New Tetrachromic VOF Stain (Type III-G.S) for Normal and Pathological Fish Tissues

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A new VOF Type III-G.S stain was applied to histological sections of different organs and tissues of healthy and pathological larvae, juvenile and adult fish species *(Solea senegalensis; Sparus aurata; Diplodus sargo; Pagrus auriga; Argyrosomus regius and Halobatrachus didactylus)*. In comparison to the original Gutiérrez´VOF stain, more acid dyes of contrasting colours and polychromatic/metachromatic properties were incorporated as essential constituents of the tetrachromic VOF stain. This facilitates the selective staining of different basic tissues and improves the morphological analysis of histochemical approaches of the cell components. The VOF-Type III G.S stain is composed of a mixture of several dyes of varying size and molecular weight (Orange G< acid Fuchsin< Light green<Methyl Blue<Fast Green), which are used simultaneously, and it enables the individual tissues to be selectively differentiated and stained. Muscle fibers, collagen, reticulin and elastin fibers, erythrocytes, cartilage, bone, mucous cells, oocytes and larvae were selectively stained and differentiated. Dyes with small size and molecular weight (i.e Orange G), penetrate all tissue structures rapidly, but are only tightly retained in densely textured tissues (i.e erythrocytes). Methyl Blue is an interesting triarylmethane dye (large size and molecular weight), which is incorporated in this new VOF tetrachrome stain, and acquires histochemical significance when used at acid pH (2.8) because collagen and reticulin fibers, as well basophilic and metachromatic substances (strongly ionized sulphated glycoconjugates) can be identified. Muscle tissues show an evident green colour (Fast Green or Light Green affinities), even those isolated and/or diffuse muscle fibers present in the digestive submucosa layer. Connective tissues showed a specific and strong blue colour (Methyl Blue affinity) or mixed blue-red staining (Methyl Blue and Acid Fucshin affinities). Very noticeable is the staining of the mucous cells, as well as the hyaline capsule of the viral lymphocystic cells, which were stained blue-purple (carboxylated and/or strongly ionized sulphated groups). Cartilaginous tissues showed a blue or purple (Methyl Blue affinity) staining, and a specific red colour (Acid Fucshin affinity) was evident during calcification or in bone structures (i.e skeleton, fins, gills, teeth).

Key words: Tetrachromic stain, cartilage, bone, fibers, digestive tract, ovary, skin, teeth, fish species.

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Trichrome methods invariably use dyes in acid pH solvents, usually diluted in aqueous acetic acid, and the concentration of this acid matches the concentration of dye. Staining depends largely on the attachment of dyes to proteins. The acid pH itself is necessary to maximise the amount of dye that will attach to tissue amino groups. Proteins have both positively (amino groups) and negatively (carboxyl and hydroxyl) charged groups. Usually one predominates and this will have an overall negative or positive charge (being an acid or a basic protein). These charges can, however, balance each other out to some degree. Phosphate groups of DNA and binding-proteins are important in nuclear staining. The ionisation of basic groups of proteins predominates at acid pH, and corresponding tissues will have affinity to acid dyes (Gutiérrez, 1990). In addition, dyes also have the same groups as the proteins, but may include the sulphonic group as well. Which of these groups is involved in any particular case depends on the circumstances, including the pH of the staining solution (Culling, 1976; Lillie, 1977; Pearse, 1985; Gutiérrez, 1990; Kiernan, 1999).

Trichrome stains can be applied as a one-step technique (i.e. van Gieson's and Gomori's methods) or as a multi-step method (i.e Masson's trichrome), where dyes and reagents are applied sequentially, and staining is optimised at each step (Culling, 1976; Lillie, 1977, Pearse, 1985; Bancroft and Cook, 1984; Gutiérrez, 1990; Kiernan, 1999).

The original Haematoxylin-Gutiérrez´VOF stain (Gutierrez, 1961,1967) is a one-step trichromic stain, useful for histological purposes and histopathological diagnosis (Gutiérrez *et al.*, 1963; Gutiérrez, 1990). Specially interesting is the maturation of the protozoan parasite, Martelia refrigens detected in the digestive gland of mussels, Mytilus edulis, by using the original Haematoxylin- VOF stain (Gutiérrez, 1977a, b), because mature stages of this parasite were clearly evidenced by a specific Orange G affinity, while inmature stages stain blue

due to a specific Haematoxylin affinity. Also, acidophilic zymogen granules secreted by the basophilic exocrine pancreas, erythrocytes accumulated in vascular systems, and yolk (larvae and oocytes) showed a strong or specific affinity to Orange G dye (Gutiérrez *et al*., 1985; Sarasquete *et al*., 1993, 1995, 2002; Ribeiro *et al*., 1999; Gisbert *et al*., 1999; Ortiz-Delgado *et al*., 2003; Santamaria *et al*., 2004). On the other hand, and interestingly, the mucous secreting cells containing neutral and/or carboxylate- and/or sulphate-rich glycoconjugates are present in the digestive tract, gills and skin of different larvae and adult fish species (Gutiérrez *et al*., 1986; Sarasquete *et al*., 1995, 1996, Gisbert *et al*., 1999; Ribeiro *et al*., 1999, Ortiz-Delgado *et al*., 2003; Arellano *et al*., 2001, 2004). Usually, these secretive cells are PAS and Alcian Blue positive, but they are unstained with Haematoxylin-eosin or Haematoxylin-Gutiérrez´VOF morphological techniques (Sarasquete *et al*., 1995, 2001; Arellano *et al*., 2001, 2002, 2004; Ortiz-Delgado *et al*., 2003).

