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Case report
Κλινικό περιστατικό

Squamous Cell Carcinoma (SCC) in Brown meagre (*Sciaena umbra* Linnaeus, 1758), a new candidate species for aquaculture in Mediterranean

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ABSTRACT: A case of epithelial tissue tumors on the body of two adult Brown meagres (*Sciaena umbra* Linnaeus, 1758) is reported. Brown Meagre population of 20.000 was imported to sea cages on September of 2003 at 3g, to check the potential use of this species as a new candidate in Mediterranean aquaculture. The population had been kept for 8 years to serve as a genetic pool for further zootechnical investigation. The main pathology observed after 8 years in the sea cages were mainly ocular lesions (cataract) possibly due to natural aging and/or long culture period under high natural light intensity (UV radiation) and/or net injuries at a percentage of 1,32% of total cultured population. Apart from that, on two fish, reddish- white tumorous growths were evident on skin, macroscopically. Histologically, the tumorous growths were diagnosed as Squamous cell carcinoma. Numerous rounded pearls and nests of epidermal proliferated cells were evident, some of which had a non-cellular center. There were also foci of necrosis and irregular deposits of keratin within the tumor tissue. Special stains (Ayoub-Shklar, Haematoxylin-Eosin & Schiff's Periodic Acid) were applied to the tissue for comparison. To our knowledge this is the first presented report of SCC in this species.

Keywords: Squamous cell carcinoma, Ayoub-Shklar Stain, keratin, *Sciaena umbra*, Brown meagre

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INTRODUCTION

The epidermis in teleost fish is a non-cornified stratified squamous epithelium that varies in thickness (from 3 up to 25 cells) (Genten et al., 2009) and is capable of producing intermediate filaments (tonofilaments) of keratinous origin (Garcia et al., 2005)which represent the most abundant proteins (nearly 80% of total protein content) in stratified epithelia of vertebrates (Rao et al., 2015).Intermediate filaments (IFs) are cytoskeletal proteins generating complex networks that support the cell's shape and conjunctions to other cells (maculae adherents or desmosomes) (Schaffeld& Markl on Omary&Coulomb 2004). In cases of abnormal manifestations on fish epidermis, IFs and especially cytokeratins can be a major diagnostic tool on malignancies' differentiation, characterization and origin.

Teleost fish, like other vertebrates can develop benign, premalignant and/or malignant cellular deformities. The etiological agents or suspected factors of such manifestations are contributed to environmental (pollution, carcinogenic chemicals, radiation, toxins), hereditary (genetic predisposition), enzymatic, immunological, traumatic, viral& other constituents(Coffee et al. 2013,Pinkney *et al.* 2004, Landsberg 2002, Pinkney *et al.* 2001, Morrison *et al.* 1996, Harshbarger *et al.*1993, Ahne *et al.* 1990, Sano *et al.* 1985, Harhsbarger 1972), while main predisposing factors are considered to be age, sex and immunological competence.

In fish, protruding skin tumors are more common in freshwater species (Ribelin & Migaki 1975) while malignant tumors are rarer than papillomata (Poochirian et al. 2011). Squamous cell carcinoma a malignant neoplasm, has been reported in many fish species (Table 1) in a variety of loci.

