THYROID GLAND DEVELOPMENT IN A MARINE TELEOST, *SPARUS AURATA* (LINNAEUS, SPARIDAE)

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With 2 plates

ABSTRACT. In the present study the development of the thyroid gland is described in cultivated sea bream (*Sparus aurata*) larvae. Follicles were readily identified using an adaptation of the Cleveland-Wolfe trichrome staining method, which renders the eosinophilic colloid brilliant red and the basophilic epithelial cells purple/blue. In *S. aurata* thyroid cells were first distinguished 4 days post-hatch (DPH) as small colloid rich cells. By 8 DPH occasional follicles containing dense homogeneous colloid in the lumen were observed. Follicles became more numerous and larger from day 11 onwards, although they still contained dense homogenous colloid, and the endothelial cells were squamous. By 36 DPH the thyroid follicles were numerous and appeared very active, the epithelial cell layer was columnar and little or no colloid was present in the follicle lumen.

KEY WORDS: Cleveland-Wolfe, follicles, sea bream, thyroid gland.

RESUMO. No presente estudo é descrito o desenvolvimento da glândula tiroide em larvas cultivadas de dourada (*Sparus aurata*). Os folículos foram prontamente identificados, usando uma adaptação do método de coloração tricrómico de Cleveland-Wolfe, o qual torna o coloide eosinófilo vermelho brilhante e as células epiteliais basófilas púrpura/azul. Em *S. aurata* as primeiras células da tiroide tornaram-se distintas ao fim de quatro dias após a eclosão (DAE), sob a forma de pequenas células ricas em coloide. Ao oitavo DAE, foram observados

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folículos ocasionais, contendo coloide homogéneo e denso no lúmen. Os folículos tornaram-se mais numerosos e maiores a partir do 11º DAE, embora ainda contivessem coloide denso e homogéneo e as células endoteliais fossem escamosas. Ao 36º DAE, os folículos da tiroide tornaram-se numerosos e pareceram muito activos, com a camada epitelial de células colunar e pouco ou nenhum coloide presente no lúmen do folículo.

INTRODUCTION

The principal function of the thyroid gland in vertebrates is the production of its two iodinated amino acid hormones, L-thyroxine (tetraiodo-L-thyronine) and 3,5,3'-triiodo-L-thyronine. Two principal types of activity have been attributed to thyroid hormones (THs), stimulation of metabolism and maturational effects (for example, acceleration of metamorphosis in amphibian tadpoles; HADLEY, 1992). Relatively little information exists about the maturational effect of thyroid hormones in fish larvae. However, several studies have indicated the importance of thyroid hormones in bony fish development and particularly in flat fish metamorphosis (BROWN, 1997; INUI & MIWA, 1985; LAM, 1980). The source of the THs early in development (maternal or larval) still remains to be resolved (BROWN *et al.*, 1989; POWER *et al.*, 2001; SULLIVAN *et al.*, 1987; TAGAWA & HIRANO, 1987, 1990).

The thyroid gland in all vertebrates is composed of follicles, these are ball-like structures which consist of a single layer of cuboidal epithelial cells enclosing a fluid filled lumen which contains colloid which is the source of thyroxine. In mammals, the thyroid follicles are organised in a discrete gland, which is bilobed, lies over the trachea and is richly supplied by blood. The thyroid gland has a more variable morphology in nonmammalian vertebrates. For example, the amphibian thyroid appears as two rounded lobes, which may lie far apart, the reptilian thyroid is relatively poorly studied but in turtles it is a single discoid structure located in front of the heart. In the cyclostomes and teleosts, however, no organised gland is present and the follicles are generally scattered singly or in small groups throughout the loose connective tissue of the pharynx, making identification difficult. Moreover, in teleost fish the absence of an encapsulated gland means follicles are not restricted to the pharyngeal region and frequently migrate to unusual sites such as the kidney and heart and sometimes also the brain, eye, oesophagus and spleen (GORBMAN *et al.*, 1983).

The embryological origin of the thyroid is well documented in vertebrates and arises as an outgrowth of the pharyngeal endoderm. However, the ontogeny of the thyroid during teleost development is poorly described and with the exception of lamprey ammocoetes (LEATHERLAND, 1994) there is a scarcity of published data. The small

size of fish embryos and larvae and the disperse nature of the follicles within the developing larval pharynx make identification difficult. In the present study, a rapid, simple method for staining thyroid follicles is described; the method specifically stains colloid and in this way makes it possible to assess the activity of the follicles. The method has been used to study the development of the thyroid gland in *S. aurata* larvae, an important aquaculture species but may be readily adapted for use with a range of other fish larvae.