Pituitary glands of vertebrates have been largely used for researching trichromic stains. In fact, by using both Gutiérrez´VOF stain (Gutiérrez, 1961, 1967, 1990) and Slidders´method (Slidders, 1961), which are composed of three (Light Green, Orange G and Acid Fucshin) isolated or mixed dyes, pituitary glands of vertebrates present an interesting colour variability of hormone-secreting cells; showing the acidophilic cells as orange or green; the basophilic cells as magenta-red and the chromophobic/amphiphilic cells as pale grey or green (Slidders, 1961; Rendón *et al*., 1997; Sarasquete *et al*., 1997; Rodríguez-Gómez *et al*., 2001). The architectural pattern of pituitary or stroma of different organs/tissues (Culling, 1976; Lillie, 1977; Rendon *et al*., 1997) is well demonstrated with reticulin stains (i.e Methyl Blue); and this triarylmethane (large size and molecular weight) and polychromatic dye (positive and negative charges) is an essential component of the now proposed tetrachromic VOF Type III -G.S stain.

In this paper we described a variant of the original trichromic Gutiérrez´VOF technique, which is a one-step tretrachrome stain (VOF Type III-G.S) composed of Light Green SF/or Fast Green FCF, Methyl Blue, Orange G and Acid Fuchsin. This stain was applied to histological sections of whole fish larvae and to sections of normal and pathological tissues from different marine fish species.

Material and Methods

Fixation and embedding samples

Samples (8-15x3-4 mm) of different tissues (ovary, digestive tract, gills, kidney, spleen, liver, heart) of healthy and pathological organisms (viral infected skin) and whole larvae and juvenile specimens (*Senegales sole, Solea senegalensis; seabream, Sparus aurata; sargo, Diplodus sargo; redbanded seabream, Pagrus auriga;* meagre, *Argyrosomus regius and toadfish, Halobatrachus didactylus*) are fixed in Helly´s or Bouin´s fluids during 2-10 h, depending on their thickness. Most trichrome stains benefit from picric acid or mercuric chloride fixation. If tissues have been fixed in formalin 10% buffered with phosphate (0.1 M at pH 7.2), fixed sections can be postfixed in Bouin's fluid or treated in picro-mercuric-alcohol (a saturated solution of picric acid in absolute alcohol containing 3% mercuric chloride) overnight. Subsequently, mercuric pigment must be removed with iodine/thiosulphate and the sections washed in water to remove picric acid. After fixation, samples are washed in 80% ethanol, and dehydrated through increasing ethanol series (or acetone), cleared and embedded in 56-60°C paraffin. Sections are routinely made at 4-6 μm, stained and mounted in Eukit medium.

Several triarylmethane derivatives: Light Green SF ($C_{37}H_{34}N_2O_9S3Na_2$, MW 792.875)/or Fast Green FCF ($C_{37}H_{34}N_2O_{10}S_3Na_2$, MW 808.86); Methyl Blue ($C_{37}H_{27}N_3O_9S_3Na_2$, MW 799.81) and Acid Fuchsin (C20H17N3O9S3Na2, MW 585.55) and a monoazoic dye: Orange G ($C_{16}H_{10}N_2O_7S_2Na_2$, MW 452.386) are used to prepare the original VOF´Gutiérrez, VOF Type-II and VOF Type III G.S stains (For review Lillie, 1977 and Zollinger, 1991; http://members.pgonline.com/~bryand/StainsFile/dyes/).

Tetrachromic VOF Type III -G.S stain solution

1. Dissolve Fast Green FCF (260 mg) or Light Green SF (260 mg), Methyl Blue (140 mg), Orange G (500 mg) and Acid Fuchsin (600 mg) completely in freshly boiled distilled water (100 ml), and let the solution cool down to room temperature.

