acidic pI to the protein. The occurrence of foetal *fTnT* splice variants in teleosts and terrestrial vertebrates (Briggs et al., 1994; Briggs and Schachat, 1993; Morgan et al., 1993; Wang and Jin, 1997), may suggest that the foetal/embryonic-specific exon arose before fish and terrestrial vertebrates diverged and that similar constraints exist in early muscle development in aquatic and terrestrial vertebrates. In fact, in *fTnT* of *Fugu*, *Tetraodon* (this work, Fig. 3), rat and quail (Bucher et al., 1999) the alternatively spliced exons of the N-terminal region share exactly the same codon splitage combination. Moreover, a similar situation is also observed for the last two *fTnT* exons, XIII and XIV, in *Fugu* and *Tetraodon* (Fig. 3), which share the same codon splitage combination as the mutually exclusive exons 16 and 17 and the last C-terminal constitutively spliced exon (18) in rat and quail (Bucher et al., 1999).

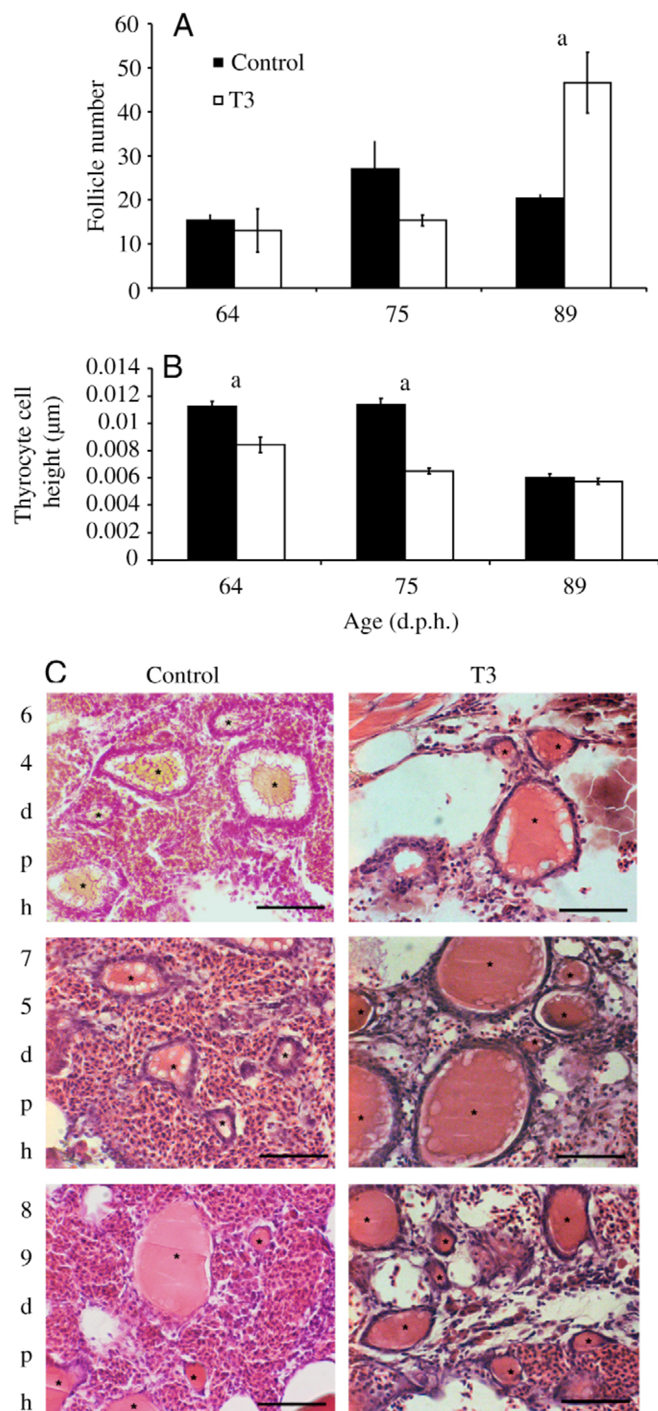

Fig. 9. (A) Follicle number per slide at the junction of the hypohyal bones (*N*=3 animals per treatment) in T3 and control sea bream after 7 (64 d.p.h.), 18 (75 d.p.h.) and 31 (89 d.p.h.) days of treatment. (B) Thyrocyte cell height was measured in order to determine thyroid activity. [a]Statistical significant differences between the T3 treated and the control group. (C) Thyroid follicles in control and T3-treated animals at each sampling point. Scale bar, 100 µm. Black asterisks denote a thyroid follicle.