MATERIALS & METHODS

Case history

A cultured population of brown meagres reared in sea cages, had been reported to present bilaterally clouded-opaque eye lenses (Fig.1d) in a percentage of 1,32% of total cultured population, which was subsequently affecting the wholesale price. This population of 20.000 brown meagres was transferred to sea cages on September of 2003 at 3g, to check the potential use of this species as a new candidate in Mediterranean aquaculture. The population had been kept for 8 years to serve as a genetic pool for further zootechnical investigation. After 8 years, when the G2 generation was ready to substitute the G1 broodstock, the whole population of almost 20.000 brown meagres was harvested. The main pathology observed after 8 years in the sea cages were mainly ocular lesions(cataract) possibly due to natural aging and/or long culture period under high natural light intensity and/or net injuries. Apart from that, tumors were evident. During the scheduled harvesting, fish sampling took place during the packaging process in order to investigate the cause of the problem.In the course of fish sampling, two (2)brown meagres had distinctive solitary protruding lesions (one on each fish) similar in texture (Fig.1). More specifically, the first fish (fish No1) had a protruding formation of solid texture on the proximal ventral left site of juncture between *os angulo-articulare* and *os dentale* (Fig1b, arrow). Circumferential skin was pale with no further obvious anomalies (ulcers or trauma). The second fish (fish No2) had a large hemispherical, ulcerative mass (≈5,5x3,1x2,9cm) that was posteriorly covering the biggest area of the left pectoral fin and anteriorly was extended up to the distal end of the left operculum. The dorsal site of the mass extended near the fish's left lateral line. The lesion was strongly attached to the subjacent tissue, with no indication of fluctuated content, but with multifocal necrosis (Fig1c, arrows) and ulcerative processes (Fig1c, white asterisk).

Microbiology

Prior to parasitological examinations, tryptone soy agar (TSA) and thiosulphate citrate bile salt agar (TCBS) media, inoculated from kidney and spleen samples, while *Flexibacter Maritimus Medium* (FMM) and Marine Agar (MA) media inoculated from skin and gill samples. All petri dishes were incubated at 21^0C (sea water temperature). Bacterial Identification was assessed biochemically using API 20E®, BioMérieux®.

Parasitology

Skin examination was initially conducted macroscopically. Microscopic examination of wet mount scrapings was performed only from suspicious skin loci. Parasitological examination was conducted in gills, skin, intestine and bile. Specially, bilateral gill epithelium scrapings from the first and second gill arches, was examined under light microscope for parasites. Concerning to intestine's parasitological examinations, wet mount scrapings from pars anterior, medial and posterior *canalis intestinalis* was performed. Bile was excised and handled similarly to the intestine.