2. Add phosphotungstic acid (1.5 g); to dissolve it, add glacial acetic acid (3 mL) and absolute ethanol (200 mL).

3. Mix, filter and transfer to three 125 ml brown-

Table 1. Histological colour results and tinctorial affinities

Tissue	Colour	Tinctorial affinity
Bone	Reddish	Acid Fuschin
Calcificating Structures	Purple-Reddish	Methyl Blue-Acid Fuschin
Cardiac Musculature	Green-Bluish	Fast Green or Light Green/Methyl Blue
Cartilage	Blue-Purple	Methyl Blue
Chromatin	Bluish or Green	Methyl Blue or Fast Green/Light Green
Connective Tissue	Blue/Red	Methyl Blue/Acid Fuchsin
Cytoplasm of Oocytes	Blue/Bluish-Red	Haematoxylin/Methyl Blue-Acid Fuschin
Cytoplasm of Fibroblasts/Lymphocystis	Blue-Purple/Green	Methyl Blue/Fast Green or Light Green
Dentin Mineralization/Teeth	Purple-Red	Methyl Blue-Acid Fuschin
Digestive Musculature	Green	Fast Green or Light Green
Elastin	Red/Blue	Acid Fuschin/Methyl Blue
Erythrocytes	Yellowish	Orange G
Exocrine Pancreas/Zymogen	Orange-Reddish	Orange G-Acid Fuchsin
Gill Skeleton	Reddish	Acid Fuschin
Hyaline Capsule of Lymphocystis	Blue-Purple	Methyl Blue
Mandible Bone	Reddish	Acid Fuschin
Mature Collagen (I)	Red	Acid Fuschin
Mucous Cells	Blue-Purple	Methyl Blue
Nuclei	Blue	Haematoxylin
Nucleoli	Red-Orange	Acid Fuschin-Orange G
Reticulin Fibers	Blue	Methyl Blue
Skeletal Musculature	Green/Red	Fast Green or Light Green/Acid Fuschin
Viral Intracytoplasmic Inclusions	Red	Acid Fuschin
Zona Radiata of Oocytes	Red-Blue	Acid Fuschin-Methyl Blue
Yolk-Granules of Oocytes	Orange	Orange G
Yolk-Sac Larvae	Green-Bluish/Orange G (heterogeneous yolk)	Fast Green-Methyl Blue/Orange G
Young Collagen (II)	Blue-Purple	Methyl Blue

glass bottles with a screw-capped dropper (this avoids evaporation).

All dyes and reagents were purchased from Sigma or Merck.

Staining procedure

1. Sections are deparaffinized and placed in distilled water. If they have a yellowish colour, they are washed with a solution of ammonium hydroxide (two drops in 100 mL of distilled water), and then washed with distilled water.

2. Stain the nuclei in acetic Harris's Haematoxylin (three drops of acetic acid in 10-12 ml of Haematoxylin) and leave for 3-4 min. They can be also stained in freshly-prepared Ferric or Mayer Haematoxylin (Krutsay, 1962; Pearse, 1978; Gutiérrez, 1990). VOF Type III-G.S stain may be performed both after or before the PAS reaction. In addition, Haematoxylin- Gutiérrez´VOF and VOF-Type II stains (Gutiérrez, 1990; Rendon *et al.*, 1997; Sarasquete *et al.*, 1997) may also be performed for comparative studies.

3. Wash with tap water for 8-10 min and then with distilled water. Stain with polychromic VOF Type III -G.S (pH 2.8) stain for 3-5 min.

4. Wash with 80% ethanol, dehydrate with absolute ethanol, clear with xylene and mount in synthetic resin according to standard procedures.

Manual procedures (embedding and staining) or automatized paraffin embedding system (Leica EG1140C) and Autostainer (XL, Leica) can be used.

Results

Table 1 and Figures 1-3 summarize and show different staining tinctorial affinity and colour variability in several organs and tissues of different fish species stained with with Haematoxylin-VOF Type III-G.S technique.

Oogenesis

In *Solea senegalensis* ovaries stained with Haematoxylin VOF-III-G.S, oogonia and cytoplasm of previtellogenic oocytes are basophilic and stain blue (Haematoxylin affinity) due to the presence of high amounts of RNA. Progressively, during the vitellogenesis phase, a decrease in cytoplasmic basophilia and RNA and a progressive increase of basic protein synthesis and parallelly increased