Developmental expression from embryonic stages to adult of fTnTsb

In common with mouse (Wang et al., 2001) and zebrafish (Hsiao et al., 2003), the expression of *fTnT* in the sea bream occurs only after the most anterior somites are formed (36 h.p.f.; Fig. 4). This correlates well with the fact that in the zebrafish, and probably other teleosts, the migration of the adaxial cells (progenitors of the red muscle layer) (Devoto et al., 1996) from their position immediately adjacent to the notochord to the surface of the developing somite constitute the signal for white muscle differentiation (Henry and Amacher, 2004). The *efTnTsb* isoform is dominant in late embryonic stages but is downregulated immediately after hatching and is substituted by *LfTnTsb* that becomes the most abundant isoform in larvae and early juvenile stages whereas in adult white muscle *afTnTsb* is the most abundant isoform (Fig. 4). Studies of muscle protein in juvenile flounder (Yamano et al., 1991), sole *Solea solea* and turbot *Scophthalmus maximus* (Focant et al., 2003), reveal that in common with sea bream two TnT isoforms (molecular mass range 34–32.5 kDa) exist in white muscle. In juvenile post-metamorphic sole the lower molecular mass TnT isoform is predominant, whereas in adult white muscle both isoforms are present in similar amounts (Focant et al., 2003), which is reminiscent of the pattern of transcript expression in sea bream (Fig. 4). The pattern of *fTnT* isoform expression in flounder is somewhat different from sea bream and repression of the higher molecular mass embryonic form only occurs during the larval to juvenile metamorphosis (Yamano et al., 1991). Differences in *fTnT* expression in fish probably reflect differences in their developmental ontogeny resulting from the different functional and physiological constraints that pelagic and flatfish face. Remarkably, the sea bream *fTnT* isoform expression profile bears more similarities to isoform ontogeny in chicken breast muscle (Yao et al., 1992) where there is a gradual transition from an embryonic to chick *fTnT* isoform immediately after hatching, and a subsequent switch during maturation to an adult type *fTnT* isoform. The change in *fTnT* isoforms in chickens is predicted (as probably also occurs in sea bream) to result in a change in the pI of the expressed proteins from acidic to basic (Yao et al., 1992). The shift from acidic to basic *fTnT* proteins

in fast skeletal vertebrate muscle has been related to changes in pH and Ca^{2+} sensitivity necessary for correct contraction, and is directly related to the hypervariable N-terminal region of *fTnT* isoforms (Jin et al., 2000; MacFarland et al., 2002; Nosek et al., 2004; Wang and Jin, 1997). The presence of an acidic exon in chicken pectoralis *fTnT*s is responsible for its higher tolerance to pH changes and for the decrease in both the interaction and assembly of *fTnT* with troponin I (TnI) and tropomyosin (Tm) (Jin et al., 2000).

The change in fTnT isoforms during ontogeny in vertebrates appears to be important for functional adaptability (Nosek et al., 2004) and the impact of different fTnT isoforms on muscle function in fish remains to be established but is probably associated with changes in the hydrodynamic environment as well as different locomotive strategies, respiration and intracellular environments (Johnston, 1994; Johnston et al., 1997; Koumans and Akster, 1995; Osse, 1990; Osse and Boogaart, 1999; Patruno et al., 1998; Verhagen, 2004; Watabe, 1999). In fact, until the gills are fully functional, which only occurs at the end of the larval stage, most gas exchange occurs through the skin and muscle that constitute the major respiratory surface of teleosts fish larvae. In fact muscle tissue in teleost embryos and larvae has different metabolic regimes than adult muscle and consequently a different cellular environment. During the larval stage white muscle is mainly aerobic and rich in mitochondria, which contrasts with the anaerobic adult white muscle (Johnston, 1994; Johnston et al., 1997; Watabe, 1999). The *fTnT* isoform switching in sea bream appears to accompany the transition from larva to juvenile and probably allows white muscle to adapt to the changing functional and physiological demands during development.

Sea bream TnT genes TH responsiveness

THs have been shown to play a role in vertebrate muscle development and muscle gene expression. In hyperthyroid newborn rats the transition of fast myosin heavy chains (MHC) from embryonic and perinatal to adult isoforms is accelerated and the opposite occurs in hypothyroid newborn rat. In general, in adult rats hypothyroidism increases slow MHC expression in skeletal and cardiac muscle and hypothyroidism induces an opposite effect (Adams et al., 1999; Soukup and Jirmanova, 2000). In mammals, the responsiveness of muscle to THs is variable, and slow muscle is more sensitive than fast muscle. Moreover, in rats THs are an important factor in MHC isoform expression in slow muscle (Soukup and Jirmanova, 2000) and normal TH levels are necessary for the formation of normal skeletal muscle (Vadaszova et al., 2004).

