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# Sarcoma in the thymus of juvenile meagre *Argyrosomus regius* reared in an intensive system

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ABSTRACT: Juvenile meagre *Argyrosomus regius* (Asso, 1809) maintained in experimental conditions developed lateral and/or bilateral circular-shaped sarcoma within the opercular cavity. The sarcoma was dense, reddish and its growth from the branchial arch exerted pressure on the operculum forcing it to open. Histologically, the neoplasm exhibited marked proliferation of mesenchymal connective tissue composed largely of fusiform cells, which developed in a solid pattern accompanied by abundant mononuclear cell types. Multifocal areas of discrete necrosis were also observed, compatible with a sarcomatous proliferation. The immunological parameters analysed suggested an inflammatory response. No bacteria were isolated from the hematopoietic organs. However, *Vibrio* species, components of the normal seawater flora, were isolated from the tumour, which may have had a role in eliciting the immune response. No evidence of viral pathogens was found by electron microscopy. In order to look for cytogenetic alterations often linked to sarcomas, the diploid number and karyotype of this species were determined for the first time. An increase in the aneuploidy level was observed in sarcoma cell metaphase stages compared to other tissues. The aetiology of this tumour remains unknown.

KEY WORDS: Neoplasm · Bacteria · Blood parameters · Karyotype · Chromosomal abnormalities



## INTRODUCTION

In fish, as in mammals, the thymus plays an important immunological role for the production and development of T lymphocytes involved in the host recognition of antigens. The thymus is a paired gland localised bilaterally under the operculum, posterior to the gill arches, and is composed primarily of lymphocytic cells supported by a fine connective tissue stroma and normally surrounded by a thin capsule of connective tissue and external epithelium.

The size and growth of the thymus are dependent on season, hormones, sexual maturity, age and the presence of stressors (Chilmonczyk 1982). Commonly this organ becomes more flattened or involuted in response to various physiological or external stimuli, but it is not clear if the thymus involutes in all fish. This is in contrast with mammals, where age-related involution is normal. The thymus is easily exposed to pathogens or pollutants because of its superficial location (Chilmonczyk 1982) when compared with other haematopoietic organs (e.g. spleen and pronephros).

Several factors can cause abnormal development and pathology of the thymus, including environmental stressors (Chilmonczyk 1982) and parasites (e.g. myxosporeans) (Honma & Tamura 1976). Bacterial and viral infections have not been implicated with abnormal development or pathology of the thymus (Chilmonczyk 1982). However, the neoplastic lesions lymphoma and lymphosarcoma affecting the organ have been described (Dawe & Berard 1971, Papas et al. 1976, Okihiro & Hinton 1989).

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In fish, neoplasia of haematopoietic origin, most frequently from lymphoid tissues (Harshbarger 1977), has been reported for several species of freshwater fish (Chilmonczyk 1982). The occurrence of thymic lymphoma has been reported for muskellunge *Esox masquinongy* Mitchill, 1824, northern pike *Esox lucius* Linnaeus, 1758 (Mulcahy 1970, Sonstegard 1975, Wolf 1988), Atlantic salmon *Salmo salar* (Linnaeus, 1758) (Roald & Hastein 1979), Japanese medaka *Oryzias latipes* (Temminck & Schlegel, 1846) (Okihiro & Hinton 1989, Battalora et al. 1990, Hayashi et al. 2008), channel catfish *Ictalurus punctatus* (Rafinesque, 1818) (Chen et al. 1985) and rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) (McArdle & Roberts 1974, Bernstein 1984, Warr et al. 1984). The origin of these neoplastic lesions is often unknown, and usually there is no associated mortality. However, the occurrence of lymphosarcoma on wild *Esox* spp., caused by a retrovirus-like agent (Papas et al. 1976), has assumed epizootic proportions (Mulcahy 1970, Papas et al. 1976, Sonstegard 1976, Thompson 1982). Bernstein (1984) also found a Type C virus in pike lymphoid tumours. Plasmacytoid leukemia caused by a retroviral agent (Kent et al. 1990, Eaton & Kent 1992, Eaton et al. 1993, Kent & Dawe 1993) has caused mass mortalities in cultured chinook salmon *Oncorhynchus tshawytscha* (Walbaum, 1792) (Kent et al. 1990).