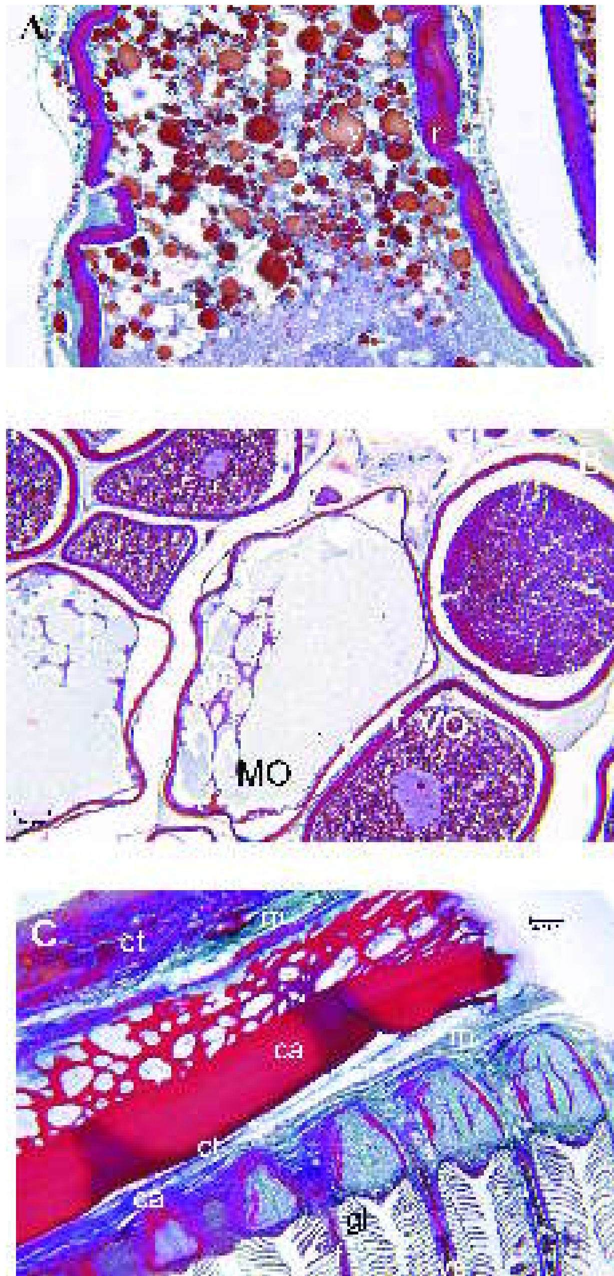

Figure 1. Histological sections of the ovary and gills. Haematoxylin-VOF Type III-G.S. A. Atretic vitellogenic oocyte of the Solea senegalensis ovary. Degeneration of the oranginophilic yolk granules, resorption of cytoplasmic content and hypertrophy of follicular envelope and desorganization and fragmentation of the strongly acidophilic zona radiata are observed. B. Solea senegalensis maturating ovary. Coalescence of yolk-granules and globules (neutral lipids) and hydration of maturating oocytes, which show a green cytoplasm (light green affinity). Vitellogenic oocytes containing lipid globules (vacuoles) and oranginophilic yolk-granules, as well as zona radiata and follicular envelopes of these oocytes are observed. D. Halobatrachus dydactylus gills. Calcified skeleton (red) of gill arches, gill filaments and lamellae, muscle, connective tissue, and vascular system containing yellow erythrocytes are observed. ca: calcificating areas; ct: connective tissue; f : follicular envelope; gc: globule coalescence; gl: gill lamellae; m: muscular; MO: maturating oocyte; r: radiata envelope; VO: vitellogenic oocyte; vs: vascular system; yc: yolk-coalescence; y: yolk-granules.

affinity to acid dyes were detected. The cytoplasm of the Solea senegalensis vitellogenic oocytes stains green when applying the Haematoxylin Gutiérrez´VOF technique and it shows a Methyl Blue affinity when Haematoxylin-VOF III-G.S stain was performed. Yolk granules of these vitellogenic oocytes stain orange (Orange G affinity) and the vitelline envelope or zona radiata shows red (external portion) and blue (internal portion) staining. In maturating *Solea senegalensis* oocytes, characterized by coalescence of the acidophilic yolk-granules, migration of nuclei towards the animal pole and its breakdown and hydration of oocytes, a homogeneous and weak green colour was detected within cytoplasms/yolk coalescence. Atretic oocytes (Figure 1A) are clearly identified by resorption of cytoplasmic content, yolk degeneration, hypertrophy of follicular cells and fragmentation of the vitelline envelope. A light orange colour was detected in hypertrophied and disintegrated zona radiata, in degenerated yolk-granules, as well in erythrocytes accumulated within the follicular cavity. Ovarian stroma is stained blue or reddish, and muscle tissue appeared green when Haematoxylin- VOF III-G.S was performed. In general, nucleoli stain red-orange and chromatin appears green or bluish (Table 1, Figure 1 A; Figure 1B).

Larvae Development

By using Haematoxylin-VOF Type III-G.S stain during larval development of *Sparus aurata, Diplodus sargo, Solea senegalensis and Pagrus auriga*, the homogeneous yolk-sac at hatching stains bluish-green. As resorption proceeded, the acidophilic yolk appeared granular and began to fragmentise into heterogeneous drops stained orange, until its complete resorption, at which time basophilic (RNA) hepatic tissue was evident surrounding the remnants of the yolk-sac envelope (3-4 days posthatching).

Interestingly, intestinal supranuclear vacuoles located in the posterior intestine of different fish larvae stain red-orange. During endogenous feeding, by 2-3 days after hatching (DAH), primordial gill arches and first chondroblasts were observed. Gill filaments and primordial lamellae were observed around 10-15DAH. From this time onwards, four pairs of gill arches are visible. When Haematoxylin-VOF-III G.S stain was performed, gill cartilage matrix (containing collagen type II) stains blue (Methyl Blue affinity) and a progressive

colour change (blue-purple-red) was evidenced during mineralization and ossification processes; in calcified structures and bone (containing collagen type I) of skeleton, gills, fins and mandible/teeth from different fish species, a specific reddish colour due to Acid Fucshin affinity was observed (Table 1; Figure 1 C, Figure 2A, 2D, Figure 3B, 3C, 3D).

During larval development of fish species, skeletal musculature shows, in general, a green colour and in some muscular areas appear fibers stained green-reddish (Figure 2D). When mucous cells appeared in gills, skin and digestive tract, they were weakly stained blue-purple (Methyl Blue affinity) when maturating, such as was observed in mucous cells from adult fish tissues.

Digestive Tract

An interesting differentiation was detected in the digestive tract of adult fish species (Halobatrachus didactylus, Argyrosomus regius) when the Haematoxylin-VOF III-G.S technique was performed (Figure 2B, 2C). Muscle layers showed an evident green colour, even those isolated and diffuse muscle fibres present throughout connective tissue from the submucosa layer. This connective tissue layer showed a specific and strong blue colour (Methyl Blue affinity). A very interesting observation is the staining of the oesophageal and intestinal mucous cells, which are not stained by different morphological stains (Haematoxylin-Eosin, Haematoxylin-Gutiérrez´VOF), but stain blue or light purple to Haematoxylin-VOF Type III- G.S (Table 1, Figure 2B. 2C).