Fish muscle has also been shown to be responsive to THs, and treatment changes the histological properties of developing zebrafish larvae muscle (Liu and Chan, 2002) and locomotion in *E. coioides* larvae (de Jesus et al., 1998). More specifically, in sea bream juveniles T4, but not T3, treatment increases myosin light chain 2 (MLC2) expression (Moutou et al., 2001). Moreover, in flounder, a pleuronectiform, THs drive the pelagic to benthic metamorphosis (Miwa and Inui, 1987; Miwa et al., 1988) and associated muscle protein changes, which include a switch in MLC and TnT isoform expression (Yamano et al., 1991; Yamano et al., 1994). In particular, T3 treatment represses the flounder 41.5 kDa embryonic/larval fTnT isoform and induces precocious expression of a 34 kDa adult fTnT isoform whereas thiourea-induced hypothyroidism prevents these changes (Yamano et al., 1991). Although THs probably play a role in sea bream larva/juvenile developmental switch, the sea bream belongs to an order that persists as a bilaterally symmetric fish throughout its life cycle and does not suffer a dramatic metamorphosis like flatfish. In general, based upon a number of different morphological characteristics the larva/juvenile transition is proposed to be completed at about 90 d.p.h., although as the sea bream is an ectotherm differences in thermal regimes will affect this timing (Loy et al., 1999; Mascarello et al., 1995; Parra and Yufera, 2001; Patruno et al., 1998; Polo et al., 1991; Rowlerson et al., 1995). In a previous study in which THs were measured during sea bream development it was proposed that metamorphosis starts at 29 d.p.h. (start of notochord flexion); however, the limited information provided in the study about the way animals were reared and the staging criteria utilised (Szisch et al., 2005) does not permit comparison with previous morphological studies or the present study. Based upon previous morphological studies, the timing of TH treatment was initiated well in advance of the larva/juvenile transition. T3-treatment had no effect on *efTnT* expression in sea bream which contrasts with flounder, where T3 treatment accelerates *fTnT* pre- to post-metamorphic isoform changes and in which thiourea-induced hypothyroidism in pre-metamorphic larvae prevents metamorphosis and maintains embryonic/larval *fTnT* expression even though in control animals this expression had already terminated (Yamano et al., 1991). Importantly, in sea bream, T3 treatment did not anticipate the adult pattern of *fTnT* isoform expression where *afTnTsb* is predominant. Moreover, the change in the ratio of *afTnTsb*:*LfTnTsb* to an adult pattern only occurred at 89 d.p.h. and after 31 days of T3 treatment and at the same time as in control animals, suggesting that in the sea bream, *fTnT* splice variant expression during development is mediated by factors other than TH and seems to indicate that THs might not be involved at all in the developmental regulation of *fTnT* isoforms in sea bream. This is in striking contrast to the flounder (Yamano et al., 1991). This fact taken together with the observation that in sea bream *efTnT* is downregulated immediately after hatching and that the differences found in the responsiveness of *fTnT* to T3 treatment between the sea bream and the flounder (Yamano et al., 1991), clearly reinforces the notion that larval muscle development in teleosts may be species specific and associated with functional demands.

As has been previously reported (Campinho et al., 2005) and in contrast to what occurs in tetrapods, sea bream *sTnT* genes have a coordinated expression pattern during sea bream development (Barton et al., 1999; Briggs and Schachat, 1993; Bucher et al., 1999; Farza et al., 1998; Gahlmann et al., 1987; Huang et al., 1999; Jin et al., 1998a; Jin et al., 2000; Nakada et al., 2002; Perry, 1998; Samson et al., 1994; Wang and Jin, 1997; Wang et al., 2002; Yonemura et al., 2000; Yonemura et al., 2002). Gene expression of *sTnT1sb* (Fig. 7) and its splice

variants in sea bream, but not *sTnT2sb* and *iTnT* (Fig. 6), are affected by T3 treatment. T3 promotes an *sTnT1sb* expression profile characteristic of adult red muscle (Campinho et al., 2005). The results obtained for *sTnT1sb* expression and the effect on the *sTnT1sb* isoform profile argue that T3 might be an important factor for sea bream, and probably other teleost, slow muscle final differentiation. The results from the present study suggest that *sTnT1sb* is the only sea bream *TnT* gene sensitive to T3 and may indicate that in common with rats (Everts, 1996; Soukup and Jirmanova, 2000), teleost slow muscle is more TH sensitive than white muscle.