Changes in haematological parameters have been widely used for assessing the health status of fish (Austin et al. 1993, Lamas et al. 1994) and thymic tumours in humans (Johns & Reinhardt 2009). Lysozyme, or muramidase, is a glycosidic enzyme produced by leucocytes (neutrophils and macrophages) involved in the non-specific immune defence mechanisms of fishes (Tort et al. 2003). Lysozyme acts as an acute-phase protein, being released not only in response to bacterial antigens but also to other alarm situations such as stress (Demers & Bayne 1997). Consequently, the level or activity of this enzyme has been widely used as an index to evaluate fish defensive capacity, towards disease and/or any other challenging situation (Bowden et al. 2004, Wu et al. 2007, Costas et al. 2011). The nitroblue tetrazolium (NBT) assay is mostly used to measure the oxidative radical production by leukocytes in defence against pathogens (Jeney & Anderson 1993, Jeney et al. 1997, Castro et al. 1999, Cook et al. 2003, Sahoo et al. 2005). Sarcomas constitute a heterogeneous group of rare tumours that present a remarkably high incidence of specific and primary chromosomal aberrations (Bridge 2008), and cytogenetic analysis can be used for the precise classification of certain tumours.

In a previous note, Soares et al. (2011) reported that 3.6% of meagre *Argyrosomus regius* (Asso, 1801) juveniles maintained in experimental conditions developed a sarcomatous proliferation beneath the opercula. No mortality was associated with the presence of these lesions; still, signs of tissue inflammation were evidenced using histological techniques. Therefore, the objective of this study was to provide additional information on the sarcoma, on the histopathology and on the ultrastructure of the tumour. Haematology and possible bacterial and viral aetiology of affected fish were also investigated. Moreover, both diploid number and karyotype composition were determined for the first time in this species. A comparison between the tumour and other tissues from affected fish as well as tissues from control individuals was made, and specific cytogenetic alterations were identified.

This study provides new information on the possible aetiology of the neoplastic lesions in meagre and contributes to the (general) knowledge in comparative pathology of thymic lesions.

## MATERIALS AND METHODS

### Animals

This study was carried out over 3 mo at the Aquaculture Research Centre facilities of IPMA (Portugal). Fish were reared at a density of 5.5 kg $m^{-3}$ and were fed with a commercial diet twice a day ad libitum.

Of a total of 972 fish, 35 fish were found to be affected with thymic sarcoma. Hereafter this group will be referred to as the 'sarcoma group'. The total number of fish with sarcoma was used for total weight and length measurements, with 10 fish sacrificed for morphological, histological, microbiological and blood analyses, and 10, for cytogenetic parameters.

Fish from 2 tanks in which sarcoma was not detected were used as controls and designated the 'control group'. For total weight and length measurements 142 fish were used, with 10 fish sacrificed specifically for blood analyses.

### Analytical procedures

Fish were anesthetized in a solution of 2-phenoxyethanol (ethylene glycol monophenyl ether), and blood was taken immediately by caudal vein puncture with a 1 ml heparinized syringe for the determination of blood parameters. Collection of blood samples was completed within 5 min of capturing the fish to mini-

mize handling stress. Afterwards, fish were euthanized by cutting the spinal cord immediately posterior to the head. Thymic sarcoma tissue was sampled for morphological, bacteriological, histological and ultrastructural analyses. Lesions were photographed, measured and dissected from adjacent tissues.

### Histology

Following fixation for a minimum of 24 h in Bouin's fixative, tissues were transferred to 70% industrial methylated spirit (IMS) and processed to wax blocks using an automatic vacuum infiltration tissue processor (Vision Biosystems Peloris tissue processor). Sections were cut at 3 to 5 µm and stained with haematoxylin and eosin (H&E) in an automatic tissue stainer. Tissues were examined on a Nikon E800 microscope using bright-field illumination for the presence of pathogens and pathological changes. Representative images were captured using LUCIA™ screen measurement system. Stained sections were deposited in the Registry of Aquatic Pathology (RAP) at Cefas Weymouth Laboratory, UK.