Interestingly, glandular epithelial border shows also an evident purple (Figure 2B) colour similar to staining detected in mucous cells; and brush border of the digestive epithelium stains green to Haematoxylin-VOF Type III-GS.

On the other hand, most cardiac muscle fibers from larvae and adult fish species stain green or green-bluish. Skeletal musculature stains green and in some muscle areas red fibers appear (Figure 2D, 3B). Reticulin fibers of the liver, spleen and kidney stain blue (Methyl Blue affinity). Elastin fibers abundant in endothelia of the vascular system stain red (i.e gills) or bluish (Table 1).

Viral Lymphocystis Disease/Fibroblastic Cells

In skin of lymphocystis- (Iridovirus, DNA) infected seabream, *Sparus aurata* juvenil specimens, an enlargement of fibroblasts and massive hyperprophy and encapsidation of host cells by an induced extracellular hyaline matrix were detected. Hypertrophied fibroblasts are always located in the collagenous connective tissue of the dermis, but never in epidermis. The host reaction take place in the connective tissue, with macrophages and erytrocytes (yellow) surrounding lymphocystis cells. A hyaline capsule formed from extracellular matrix of infected cells, as well as acidophilic viral intracytoplasmic inclusions are observed progressivelly.

Interesting results were observed (hyaline capsule, cytoplasm, nuclei, intracytoplasmic inclusions) in the fibroblastic viral infected cells of Sparus aurata juvenile specimens when Haematoxylin-VOF III-G.S staining was performed (Table 1, Figure 3A). Cytoplasm of the fibroblastic viral infected cells stain blue-red (Methyl Blue and Acid Fucshin affinities). Hyaline capsule of these lymphocystis cells stains blue-purple; the peripheral zone of cytoplasm showing a green colour, and DNA-viral intracytoplasmatic inclusions stain red (Figure 3A). Connective tissue stains blue or red-bluish and the muscle layer appears green with scarce fibers stained reddish. Hypertrophied mucous cells present in epidermis of these viral infected seabream juvenile specimens stain blue or purple.

Developing bone

By using Haematoxylin-VOF Type-III-G.S, during larvae development and juvenile specimens, notochord, vertebrae, axial and appendicular skeleton and nervous system are well differentiated from other cell structures (Figure 3B).

During development of the *Argyrosomus regius* teeth, superficial cells of the dental papilla differentiate into odontoblasts. As soon as the enameloid matrix formation was finished, odontoblasts began to secrete dentin matrix continuously, then mineralization occurrs and spreads in both dentin and enameloid. In a completely formed *Argyrosomus regius* tooth, the pulp was composed mainly of connective tissue and occupied the center of the tooth. The odontoblasts were arranged at the outermost region of the pulp and they secrete dentin.

By using Haematoxylin-VOF-Type III G.S and Gutiérrez´ VOF stains, dentin (composed mainly of collagen fibers), was structurally similar to mandible acellular bone, and strongly stained reddish (Acid Fuchsin affinity), its extracellular matrix (which is not fully mineralized) showing focal bluish-purple-stained areas (due to Methyl Blue