Table 1. Cases of Squamous cell Carcinoma in fish

Species		Habitat	Reference
Common name	**Scientific name**		
Bream	*Abrami ssp.*	Freshwater	Hanjavanit & Mulcahy 2004
Yellowbullhead	*Ameiurus natalis*	Freshwater	Hanjavanit & Mulcahy 2004
Brownbullhead	*Ameiurus nebulosus*	Freshwater	PInkney et al 2011,
Blackbullhead	*Ameiuru smelas*	Freshwater	R.T.L.A. (Sterling VA) Hanjavanit & Mulcahy 2004
American eel	*Anguilla rostrata*	Catadromous	Hanjavanit & Mulcahy 2004
Oscar	*Astronotus ocellatus*	Freshwater	Rahmati-Holasoo & Hobbenaghi 2010
Barbel	*Barbus barbus*	Freshwater	Keysselitz 1908, Clunet 1910 (on Ribelin & Migaki 1975)
Gulf menhaden	*Brevoortia patronus*	Marine	Fournie et al. 1987
Goldfish	*Carrassius auratus*	Freshwater	Hanjavanit & Mulcahy 2004
Whitesucker	*Catostomus commersoni*	Freshwater	Hanjavanit & Mulcahy 2004
-	*Chondrostoma soetta*	freshwater	Mazzarelli, 1910 (on Ribelin & Migaki 1975)
Mirrorcarp	*Cyprinus carpio*	Freshwater	Manera & Biavati 1994, Wildgoose 1992
Malawicichlid	*Cyrtocara moorii*	Freshwater	Hanjavanit & Mulcahy 2004
Saithe	*Gadus virens*	Marine	Williams 1929 (on Ribelin & Migaki 1975)
Gudgeon	*Gobio gobio*	Freshwater	Mawdesley-Thomas & Bucke 1967
Black Tetra	*Gymnocorymbus ternetzi*	Freshwater	Sharifpour et al. 2014
Hybrid sunfish	*Lepomi s*sp.	Freshwater	Fitzgerald et al. 1991
Ide	*Leuciscus idus*	Freshwater	Mawdesley-Thomas 1972b (on Ribelin & Migaki 1975)
Whiting	*Merlangius merlangus*	Marine	Johnstone,1923-25,1924,1924-25 (on Ribelin & Migaki 1975)
Grey mullet	*Mugil cephalus*	Marine-Freshwater	Poochirian et al. 2011,
Chinooksalmon	*Onchorhynchus tshawytscha*	Anadromous	Hanjavanit & Mulcahy 2004
Medaka	*Oryzias latipes*	Amphidromous	Hanjavanit & Mulcahy 2004
European smelt	*Osmerus eperlanus*	Marine-Freshwater	Breslauer, 1915-16 (on Ribelin & Migaki 1975)
Atlantic rainbow smelt	*Osmerus mordax*	Anadromous	Hanjavanit & Mulcahy 2004, Herman 1988, Morrison & MacDonald 1995
Pudgy cuskeel	*Parabassogigas crassus* (Invalid) *Spectrunculus grandis* (valid)	Marine	Mawdesley- Thomas 1971a (on Ribelin & Migaki 1975)
Congo tetra	*Phenacogrammus interruptus*	Freshwater	Hanjavanit & Mulcahy 2004
Roach	*Rutilus rutilus*	Freshwater	Hanjavanit & Mulcahy 2004
Salmon	*Salmo salar*	Anadromous	Roberts 1972
Rudd	*Scardinius erythrophthalmus*	potamodromous	Hanjavanit et al. 1990, Hanjavanit & Mulcahy 2004
Brown meagre	*Sciaena umbra*	Marine	Kolygas et al. - (Current article)
Sea stickleback	*Spinachia spinachia*	Marine	Murray, 1908 (on Ribelin & Migaki 1975)
Alaska pollock	*Theragra chalcogramma*	Marine	Takahashi, 1929 (on Ribelin & Migaki 1975)