### Electron microscopy

Small samples of tissue fixed with neutral buffered formalin were carefully removed from blocks of tumour tissue and rinsed 3 times in 0.1 M sodium cacodylate buffer, each rinse for 30 min. The samples were then fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h and rinsed in the same buffer before dehydration through a graded acetone series. They were then embedded in Agar 100 epoxy resin (Agar Scientific, Agar 100 premix kit, medium) and polymerised overnight at 60°C. Semi-thin sections (1 to 2 µm) were stained with toluidine blue for viewing by light microscopy, and selected regions were identified for ultrastructural study. Ultrathin sections (70 to 90 nm) were obtained and stained with uranyl acetate and Reynold's lead citrate. Grids were examined on a JEOL JEM 1210 transmission electron microscope, and digital images were captured using a Gatan Erlangshen ES500W camera and Gatan Digital Micrograph™ software.

### Bacterial sampling, isolation and identification

Samples were collected from 10 meagre with thymic sarcoma and 10 meagre from the control group. They were opened aseptically, and the internal organs of fish and sarcoma were routinely sampled for bacteriological analyses. Anterior kidney, liver, spleen and thymus sarcoma were cultured on Tryptic Soy Agar (TSA, OXOID®) supplemented with 1.5% NaCl and thiosulfate citrate bile salts sucrose agar (TCBS, OXOID®), and the plates were incubated at 24°C for up to 72 h.

Pure cultures of the isolates, obtained by plating on TSA were identified by physiological and biochemical characterisation as described by Smibert & Krieg (1981), Holt (1994) and Buller (2004).

The following tests were performed: Gram-negative test, cell morphology and motility (microscopical observation in a Zeiss® Axiostar Plus microscope); luminescence; cytochrome-oxidase (OXOID®-BR64A sticks oxidase); catalase; O-F test (OF basal medium Difco®, with D-glucose [Merck®] as added sugar); growth on TCBS agar (OXOID®) for *Vibrionaceae* determination; swarming on TSA-SW; growth in 0, 5 and 10% NaCl; gas production from glucose (triple sugar iron agar, Merck®); dihydrogen sulfide ($H_2S$) production (triple sugar iron agar, Merck®); arginine dihydrolase; decarboxylation of lysine and ornithine; nitrate reduction (nitrate broth, Merck®); sucrose, dextrose and lactose fermentation (triple sugar iron agar, Merck®); and the extracellular enzymatic activities of gelatinase, lipase and amylase. All tests were incubated at 24°C. Drug sensitivity of the isolates was determined on Mueller-Hinton agar (OXOID®, supplemented with 1.5% of sodium chloride) by the disc diffusion method using the following chemotherapeutic agents (µg $disc^{-1}$): Ampicillin (10 µl, OXOID®), novobiocin (30 µl, OXOID®) and the vibriostatic agent O129 (10 µl, OXOID®). Antibiogram readings were performed 48 h after incubation at 24°C.

### Blood sample preparation

Blood samples were collected from 20 meagre from both groups. Two different aliquots of blood were used for different analyses. The first aliquot of blood was used for NBT determination as described by Anderson (2004) for measuring the activity of circulating neutrophils. The second aliquot was transferred to a plate and was used for haematological determination. Blood cells were fixed with methyl alcohol, air dried and stained with Giemsa. The number of leucocytes and erythrocytes were counted microscopically.

A blood sample portion was transferred to micro-haematocrit-tubes (plain capillary tubes, 75 mm;

Super Rior) upon centrifugation (5000 × *g*, 5 min) with a haematocrit centrifuge (EBA 21 Hettich) to determine the haematocrit value.

The remaining blood was centrifuged for 10 min at 2500 × *g*, and the plasma was stored at –20°C until analysis.

### Lysozyme activity

Lysozyme activity was assayed using the turbidometric assay, based on the methods described by Ellis (1990) and modified by Costas et al. (2011). Briefly, lysozyme of chicken egg white (Fluka) was serially diluted with 0.05 M phosphate buffer (pH 6.2) and used as the standard solution. *Micrococcus lysodeikticus* (0.5 mg $ml^{-1}$ of 0.05 M phosphate buffered saline [PBS]; pH 6.2) was used as the substrate. Diluted lysozyme (15 µl; standard solution) or 15 µl fish plasma was added to a 175 µl suspension of *M. lysodeikticus* (Sigma-M). The reaction was carried out at 25°C, at 450 nm, for 5 min, with readings taken every 30 s. The amount of lysozyme in the sample was calculated using the formula of the standard curve.