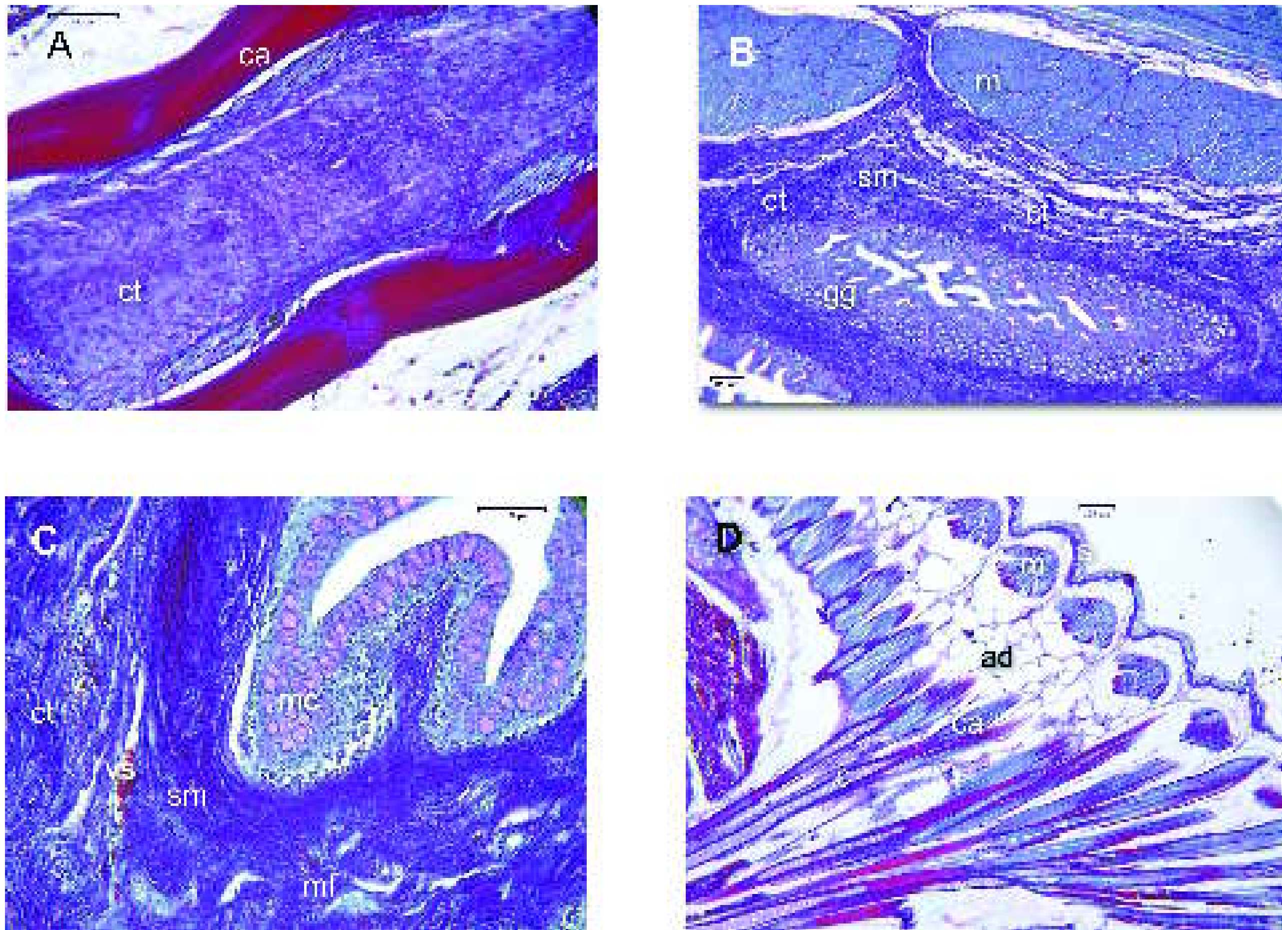

Figure 2. Histological sections of juvenile specimens and digestive tract of adult fish species. Haematoxylin-VOF Type III-G.S. A. Sparus aurata juvenile specimen. Histological section of fin showing calcificating areas (red) in the external portion, and connective central portion containing blue collagenous fibers. B. Argyrosomus regius stomach. Stomach mucosa containing gastric glands; submucosa of connective tissue and muscle are observed. C. Argyrosomus regius intestine. Intestinal mucosa containing evident purple mucous cells and submucosa of connective tissue (blue) containing diffuse muscle fibers (green) are identified. D. Diplodus sargo juvenile specimen. Longitudinal histological section showing the skin, adipose tissue, skeletal musculature and intramuscular spines with calcificating cartilagenous areas. ad: adipose tissue; c: cartilage; ca: calcificating areas; ct: connective tissue; gg: gastric glands; m: muscle; mf: muscle fibers; mc: mucous cells; s: skin; sm: submucosa; vs: vascular system.

affinity). When finishing mineralization of extracellular matrix (red colour), osteoblasts dissapear and lacunae/endosteal cells are evidenced (Figure 3C, Figure 3D).

Discussion

The physico-chemical and histochemical mechanisms of the trichromic VOF´Gutiérrez staining were discussed previously (Gutiérrez, 1990). When applying the proposed VOF Type III-G.S stain, a mixture of several dyes of varying size and molecular weight (Orange G< Acid Fuchsin< Light Green<Methyl Blue<Fast Green) are used simultaneously, which enables the individual tissues to be selectively differentiated and stained (i.e. muscle fibers, collagen, reticulin, erythrocytes, cartilage, bone, etc.). The acid nature of Orange G and the amphoteric character of Light Green and Acid Fucshin were pointed out. These dyes are hydrophilic, and at acid pH enhances the protonization of basic groups of proteins (Gutiérrez, 1990; Sarasquete *et al.*, 1993).

As for other methods (i.e. Gutiérrez´VOF and Masson stains), this tetrachromic VOF Type III-G.S stain also incorporates polyacids or *colourless dyes* (phosphotungstic or phosphomolybdic acids), which are high molecular weight compounds producing displacement in chemical reactions. In practical histological terms, this is referred to as differ-