In summary, three sea bream *fTnT* cDNAs have been isolated and shown to be the result of alternative splicing of a single gene. Furthermore, database studies suggest that alternative splicing may be a common feature of teleost *fTnTs*. The expression of *fTnT* during the larval/juvenile transition in sea bream does not seem to be regulated by THs, which contrasts with the TH responsiveness found in flounder (Yamano et al., 1991). by contrast, *sTnT1sb* in sea bream seems to be under the control of THs during the larva/juvenile transition, although *sTnT2* and *iTnT* (Campinho et al., 2005; Hsiao et al., 2003) are not affected. It seems likely that despite the lower isoform heterogeneity of sarcomeric proteins and a lower number of specialised muscles in teleosts, in common with tetrapods, they have diverse and complex muscle development programs regulated by as yet unidentified genetic and molecular mechanisms.

Appendix

A

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efTnTsb  1    GCAGCTAAAGGCTCCAGGTTCGACAAGTCCACTCTCACCATGTCTGACACTGAGGAAGTTGATCAGGTCGAGGAATACGATGCTGTAGAA 90
                                                         M  S  D  T  E  E  V  D  Q  V  E  E  Y  D  A  V  E

efTnTsb  91   GAGGAGGTAGTAGAGGAAGTAGAGGTGGCCCCTGAGGCGGCCCCTGAGCCAGAGCCAGAGCCAGAACCAGAGCCAGAGCCAGAACCAGTG 180
               E  E  V  V  E  E  V  E  V  A  P  E  A  A  P  E  P  E  P  E  P  E  P  E  P  E  P  E  P  V

efTnTsb  181  GTAGAACCAGAGCCAGAACCAGAACCAGAGCCTGAGCCTGAGCCTGAAGAGGCCGTTGAAGAGGAAGAGGAGAAGCCAAAGTTCAAGCCC 270
               V  E  P  E  P  E  P  E  P  E  P  E  P  E  P  E  E  A  V  E  E  E  E  E  K  P  K  F  K  P

efTnTsb  271  AGCGCTCCCAAGATCCCTGATGGTGAGAAAGTGGACTTTGATGACATCCAGAAGAAACGTCAGAACAAGGACCTGGTTGAGCTGCAGGCC 360
               S  A  P  K  I  P  D  G  E  K  V  D  F  D  D  I  Q  K  K  R  Q  N  K  D  L  V  E  L  Q  A

efTnTsb  361  CTCATTGATGCCCACTTTGAGTGCAGGAAGAAGGAGGAGGAGGAGCTGATCGCCCTCAAGGACAGGATTGAGAAGCGTCGTGCCGAGAGG 450
               L  I  D  A  H  F  E  C  R  K  K  E  E  E  E  L  I  A  L  K  D  R  I  E  K  R  R  A  E  R

efTnTsb  451  GCCGAGCAGCAGAGGGTCCGTGCTGAGAAGGAGAAGGAGCGCCAGGCGAGACGTGAGGAGGAGAGGCGGATCAGGGAGGAGGCTGACGCC 540
               A  E  Q  Q  R  V  R  A  E  K  E  K  E  R  Q  A  R  R  E  E  E  R  R  I  R  E  E  A  D  A

efTnTsb  541  AAGAAGAAGGCTGATGAGGATGCCAAGAAGAAGTCGGCTCTGTCCAGCATGGGCTCCAACTACAGCAGCCACCTGCAGAGAGCCGACCAG 630
               K  K  K  A  D  E  D  A  K  K  K  S  A  L  S  S  M  G  S  N  Y  S  S  H  L  Q  R  A  D  Q

efTnTsb  631  AAGAGAGGAGGCAAGAAAGAGACTGAGAGAGAGAAGAAGAAGAAGATCCTGGCCGCCAGACGCAAGCAGCTGAACATCGACCATCTGAAC 720
               K  R  G  G  K  K  E  T  E  R  E  K  K  K  K  I  L  A  A  R  R  K  Q  L  N  I  D  H  L  N

efTnTsb  721  GAGGACAAGCTGAAGGATAAGATCAATGAGCTGCATGAATGGATGGTCACGCTGGAGTCTGAGAAGTTCGACCACATGGAGAGACTGAAG 810
               E  D  K  L  K  D  K  I  N  E  L  H  E  W  M  V  T  L  E  S  E  K  F  D  H  M  E  R  L  K