### Cytogenetic analysis

Fish were injected in the intraperitoneal region with 0.025% colchicine solution (2 µl $g^{-1}$ body weight) 75 min before sacrifice. Cephalic kidney, spleen and thymus sarcoma were removed and dissociated directly in 0.9% sodium citrate, and were kept 20 min at 37°C for hypotonic treatment. Afterwards the suspension was centrifuged, and the pellet was subsequently fixed in a freshly prepared mixture of absolute alcohol–acetic acid (3:1).

Chromosomal slides were performed following the air-drying technique of Thiriot-Quiévreux & Ayraud (1982) and stained for 10 min with Giemsa (4%, pH 6.8). Chromosome counts were made directly on images of Giemsa-stained metaphases acquired with a digital camera (Nikon DSFi 1) coupled to a light microscope (Nikon Eclipse 80i). Digital images were processed with Adobe Photoshop (Version CS5) using functions affecting the whole of the image only.

Karyotypes of diploid and aneuploid metaphases were performed taking into consideration standard measurements of chromosome pairs (measurements of size and centromeric index); terminology relating to centromere position followed that of Levan et al. (1964).

## RESULTS

Meagre *Argyrosous regius* specimens with sarcomas showed a significantly lower weight and condition factor ($K$) ($p < 0.05$) than fish without sarcomas (Table 1), but mean lengths were identical between the 2 meagre groups.

Sarcomas were located in the upper quadrant of each branchial chamber, just under the operculum (Fig. 1). In this study, 97.1% of fish exhibited bilateral sarcoma-like structures; no side preference was observed for the appearance of this structure. Sarcoma diameter ranged from 0.8 ± 0.4 to 1.1 ± 0.3 cm; smaller sarcomas exhibited an oedematous aspect, whereas larger sarcomas had a circular/round shape. The colour was pale red and/or with haemorrhagic spots.

Histologically, thymocyte-like cells were present in small multifocal regions and were interspersed throughout the neoplastic tissue (which was pre-

Table 1. *Argyrosomus regius*. Meagre length, weight, sarcoma diameter, haematocrit, number of leukocytes and nitroblue tetrazolium (NBT)-positive cells in fish from sarcoma and control groups. Different superscripts indicate differences between groups

| | Sarcoma group | Control group |
|---|---|---|
| Weight (g) | 85.7 ± 11.25$^{b}$ | 91.7 ± 13.92$^{a}$ |
| Mean total length (cm) | 20.9 ± 0.92 | 21.2 ± 1.21 |
| Sarcoma diameter, min./max. (cm) | 0.8 ± 0.4 / 1.1 ± 0.3 | – |
| Haematocrit | 18.7 ± 3.34$^{b}$ | 22.4 ± 3.55$^{a}$ |
| Leucocytes (Giemsa) | 8.7 ± 0.05$^{a}$ | 4.14 ± 0.02$^{b}$ |
| NBT-positive cells (%) | 0.15 ± 0.13 | 0.04 ± 0.01 |

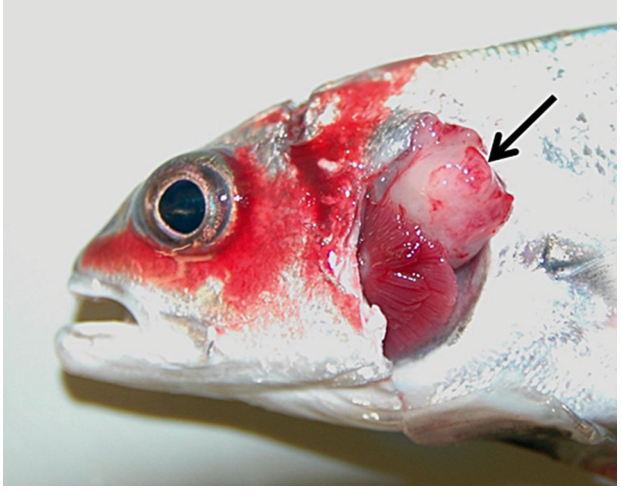

Fig. 1. *Argyrosomus regius*. Meagre thymic sarcoma attached posterior to the gill arches (arrow)