METHODS

Larval culture

S. aurata larvae were hatched and cultured in 0.2 m^3 conical tanks with a continuous flow of gently aerated sea water. Hatching occurred 40 h after fertilisation. The larvae were fed on *Brachionus plicatilis* 4 days post hatch (DPH) when the yolk sac was consumed and *Artemia* sp., was introduced into the diet from 15 DPH until 40 DPH after which dry food was introduced gradually. Light conditions followed a cycle of 12 h light: 12 h dark and the ambient water temperature was 19 ºC ± 1 ºC.

Sampling and fixation

Eggs were sampled at 12 hour intervals and the chorion ruptured with a fine needle prior to fixation. Larvae were sampled on alternate days from 1 DPH (< 2.1 mm notochord length) to 60 DPH (19.4 mm standard length), anaesthetised in MS222 (0.1% solution in sea water) and fixed overnight at 4 ºC in a 4% paraformaldehyde (PFA) solution, washed in phosphate buffered saline (PBS, pH 7.6), dehydrated through a graded series of alcohol and embedded in wax and serial sagittal and transverse sections (6 mm), cut and mounted on poly-L-lysine coated slides.

Histology

Sections were dewaxed in xylene and rehydrated through graded alcohols to water and subjected to staining using an adaptation of the Cleveland-Wolfe trichrome method (CLEVELAND & WOLFE, 1932). The component stains of the method are used separately and can be varied independently in order to achieve the desired differentiation. Experience has shown that it is necessary to adapt the method for the species being studied, by varying staining times.

Rehydrated sections were stained in Erlich's hematoxylin (5 mins) and subjected to a short immersion in alkaline alcohol to "blue", then rinsed in tap water, followed by distilled water. Slides were then immersed in erythrosin (1% aqueous solution) for 5

mins; orange G (2% in 1% phosphotungstic acid) for 8-10 seconds and finally aniline blue (0.25% aqueous, acetified to pH 3-4) for 1.5 mins. After immersion in each of the stains, slides were rapidly rinsed in water and the excess allowed to drain off. Permanent preparations were made after staining by rinsing in two changes of absolute alcohol followed by xylene and covering with DPX and a glass coverslip.

RESULTS

Thyroid follicles in *S. aurata* stained with Cleveland-Wolfe trichrome had a very characteristic appearance. In common with what has been observed in other teleosts the thyroid follicles were not arranged in a discrete gland but were scattered throughout the connective tissue of the pharynx (Figs. 1A & B). The colloid present in the centre of thyroid follicles was generally eosinophilic and stained brilliant red and the layer of surrounding epithelial cells which varied in shape were basophilic and stained blue/ purple (Fig. 1C). The loss of colloid from the lumen appeared to start at the periphery of the lumen with the appearance of clear "vesicles" and progressed centripetally (Figs. 1C & D).

The first evidence of thyroid tissue containing colloid in cultured *S. aurata* larvae was at 3 DPH, when occasional colloid containing cells were observed in the pharyngeal region (Fig. 2A). Thyroid follicles were only apparent at 7 DPH in mid-line sagittal sections. The follicles contained dense intensely staining colloid and were surrounded by a single layer of squamous epithelial cells (Fig. 2B). Follicles became larger and more numerous as the larvae developed, but did not appear very active.

Thyroid follicles were numerous and clearly very active at 36 DPH and all those present contained little or no colloid and the epithelial cells surrounding the follicle lumen were columnar (Figs. 1A & B). By 60 DPH numerous follicles were evident in the connective tissue where the gill bars join the pharyngeal region (Fig. 1C). The diameter of the follicles was generally smaller than that observed in larvae at 36 DPH, the thyroid follicular epithelium was columnar and vesicles were observed at the periphery of the lumen around dense, brightly stained colloid suggesting the thyroid follicles were also active at this stage.