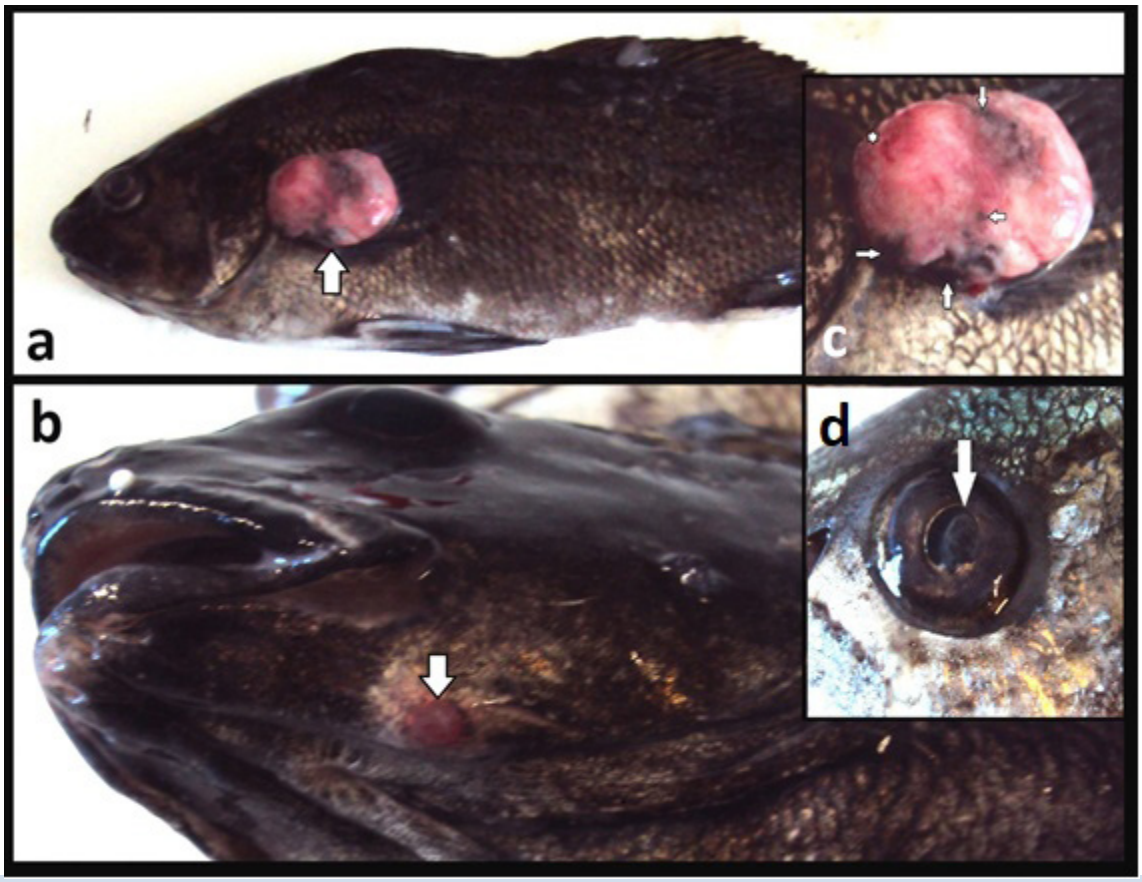

Figure 1: a) spherical, ulcerated tumor at the level of the lateral line in *Sciaena umbra*, b) small protruding formation on the left mandible, c) tumorous growth, note the necrotic loci (arrows) and the ulcerative site (white asterisk), d) cloudy-opaque eye lenses was the initial reason of examination.

Histopathology

Both lesions were excised, and tissue blocks were fixed in 10% formalin and Davidson's fixative. Tissue process was according to routine protocols. All tissue sections (5μm) were stained with Haematoxylin – Eosin (H&E), Periodic-Acid Schiff's(PAS) and Ayoub-Shklar (A-S)(Bancroft & Gamble 2008, Rao et al. 2005). In the A-S staining procedure no phosphotungstic acid(PTA) was used, the reason for this change in A-S staining protocol is explained in discussion's chapter. Sections were deparaffinized and hydrated to distilled water. Acid fuchsin5% added for 3min and slides were transferred directly to 0,5% aniline blue-1%orange G solution for 45min. Dehydration was performed on two 5min changes on 95% alcohol followed by one 3min, 100% EtOH change. After dehydration, two 3min changes on Xylene followed and eventually slides were mounted. Moreover, after necropsy, tissues from main internal organs such as liver, spleen and kidney were also selected for histopathology. Blood smears were impossible to be conducted given the fact that fish sampling took place during manufacture packaging process.

RESULTS

Microbiology

After a 72h incubation period of FMM and MA inoculated media from skin samples of both fish, colonies of distinct bacteria resembling to *Tenacibaculum maritimum* (Pazos et al., 1996) were observed and confirmed by the biochemical profile of the bacterium (Kolygas et al., 2012, Gourzioti et al., 2016). After a 48h incubation period of TSA and TCBS inoculated media from spleen and kidney, growth of *Listonella (Vibrio) anguillarum* was observed on fish No2samples.

Parasitology

Parasitological examination of the gills indicated a mild infection from monogenean parasites of the genus *Lamellodiscus* (5,25±2,12 parasites/gill arch). Wet skin and bile mounts showed no presence of parasites. In contrast, wet mounts of intestine from all three parts on both fish revealed scolices (mean intensity 0,43 parasites/ optical field).

HISTOPATHOLOGY

Microscopical studies from both fish lesions showed similar histopathological pattern of well differentiated squamous cell carcinomas (Fig.2).More specifically, multiple aggregates of tumor cells nests (Fig2.c), with pleomorphic characteristics (Fig.2a) were abundant. Certain areas of well differentiated squamous epithelial cells (resembling more or less to normal fish epidermis) forming projections into the chorium, exhibited a pattern with basosquamous cells on the nest's periphery (Fig.2a, arrow), and squamous keratinogenous cells at the center (Fig.2b, asterisk). The intermediate space between the nests, contained layers of connective tissue infiltrated by inflammatory cells (lymphocytes). Tissue from liver, spleen and kidney did not indicate any histopathological findings of neoplasm metastasis.