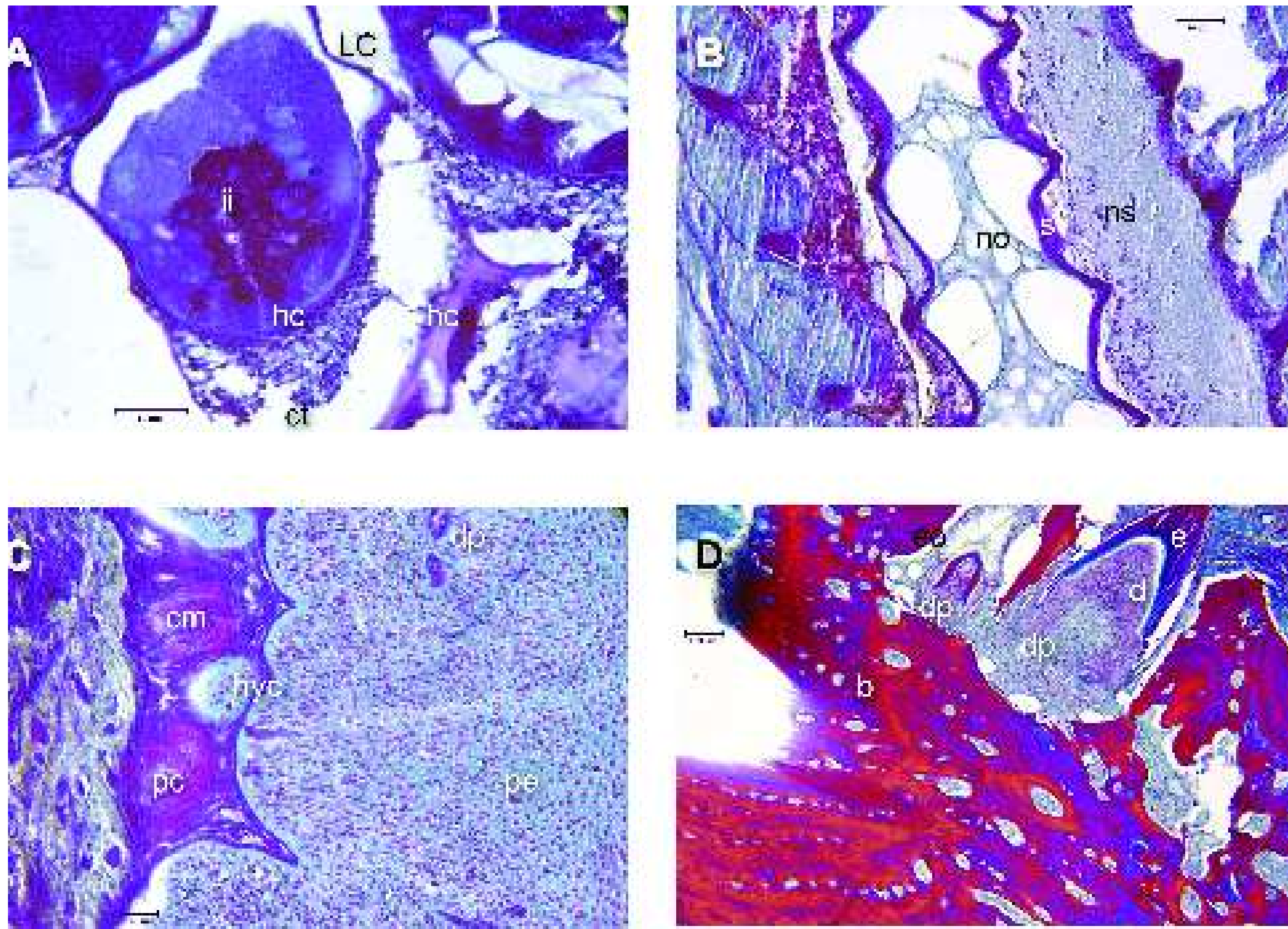

Figure 3. Histological sections stained with Haematoxylin-VOF Type III-G.S. A. Detail of lymphocystic infected cell in the skin of the Sparus aurata juvenile specimen. Hypertrophied fibroblastic cells showing the hyaline capsule and typical acidophilic intracytoplasmic inclusions, as well as the subjacent connective tissue around it. B. Histological section of Solea senegalensis larvae (33 DAH). Notochord (green), weak calcification (red) of axial skeleton and nervous system are differentiated. C.Dental papillae, calcificating areas, hypertrophied and proliferating chondrocytes are observed in the pseudostratified epithelium of Argyrosomus regius mandible. D. Developing of Argyrosomus regius. teeth showing enamel organ and dentin mineralization, which is similar in structure to acellular mandible bone (red). b:bone; cm: calcified matrix; ct: connective tissue; d: dentin; dp: dental papilla; e: enamel; eo: enamel organ; hc: hyaline capsule; hyc: hypertrophied chondrocytes; ii: intracytoplasmic inclusion; no: notochord; ns: nervous system pc: proliferative chondrocytes; s: skeleton; pe: pseudostratified epithelium.

entiation or the ability to replace a dye already attached to tissue groups by another dye which is similarly charged; in other words, within the context of polychrome staining, using one acid dye to replace another on tissue amino groups (Puchtler and Isler, 1958; Bulmer, 1962; Everett and Miller, 1974). However, the commonest Van Gieson´stain does not use a polyacid at all. The strongly acidic picric acid provides what acidity is required. There is also at least one variation of Lendrum's Picro-Mallory stain which uses trichloracetic acid rather than one of the acids above. It should be noted that trichloracetic acid differs from the other two in that it does not contain any metal atoms (Culling, 1976; Pearse, 1985; Bancroft and Stevens, 1990; Gutiérrez, 1990, Kiernan, 1999).