DISCUSSION

The Cleveland-Wolfe trichrome method has traditionally been used to differentiate pituitary gland cell types. In the present study this method was adapted for the detection of thyroid follicles in *S. aurata* larvae. The thyroid in *S. aurata* is similar to that observed in other teleosts (with the exception of the tuna fish and parrot fish). It is none "glandular", and the thyroid follicles are scattered throughout the connective tissue of the pharynx (GORBMAN *et al.*, 1983). Interestingly in early larval stages (3

DPH) of *S. aurata* no follicles were detected and instead isolated colloid containing epithelial cells were detected. It is tempting to speculate that the latter cells may be similar to type III cells of the endostyle in the lamprey ammocoete, which lacks thyroid follicles but is still able to produce THs (LEATHERLAND, 1994). In later stages of *S. aurata* larvae (7 DPH), despite the scarcity and small size of thyroid follicles they were readily identified using Cleveland-Wolfe staining as with this method the colloid is rendered bright red.

The activity of the thyroid follicles may be influenced by numerous factors, but the principal factor controlling activity is thyroid-stimulating hormone (TSH), which is produced by the pituitary gland (BAKER, 1964; LEATHERLAND & BARRETT, 1993). Alterations in the level of stimulation of thyroid follicles by TSH will be reflected by alterations in their appearance and several indices can be used to access thyroid follicle activity. For example, in unstimulated thyroid tissue, the follicles are usually large, their epithelial cells are squamous or cuboidal in appearance and the nucleus: cytoplasm ratio of the epithelial cells is high. The appearance of the colloid within the lumen may also be used to give an indication of activity. A densely staining, uniformly eosinophilic colloid is found next to squamous epithelium (GORBMAN *et al*., 1983). The colloid of active glands is non-uniform and usually contains numerous non-staining, vacuole like spaces. However, as the colloid is suggested to be prone to fixation artefacts, which result in vacuoles, interpretation of follicle activity based upon colloid is less clear. Clearly by combining Cleveland-Wolfe staining together with some simple histological measurements, an index of thyroid follicle activity can be determined.

The alteration in appearance of thyroid follicles with activity is readily explained by considering the biosynthesis of thyroid hormones. The follicular epithelial cells are responsible for the accumulation of diffusible iodide and the synthesis and exocytosis of thyroglobulin into the follicle lumen, where tyrosine residues in the thyroglobulin are iodinated. An increase in the biosynthetic requirements (stimulation) of the follicular epithelial cells will result in an increase in cytoplasmic volume as a consequence of an increase in the size and number of the organelles necessary for protein synthesis. The thyroglobulin in the lumen acts as an extracellular store of the thyroid hormones, an increased requirement for thyroid hormones will result in pinocytosis of thyroglobulin with the associated thyroid hormones and metabolism within the follicular epithelia to liberate the thyroid hormones. Clearly the latter will result in reduction in colloid (thyroglobulin) density in the follicular lumen.

The main difficulty in studying thyroid gland ontogeny occurs at the embryological stage, when cells are relatively undifferentiated and Cleveland-Wolfe and other staining methods do not permit the identification of thyroid tissue. Studies of the development of the thyroid gland in vertebrates show it has a relatively simple development from the floor of the pharynx. It appears early in development as a small pocket of tissue or a solid mass growing ventral in the midline at the level of the first

or second visceral pouches. In order to determine whether the early, undifferentiated gland produces thyroid hormones will require methods other than histological staining, such as immunocytochemistry and *in situ* hybridisation for proteins in the biosynthetic pathway of TH.