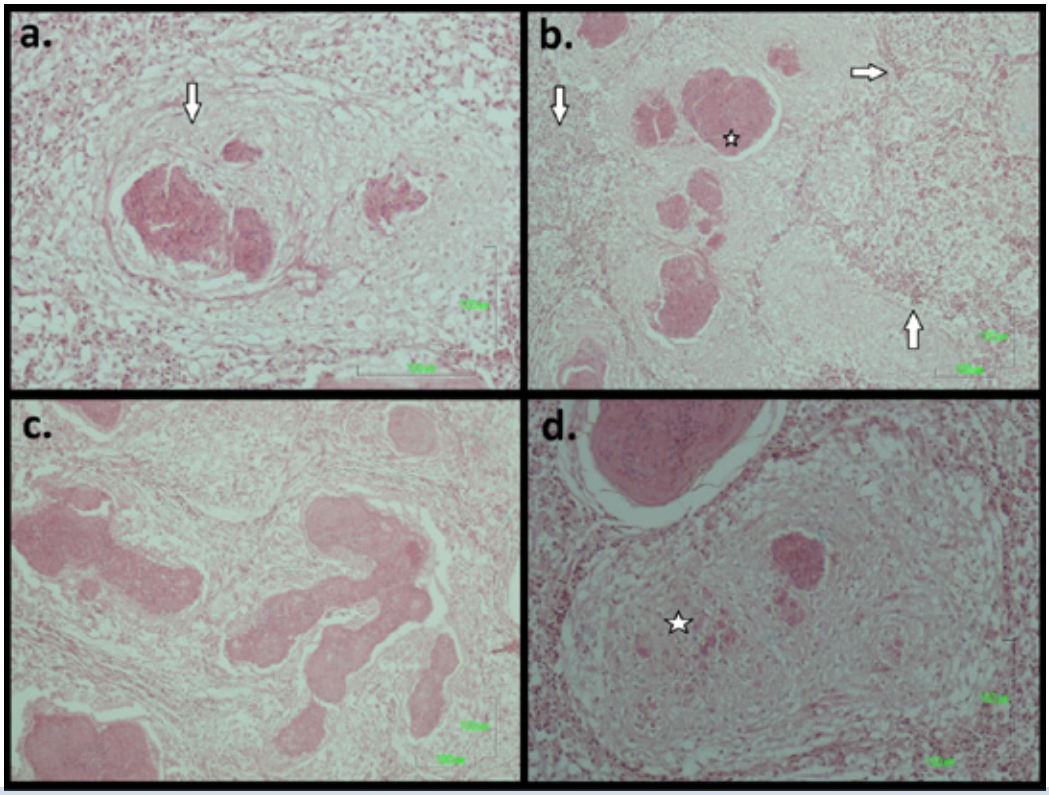

Figure 2: a) pleomorphic epithelial cells (arrow) inside a nest (H&E stain), b) wide areas of necrotic tissue (arrows) surrounding the well differentiated clusters of tumor cells (tumor cell nests). Note the squamous keratinogenous cells at the center of these nests (asterisk), (H&E stain), c) tumor's diathesis on creating digitiform tumors strands, (H&E stain), d) tumor's diathesis on creating satellite growths. The mature keratinogenous nest (center) gave early, small proximal satellite foci (asterisk) tumors (H&E stain).

Comparison between stains

Standard H&E stain on low magnifications (40x-100x) displayed a clear differentiation between the connective tissue and the tumorous growths, mainly because of the more compact distribution of epithelial cells and the hyperchromatic diathesis, of the latter. In higher magnification powers (≥400x) early development of tumorous cell nests (Fig.2d, asterisk), was difficult to diagnose. The transitional states of cell nests and keratin accumulations on the more matured ones, was problematic to distinguish. Some cells nests had higher stain accumulations than other, and that can be mistakenly attributed to respective keratin accumulation, but in fact this correlation is precarious as necrotic content within the cell nests can give the same results (Fig.3, H&E, 100x &400x). Staining with PAS, gave similar results to H&E on lower magnification. More specifically, discernment between the peri-tumorous connective tissue and the tumorous cells nests was fair in low magnifications (40x, 100x). Higher magnification powers (≥400x) gave the same disadvantages as H&E stain. Ayoub-Shklar stain displayed better diagnostic potentials in comparison to afore mentioned stains, as it was able not only to highlight early developmental tumorous cell nests and early satellite growths (Fig.3, A-S, 100x) but also to distinguish their transitional level with escalating stain intensities on keratin accumulation (Fig.3, A-S, 400x).