Acidic tissue elements, such us nuclei, would have an affinity for a basic stain (Haematoxylin), while cytoplasm, which is basic in character, will have an affinity for acid stains (eosin, VOF dyes). However, DNA (nucleoproteins, phosphate groups) and RNA contents (ribosomes/proteins synthesis), as well as the size of dyes, and the ionic strength and pH of the staining solutions often affect the staining reaction (Gutiérrez, 1990). In cytoplasm, negative charges of the synthesized RNA show affinity for basic groups of dyes (Sarasquete *et al.*, 1993). In the liver and oocytes of the different studied

species, cytoplasm can be stained blue (RNA/ribosomes) or show acidophilia (basic protein synthesis), having affinities for Haematoxylin, Acid Fucshin (hepatocytes) or Methyl Blue (oocytes) respectively. By using both Haematoxylin-Gutiérrez´VOF (Gutiérrez *et al.*, 1985; Sarasquete *et al.*, 1993, 2002; Grau *et al.*, 1996) and Haematoxylin-VOF Type III.-G.S techniques, yolk granules of the vitellogenic oocytes, and erythrocytes, stain yellow (Orange G affinity) due to high content of basic proteins and haemoglobin, respectively. Staining of the nuclear structures with VOF dyes is possibly due to DNA-protamines/histones, which are basic proteins rich in lysine and arginine (Gutierrez, 1990; Sarasquete *et al.,* 1993). When applying the Haematoxylin-VOF type III-G.S stain, nuclei stain dark blue (Haematoxylin affinity); chromatin stains green-bluish (Light Green/Methyl Blue affinities) and nucleoli stain red-orange (Acid Fucsin/Orange G affinities).

The proposed VOF Type III- G.S stain incorporates an additional dye (Methyl Blue). This triarylmethane dye, used at acid pH (2.8), acquires histochemical significance, because reticulin and collagen fibers, as well as basophilic and metachromatic substances (i.e acidic polyanionic groups) can be identified. A very interesting observation is the staining of the mucous cells, which are uncoloured (Sarasquete et al, 2001; Arellano *et al.*, 2001, 2002, 2004), with different morphological stains (Haematoxylin-Eosin, Haematoxylin-Gutiérrez´VOF dyes), but these secretive cells appeared blue or light purple when Haematoxylin-VOF Type III G.S stain was applied. This polychromatic/metachromatic property can be attributed to glycoconjugates containing strongly ionized sulphated groups, which have negative charges but also are extremely hydrophilic attracting large volumes of water and cations (Gutiérrez, 1990). In larvae and adult fish species, carboxylated, and especially sulphated, glycoconjugates (strongly ionized) are important components of the digestive, skin and gill mucous secretions (Sarasquete *et al.*, 1995, 1996; 2001; Ribeiro *et al.*, 1999; Gisbert *et al.*, 1999, Arellano *et al.*, 1999, 2001, 2002, 2004; Ortiz-Delgado *et al.*, 2003). Similarly, the hypertrophied mucous cells present in epidermis of the viral lymphocystis- infected Sparus aurata juvenile specimens, stain blue (carboxylated groups) or metachromatic purple colour (sulphated groups). Also, the hyaline capsule of these lymphocystic cells stained blue-purple, due to high content of strongly ionised sulphated glycoconjugates, as previously described (González de Canales *et al.*, 1996; Sarasquete *et al.*, 1998). Sulphated glycoconjugates give a strong alcohol-resistant metachromasia, while non-sulphated groups and nucleoproteins give a weak metachromasia that is susceptible to alcohol extraction (Pearse, 1985, Gutiérrez, 1990).

When using triarylmethane dyes (i.e. Acid Fucshin, Methyl Blue, Light Green, which are components of the VOF-Type III-G.S stain), loose/areolar and dense connective tissues, elastin, reticulin or collagen fibres, as well as specialised fibrous connective tissue, such as cartilage and bone have different composition and show different tinctorial variability. Collagen has affinity for anionic aniline dyes of large molecular size (i.e. Methyl Blue, Acid Fucshin), which have a tendency to bind through electrostatic attraction or Van der Waals forces (Herovici, 1963; Horobin and Bennison, 1973 Bancroft and Stevens, 1990). Methyl Blue stains young collagen and reticulin where the fibrils are closely knit. Type II collagen is found in both hyaline and elastic cartilage whilst the latter also contains abundant elastic fibers. Type I collagen is the most common form encountered in bone and mature collagen fibers with larger spaces being stained red by Acid Fuchsin. Type III collagen occurs in conjunction with other types of collagen fibers and is a major component of reticulin, which also contains other types of collagen, glycoproteins and proteoglycans. Collagen IV is typical in basal laminae and Type V collagen is found mainly in blood vessels (Herovici, 1963; Horobin and Bennison, 1973; Lillie, 1977; Bailey, 1978; Pearse, 1985; Bancroft and Stevens, 1990, Gutiérrez, 1990; Kiernan, 1999). In different larvae, juvenile and adult fish species (i.e. *Diplodus sargo, Sparus aurata, Pagrus auriga, Solea senegalensis, Halobatrachus didactylus*), gill cartilage matrix containing collagen Type II stains blue (Methyl Blue affinity) and a progressive colour change (blue-purple-red) was evidenced during mineralization and ossification processes, showing in calcified structures and bone (collagen Type I) of the skeleton, gills and fins, a specific reddish colour due to Acid Fucshin affinity.Interestingly, during development of the *Argirosomus regius teeth*, dentin matrix is secreted by odontoblasts and the mineralization process occurs progressively in both dentin

and enameloid (Ortiz-Delgado *et al.*, unpublished data). Dentin, structurally similar to mandible acellular bone (Sire and Huysseune, 2003) was strongly stained reddish (Acid Fucshin affinity), showing its extracellular matrix, not fully mineralizated, focal areas stained bluish-purple (Methyl Blue affinity) when Haematoxylin-VOF Type III G.S was applied.

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