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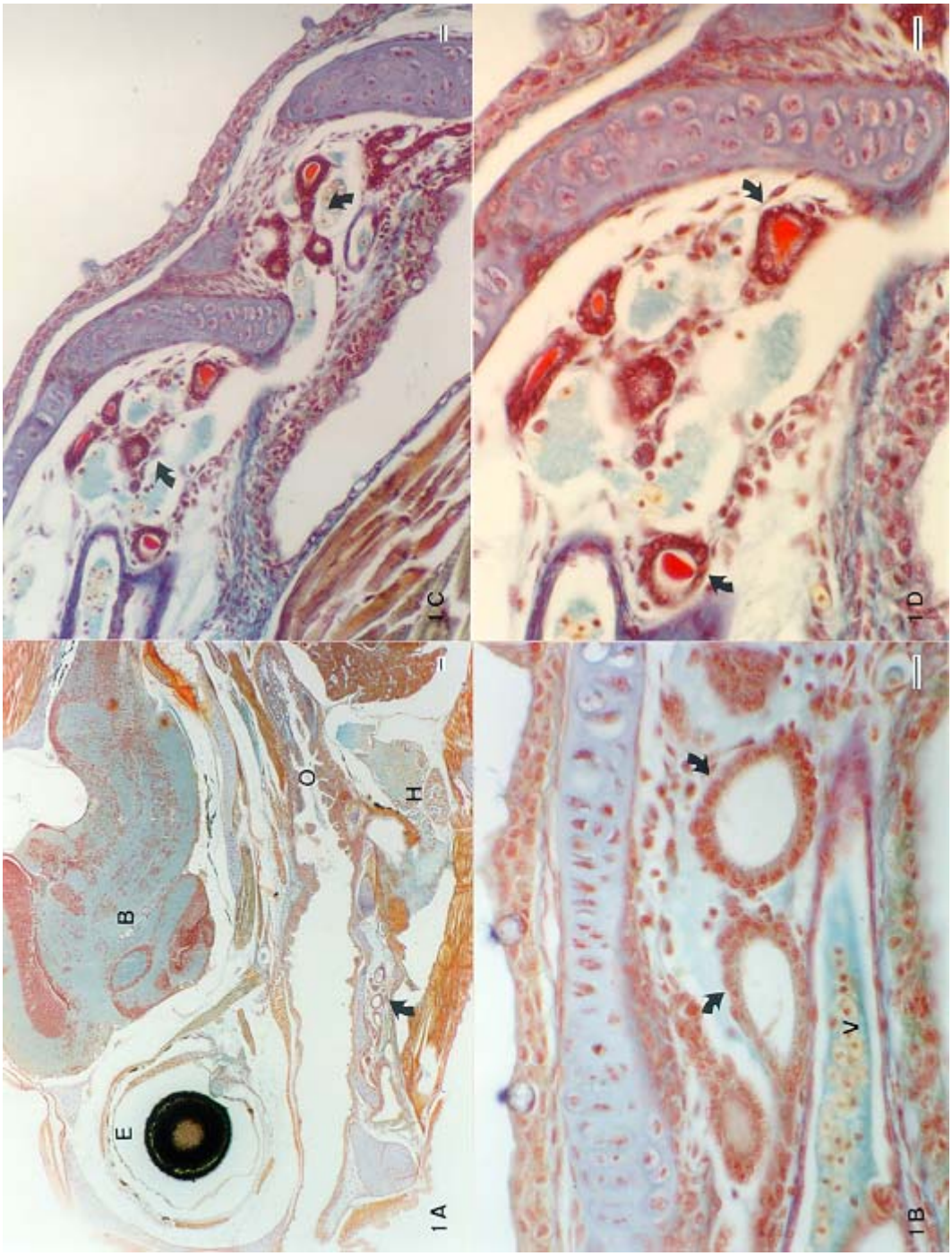
Plate I
Sagittal sections of *S. aurata* larvae after staining with Cleveland-Wolfe trichrome. A) Shows a 36 DPH larvae with the anterior region located on the left-hand-side of the plate. The thyroid follicles which are scattered in the connective tissue of the pharyngeal tissue are indicated with an arrow head. Numerous "empty" follicles are visible and major structures are indicated; E - eye, B - brain, H - heart, O - oesophagus. Scale bar corresponds to 5 mm. B) High power magnification of the thyroid follicles in A, note the variable size of the follicles and the columnar epithelial follicular cells and the absence of stainable colloid in the lumen. The area is richly supplied with blood vessels and a large vein (V) is visible. Scale bar corresponds to 1 mm. C) Thyroid follicles of a 60 DPH *S. aurata* larvae. Numerous groups of follicles (indicated by arrows) are visible in the pharynx where the gill bars are inserted. Note the variable size of the follicles and the bright red staining colloid which is depleted next to the follicular epithelial cells. Scale bar corresponds to 1 mm. D) Higher magnification of C, showing more clearly the follicles and associated blood vessels (V). The follicular cells are cuboidal and the colloid is depleted indicating the follicles are active. Interestingly the follicles are considerably smaller than those found at 38 DPH. Scale bar corresponds to 1 mm.