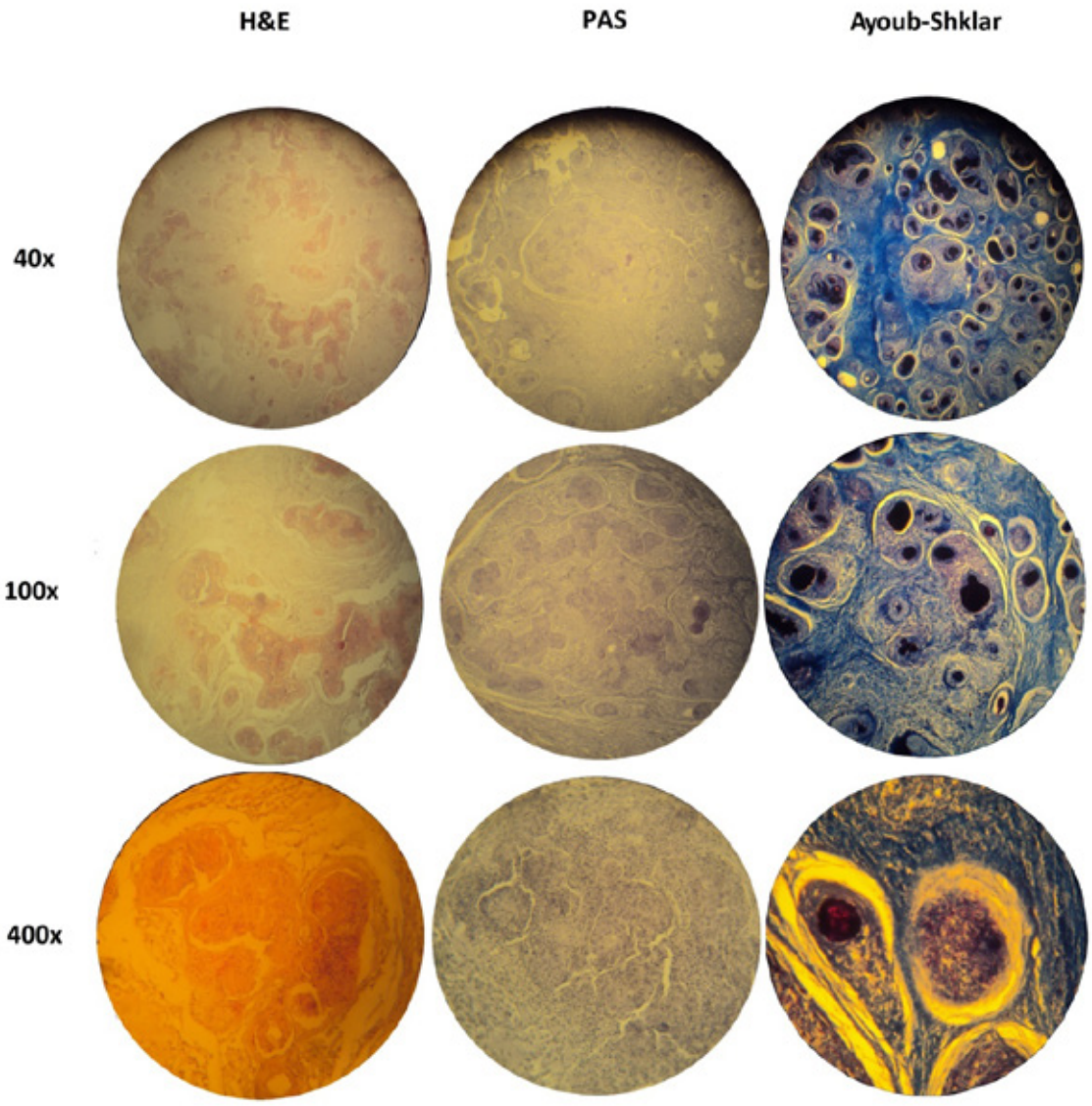

Figure 3. Pictomicrograph comparison of SCC stained with H&E, Schiff's Periodic Acid (PAS), and Ayoub-Shklar (A-S), respectively at different magnification powers.