efTnTsb  811  AGGCAGAAGTACGAGGTTACAACCCTGCGTAAGAGAATTGAGGAGCTCAGTAAATTCAGCAAGAAGGGCGCCGCCGCTCGCCGCAGAAAG 900
               R  Q  K  Y  E  V  T  T  L  R  K  R  I  E  E  L  S  K  F  S  K  K  G  A  A  A  R  R  R  K

efTnTsb  901  TAGATCGCCTCGCCCCGCCACTGCTCAGAAACCAAACTGCGATCGTCTCGGACACACGCCTGATGAAACTACGCACACTGTGGCGAAGGT 990
               *

efTnTsb  991  CGTAGCTCGGAAGAAGCCAGTCATGCTGAGGTCTACTCTGCGGTTATCAACCCACGTCTTGAAACATGCAACCAGGAGCCTTTAGCTGCT 1080
efTnTsb  1081 GCTCAATAGGAATAAAAAAAAAAAAAAAA 1107
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B

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afTnTsb  1    GGCACGAGGCCAGCAGGGAGCTCGCTTCTTTCAGCTTTTCTGAGGTTGCCACAAGTCCACTCTCACCATGTCTGACACTGAGGAAGTTGA 90
                                                                                  M  S  D  T  E  E  V  D

afTnTsb  91   TCAGGTCGAGGAATACGATGAGGAGAAGCCAAAGTTCAAGCCCAGCGCTCCCAAGATCCCTGATGGTGAGAAAGTGGACTTTGATGACAT 180
                Q  V  E  E  Y  D  E  E  K  P  K  F  K  P  S  A  P  K  I  P  D  G  E  K  V  D  F  D  D  I

afTnTsb  181  CCAGAAGAAACGTCAGAACAAGGACCTGGTTGAGCTGCAGGCCCTCATTGATGCCCACTTTGAGTGCAGGAAGAAGGAGGAGGAGGAGCT 270
                Q  K  K  R  Q  N  K  D  L  V  E  L  Q  A  L  I  D  A  H  F  E  C  R  K  K  E  E  E  E  L

afTnTsb  271  GATCGCCCTCAAGGACAGGATTGAGAAGCGTCGTGCCGAGAGGGCCGAGCAGCAGAGGGTCCGTGCTGAGAAGGAGAAGGAGCGCCAGGC 360
                I  A  L  K  D  R  I  E  K  R  R  A  E  R  A  E  Q  Q  R  V  R  A  E  K  E  K  E  R  Q  A

afTnTsb  361  GAGACGTGAGGAGGAGAGGCGGATCAGGGAGGAGGCTGACGCCAAGAAGAAGGCTGATGAGGATGCCAAGAAGAAGTCGGCTCTGTCCAG 450
                R  R  E  E  E  R  R  I  R  E  E  A  D  A  K  K  K  A  D  E  D  A  K  K  K  S  A  L  S  S

afTnTsb  451  CATGGGCTCCAACTACAGCAGCCACCTGCAGAGAGCCGACCAGAAGAGAGGAGGCAAGAAAGAGACTGAGAGAGAGAAGAAGAAGAAGAT 540
                M  G  S  N  Y  S  S  H  L  Q  R  A  D  Q  K  R  G  G  K  K  E  T  E  R  E  K  K  K  K  I

afTnTsb  541  CCTGGCCGCCAGACGCAAGCAGCTGAACATCGACCATCTGAACGAGGACAAGCTGAAGGATAAGATCAATGAGCTGCATGAATGGATGGT 630
                L  A  A  R  R  K  Q  L  N  I  D  H  L  N  E  D  K  L  K  D  K  I  N  E  L  H  E  W  M  V

afTnTsb  631  CACGCTGGAGTCTGAGAAGTTCGACCACATGGAGAGACTGAAGAGGCAGAAGTACGAGGTTACAACCCTGCGTAAGAGAATTGAGGAGCT 720
                T  L  E  S  E  K  F  D  H  M  E  R  L  K  R  Q  K  Y  E  V  T  T  L  R  K  R  I  E  E  L

afTnTsb  721  CAGTAAATTCAGCAAGAAGGGCGCCGCCGCTCGCCGCAGAAAGTAGATCGCCTCGCCCCGCCACTGCTCAGAAACCAAACTGCGATCGTC 810
                S  K  F  S  K  K  G  A  A  A  R  R  R  K  *

afTnTsb  811  TCGGACACACGCCTGATGAAACTACGCACACTGTGGCGAAGGTCGTAGCTCGGAAGAAGCCAGTCATGCTGAGGTCTACTCTGCGGTTAT 900
afTnTsb  901  CAACCCACGTCTTGAAACATGCAACCAGGAGCCTTTAGCTGCTGCTCAATAGGAATAAAAAAAAAAAAAAAAAA 973
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See next page for Fig. A1C,D