E
B
O
H
1A
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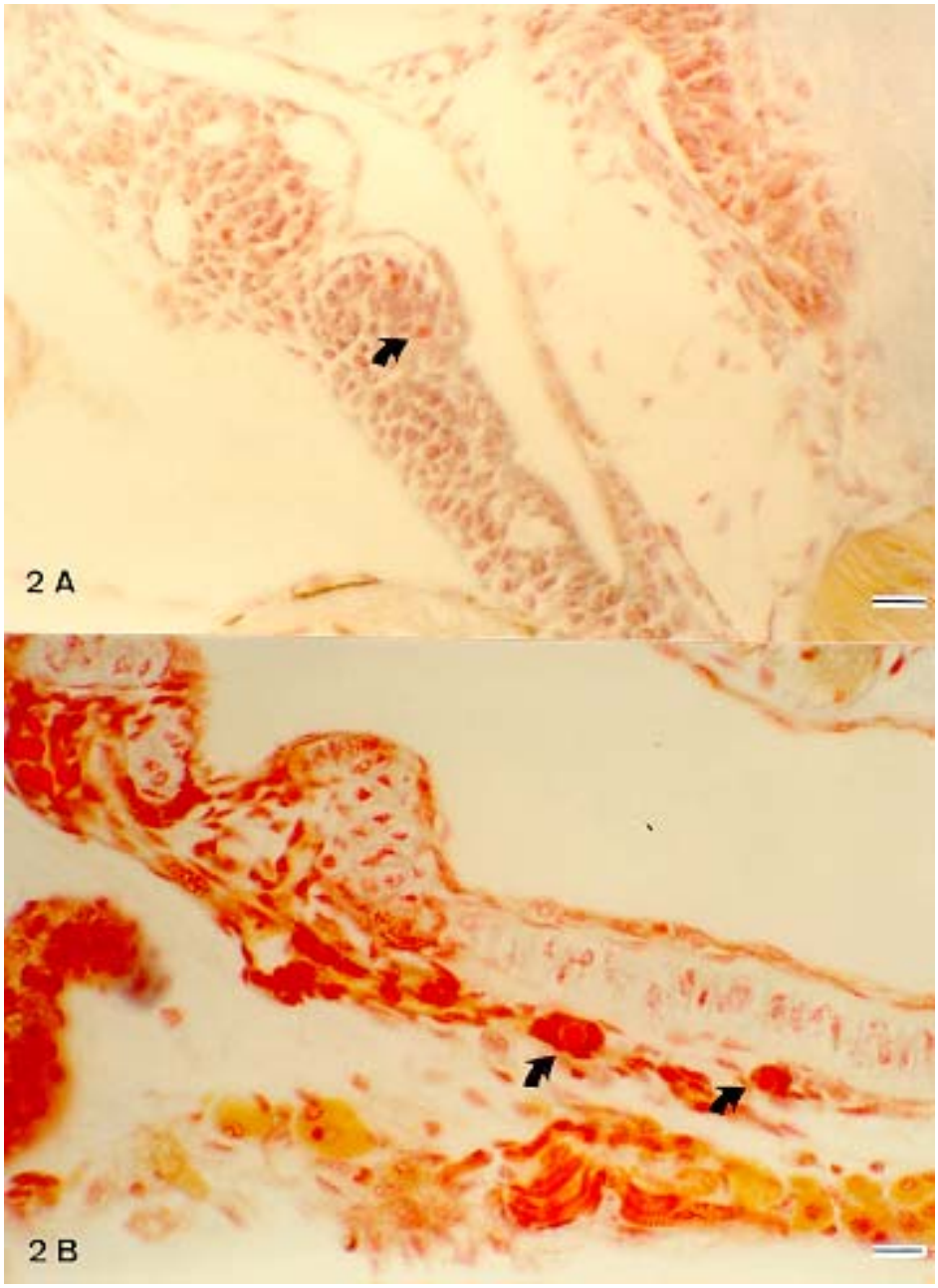
1C
1D

Plate II

Mid-line sagittal sections of *S. aurata* larvae after staining with Cleveland-Wolfe trichrome. A) 3 DPH larvae, the anterior region is located on the left-hand-side of the plate. The putative thyroid cells containing colloid are scattered in the connective tissue of the pharynx. A colloid containing cell is indicated by an arrowhead. Scale bar corresponds to 1 mm. B) 7 DPH larvae, the anterior region is located on the left-hand-side of the plate. Colloid containing follicles are highlighted with arrowheads and are evident in the connective tissue of the pharynx. Note the cuboidal epithelium which surrounds the densely staining follicle lumen. Scale bar corresponds to 1 mm.



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