DISCUSSION

Both cases of Brown meagres lesions, exhibit tumorous growth diagnosed as Squamous Cell Carcinomas. To our knowledge this is the first occurrence of malignancy of this type on Brown meagres. The causative agent of this malignant manifestation in these two fish is unknown, and only speculations on the possible etiology can be made. Many authors have indicated several possible agents that can give rise to such cellular abnormal proliferations (Roberts, 2001, Hanson et al, 2011). The microbiological and parasitological findings are considered of minor importance and at some level expected given the health status of both fish.

Regarding the staining methods, Ayoub-Shklar stain exhibited higher specificity than H&E and PAS in squamous cell carcinomas. Considering that the tumors were well differentiated, the risk of misinterpreted diagnosis with H&E and PAS stain was low, but in poor differentiated tumorous growths, an accurate diagnosis is highly compromised under the use of unspecific keratin stains. Keratin as a marker can provide not only concluding diagnosis in SCC but also key differential histological evidence between mesenchymal and epithelial malignancies (Ramulu et al. 2013). Immunohistochemical methods are more sensitive diagnostic tools for keratin demonstration (Karantza 2011) but are expensive and time consuming (Rao et

al. 2014).Moreover, specific stains can demonstrate histologically the degree of keratinization (Ramulu et al, 2013). Diagnostic misinterpretations between a) mature versus early SCC satellite keratin nests and b) keratin accumulations versus necrotic content within the nests, in histological sections are likely to occur when basic H&E protocols are used, in contrast to Ayoub-Sklar which is a quick, cost effective stain that can provide adequate specificity on keratin exposition in SCC cases and set definitive diagnosis on SCC in fish.

Another crucial parameter that always must be taken into consideration when cases of SCC are suspected is whether to apply or not phosphotungstic acid in Ayoub-Shklar staining protocol. In fact, the presence of PTA can dramatically change the final stain outcome of A-S on keratin demonstration of SCC cases. During a comparative staining trial on keratin demonstration it is mentioned that in some SCC cases, A-S stain failed to uniformly stain malignant manifestations of keratin pearl demonstrations (Ramulu *et al* 2013). Moreover, during a similar comparative staining trial of keratin demonstration, the conclusion was that H&E's staining specificity and intensity is highest when compared to special stains such as Ayoub-Shklar (Rao *et al* 2014), which is in contrast to the conclusions of the present study. The reason for these dissenting results could be that the presence of PTA during A-S stain on afore mentioned studies, possibly reduces stain's overall specificity, as far as keratin demonstration is concerned. The presence of PTA possibly transitions the staining outcome closer to Masson's Trichrome stain, a stain that is used to distinguish collagen fibers versus smooth muscles and not keratinogenous cells, keratin and prekeratin. In fact, the role of PTA in histology is to bind to prestained specific endocellular and ectocellular molecules in order to selectively decolorize them (Everett & Miller 1974). In trichrome stains like Masson & Mallory, PTA is used to mordant anionic dyes to connective tissue fibers. In the case of A-S stain protocol, PTA might have the same effect on some keratin nests of certain maturity states, reducing the overall specificity. To either accept or reject this hypothesis, further studies on different staining protocols need to be made.

Conclusively, the above A-S stain protocol can be adopted in in-house labs as a quick and cost-effective diagnostic procedure especially on broodstock facilities where early intervention is of major importance on this high-value livestock.

CONFLICT OF INTEREST

None declared by the authors.

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