C

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LfTnTsb   1   GCACGAGGCCTCGTGCCGAATTCGGCACGAGGCTTACAGCCTGTCTCGGCCAGCAGGGAGCTCGCTTCTTTCAGCTTTTCTGAGGTTGC 90

LfTnTsb  91   CACAAGTCCACTCTCACCATGTCTGACACTGAGGAAGTTGATCAGGTCGAGGAGGAGAAGCCAAAGTTCAAGCCCAGCGCTCCCAAGATC 180
                                 M  S  D  T  E  E  V  D  Q  V  E  E  E  K  P  K  F  K  P  S  A  P  K  I

LfTnTsb 181   CCTGATGGTGAGAAAGTGGACTTTGATGACATCCAGAAGAAACGTCAGAACAAGGACCTGGTTGAGCTGCAGGCCCTCATTGATGCCCAC 270
               P  D  G  E  K  V  D  F  D  D  I  Q  K  K  R  Q  N  K  D  L  V  E  L  Q  A  L  I  D  A  H

LfTnTsb 271   TTTGAGTGCAGGAAGAAGGAGGAGGAGGAGCTGATCGCCCTCAAGGACAGGATTGAGAAGCGTCGTGCCGAGAGGGCCGAGCAGCAGAGG 360
               F  E  C  R  K  K  E  E  E  E  L  I  A  L  K  D  R  I  E  K  R  R  A  E  R  A  E  Q  Q  R

LfTnTsb 361   GTCCGTGCTGAGAAGGAGAAGGAGCGCCAGGCGAGACGTGAGGAGGAGAGGCGGATCAGGGAGGAGGCTGACGCCAAGAAGAAGGCTGAT 450
               V  R  A  E  K  E  K  E  R  Q  A  R  R  E  E  E  R  R  I  R  E  E  A  D  A  K  K  K  A  D

LfTnTsb 451   GAGGATGCCAAGAAGAAGTCGGCTCTGTCCAGCATGGGCTCCAACTACAGCAGCCACCTGCAGAGAGCCGACCAGAAGAGAGGAGGCAAG 540
               E  D  A  K  K  K  S  A  L  S  S  M  G  S  N  Y  S  S  H  L  Q  R  A  D  Q  K  R  G  G  K

LfTnTsb 541   AAAGAGaCTGAGAGAGAGAAGAAGAAGAAGATCCTGGCCGCCAGACGCAAGCAGCTGAACATCGACCATCTGAACGAGGACAAGCTGAAG 630
               K  E  T  E  R  E  K  K  K  K  I  L  A  A  R  R  K  Q  L  N  I  D  H  L  N  E  D  K  L  K

LfTnTsb 631   GATAAGATCAATGAGCTGCATGAATGGATGGTCACGCTGGAGTCTGAGAAGTTCGACCACATGGAGAGACTGAAGAGGCAGAAGTACGAG 720
               D  K  I  N  E  L  H  E  W  M  V  T  L  E  S  E  K  F  D  H  M  E  R  L  K  R  Q  K  Y  E

LfTnTsb 721   GTTACAACCCTGCGTAAGAGAATTGAGGAGCTCAGTAAATTCAGCAAGAAGGGCGCCGCCGCTCGCCGCAGAAAGTAGATCGCCTCGCCC 810
               V  T  T  L  R  K  R  I  E  E  L  S  K  F  S  K  K  G  A  A  A  R  R  R  K  *

LfTnTsb 811   CGCCACTGCTCAGAAACCAAACTGCGATCGTCTCGGACACACGCCTGATGAAACTACGCACACTGTGGCGAAGGTCGTAGCTCGGAAGAA 900
LfTnTsb 901   GCCAGTCATGCTGAGGTCTACTCTGCGGTTATCAACCCACGTCTTGAAACATGCAACCAGGAGCCTTTAGCTGCTGCTCAATAGGAATAA 990
LfTnTsb 991   AAAAAAAAAAAAAAAA 1006
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D

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efTnTsb    1   --------------------------------------------GCAGCTAAAGGCTCCAGGTTCGACAAGTCCACTCTCACCATGTCT 45
 aftnTsb   1   ----------------------------AGCAGGGAGCTCGCTTCTTTCAGCTTTTCTGAGGTTGCCACAAGTCCACTCTCACCATGTCT 62
 LfTnTsb   1   GGCACGAGGCTTACAGCCTGTCTCGGCCAGCAGGGAGCTCGCTTCTTTCAGCTTTTCTGAGGTTGCCACAAGTCCACTCTCACCATGTCT 90

efTnTsb   46   GACACTGAGGAAGTTGATCAGGTCGAGGAATACGATGCTGTAGAAGAGGAGGTAGTAGAGGAAGTAGAGGTGGCCCCTGAGGCGGCCCCT 135
 aftnTsb  63   GACACTGAGGAAGTTGATCAGGTCGAGGAATACGATG----------------------------------------------------- 99
 LfTnTsb  91   GACACTGAGGAAGTTGATCAGGTCGAGG-------------------------------------------------------------- 118

efTnTsb  136   GAGCCAGAGCCAGAGCCAGAACCAGAGCCAGAGCCAGAACCAGTGGTAGAACCAGAGCCAGAACCAGAACCAGAGCCTGAGCCTGAGCCT 225
 aftnTsb  99   ------------------------------------------------------------------------------------------ 99
 LfTnTsb 118   ------------------------------------------------------------------------------------------ 118

efTnTsb  226   GAAGAGGCCGTTGAAGAGGAAGAGGAGAAGCCAAAGTTCAAGCCCAGCGCTCCCAAGATCCCTGATGGTGAGAAAGTGGACTTTGATGAC 315
 aftnTsb  99   ----------------------AGGAGAAGCCAAAGTTCAAGCCCAGCGCTCCCAAGATCCCTGATGGTGAGAAAGTGGACTTTGATGAC 167
 LfTnTsb 118   ----------------------AGGAGAAGCCAAAGTTCAAGCCCAGCGCTCCCAAGATCCCTGATGGTGAGAAAGTGGACTTTGATGAC 186

efTnTsb  316   ATCCAGAAGAAACGTCAGAACAAGGACCTGGTTGAGCTGCAGGCCCTCATTGATGCCCACTTTGAGTGCAGGAAGAAGGAGGAGGAGGAG 405
 aftnTsb 168   ATCCAGAAGAAACGTCAGAACAAGGACCTGGTTGAGCTGCAGGCCCTCATTGATGCCCACTTTGAGTGCAGGAAGAAGGAGGAGGAGGAG 257
 LfTnTsb 187   ATCCAGAAGAAACGTCAGAACAAGGACCTGGTTGAGCTGCAGGCCCTCATTGATGCCCACTTTGAGTGCAGGAAGAAGGAGGAGGAGGAG 276

efTnTsb  406   CTGATCGCCCTCAAGGACAGGATTGAGAAGCGTCGTGCCGAGAGGGCCGAGCAGCAGAGGGTCCGTGCTGAGAAGGAGAAGGAGCGCCAG 495
 aftnTsb 258   CTGATCGCCCTCAAGGACAGGATTGAGAAGCGTCGTGCCGAGAGGGCCGAGCAGCAGAGGGTCCGTGCTGAGAAGGAGAAGGAGCGCCAG 347
 LfTnTsb 277   CTGATCGCCCTCAAGGACAGGATTGAGAAGCGTCGTGCCGAGAGGGCCGAGCAGCAGAGGGTCCGTGCTGAGAAGGAGAAGGAGCGCCAG 366

efTnTsb  496   GCGAGACGTGAGGAGGAGAGGCGGATCAGGGAGGAGGCTGACGCCAAGAAGAAGGCTGATGAGGATGCCAAGAAGAAGTCGGCTCTGTCC 585
 aftnTsb 348   GCGAGACGTGAGGAGGAGAGGCGGATCAGGGAGGAGGCTGACGCCAAGAAGAAGGCTGATGAGGATGCCAAGAAGAAGTCGGCTCTGTCC 437
 LfTnTsb 367   GCGAGACGTGAGGAGGAGAGGCGGATCAGGGAGGAGGCTGACGCCAAGAAGAAGGCTGATGAGGATGCCAAGAAGAAGTCGGCTCTGTCC 456

efTnTsb  586   AGCATGGGCTCCAACTACAGCAGCCACCTGCAGAGAGCCGACCAGAAGAGAGGAGGCAAGAAAGAGACTGAGAGAGAGAAGAAGAAGAAG 675
 aftnTsb 438   AGCATGGGCTCCAACTACAGCAGCCACCTGCAGAGAGCCGACCAGAAGAGAGGAGGCAAGAAAGAGACTGAGAGAGAGAAGAAGAAGAAG 527
 LfTnTsb 457   AGCATGGGCTCCAACTACAGCAGCCACCTGCAGAGAGCCGACCAGAAGAGAGGAGGCAAGAAAGAGaCTGAGAGAGAGAAGAAGAAGAAG 546

efTnTsb  676   ATCCTGGCCGCCAGACGCAAGCAGCTGAACATCGACCATCTGAACGAGGACAAGCTGAAGGATAAGATCAATGAGCTGCATGAATGGATG 765
 aftnTsb 528   ATCCTGGCCGCCAGACGCAAGCAGCTGAACATCGACCATCTGAACGAGGACAAGCTGAAGGATAAGATCAATGAGCTGCATGAATGGATG 617
 LfTnTsb 547   ATCCTGGCCGCCAGACGCAAGCAGCTGAACATCGACCATCTGAACGAGGACAAGCTGAAGGATAAGATCAATGAGCTGCATGAATGGATG 636

efTnTsb  766   GTCACGCTGGAGTCTGAGAAGTTCGACCACATGGAGAGACTGAAGAGGCAGAAGTACGAGGTTACAACCCTGCGTAAGAGAATTGAGGAG 855
 aftnTsb 618   GTCACGCTGGAGTCTGAGAAGTTCGACCACATGGAGAGACTGAAGAGGCAGAAGTACGAGGTTACAACCCTGCGTAAGAGAATTGAGGAG 707
 LfTnTsb 637   GTCACGCTGGAGTCTGAGAAGTTCGACCACATGGAGAGACTGAAGAGGCAGAAGTACGAGGTTACAACCCTGCGTAAGAGAATTGAGGAG 726

efTnTsb  856   CTCAGTAAATTCAGCAAGAAGGGCGCCGCCGCTCGCCGCAGAAAGTAGATCGCCTCGCCCCGCCACTGCTCAGAAACCAAACTGCGATCG 945
 aftnTsb 708   CTCAGTAAATTCAGCAAGAAGGGCGCCGCCGCTCGCCGCAGAAAGTAGATCGCCTCGCCCCGCCACTGCTCAGAAACCAAACTGCGATCG 797
 LfTnTsb 727   CTCAGTAAATTCAGCAAGAAGGGCGCCGCCGCTCGCCGCAGAAAGTAGATCGCCTCGCCCCGCCACTGCTCAGAAACCAAACTGCGATCG 816

efTnTsb  946   TCTCGGACACACGCCTGATGAAACTACGCACACTGTGGCGAAGGTCGTAGCTCGGAAGAAGCCAGTCATGCTGAGGTCTACTCTGCGGTT 1035
 aftnTsb 798   TCTCGGACACACGCCTGATGAAACTACGCACACTGTGGCGAAGGTCGTAGCTCGGAAGAAGCCAGTCATGCTGAGGTCTACTCTGCGGTT 887
 LfTnTsb 817   TCTCGGACACACGCCTGATGAAACTACGCACACTGTGGCGAAGGTCGTAGCTCGGAAGAAGCCAGTCATGCTGAGGTCTACTCTGCGGTT 906

efTnTsb 1036   ATCAACCCACGTCTTGAAACATGCAACCAGGAGCCTTTAGCTGCTGCTCAATAGGAATAAAAAAAAAAAAAA     1107
 aftnTsb 888   ATCAACCCACGTCTTGAAACATGCAACCAGGAGCCTTTAGCTGCTGCTCAATAGGAATAAAAAAAAAAAAAAAAA  962
 LfTnTsb 907   ATCAACCCACGTCTTGAAACATGCAACCAGGAGCCTTTAGCTGCTGCTCAATAGGAATAAAAAAAAAAAAAAAAAA 982
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Fig. A1. Nucleotide sequence and deduced protein sequence of the sea bream *fTnT* isoforms isolated. Putative isoform efTnTsb (A), isoform afTnTsb (B) and LfTnTsb (C) are shown. The ATG translation initiation codon is shown in bold as well as the TAG STOP codon, which is also indicated by an asterisk. The Kozak consensus sequence is underlined and the double underline indicates the polyadenylation signal. (D) Clustal X (Thompson et al., 1997) multiple nucleotide sequence alignment of sea bream *fTnT* cDNAs. The position of forward and reverse primers used for RT–PCR expression analysis are indicated by arrows.

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