dominant), surrounded by a prominent marginal layer of hyperplastic epithelial cells composing the mucosal epithelium (Fig. 2). The tumour itself was composed of a solid mass of pleomorphic, generally fusiform cells, with lightly staining cytoplasm containing small vacuoles, and round to oval nuclei that were slightly irregular in shape and contained a central nucleolus. These cells developed into a pattern of growth that was solid and dense in cellularity, with moderate vascularization (Fig. 3) and exhibiting multifocal congestion. In some areas these cells were accompanied by an intense and diffuse infiltration of residual thymocytes/lymphocytes. Areas of necrosis, including granulomatous-like lesions compatible with sarcomatous proliferation, were also observed. Areas of intense proliferation of fusiform cells suggestive of a mesenchymal origin, which grew in a solid pattern accompanied by an abundant number of mononuclear-like cells, were also present. The histological picture is compatible with sarcomatous proliferation, associated with a secondary chronic inflammatory response.

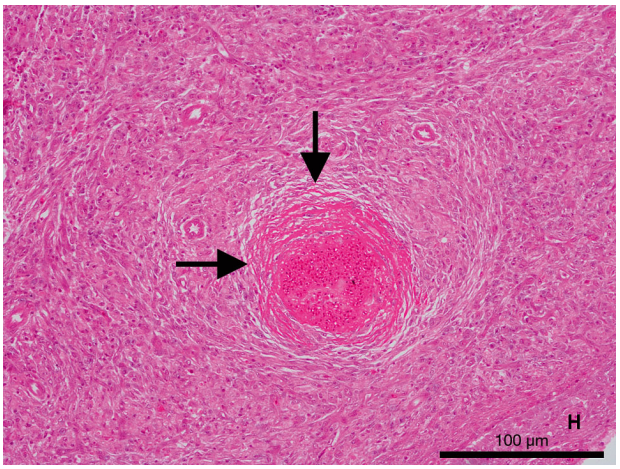


Fig. 2. *Argyrosomus regius*. Perpendicular section through the sarcoma, showing a layer of hyperplastic epithelium (H) and the underlying neoplastic tissue with a necrotic focus (arrows), haematoxylin and eosin (H&E). Scale bar = 100 µm

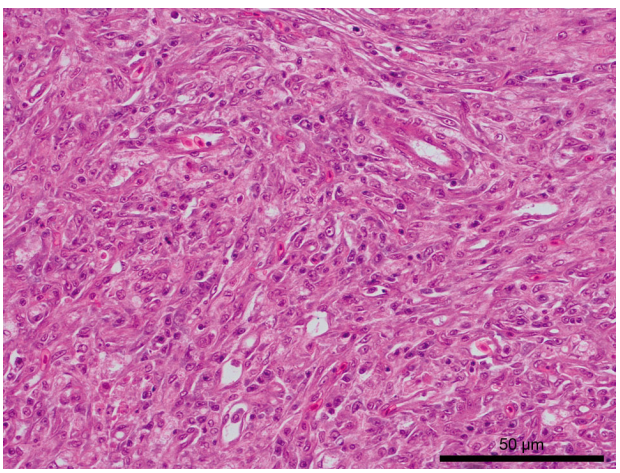


Fig. 3. *Argyrosomus regius*. Section showing prominent vascularisation of the neoplasm and the random orientation of the cellular architecture, haematoxylin and eosin (H&E). Scale bar = 50 µm

Ultrastructural examination of the tumour revealed the presence of a mixed population of cells, often lymphocytic in nature. Occasional putative granulocyte cells were also observed. Larger fusiform tumour cells were predominant, often with round to oval nuclei containing sparse heterochromatin and a prominent nucleolus. Necrotic cells were distributed throughout the tissue in low numbers. Viral assembly sites and virions were not detected in the sections examined.

Bacteria were only isolated from 4 thymus specimens of the sarcoma group, and they were found in low numbers. Different *Vibrionaceae* species (*Vibrio alginolyticus*, *V. vulnificus*, *V. proteolyticus*, *V. fischeri* and *V. mediterranei*) were identified, with only *V. alginolyticus* being found in all tumours examined. No bacterial growth was observed in any of the other analysed organs (anterior kidney, liver and spleen), either in the control or sarcoma group. Meagre from the sarcoma group exhibited a lower number of erythrocytes ($p < 0.05$) than the control group, based on haematocrit and counts from Giemsa-stained smears. However, this difference was less clear using the NBT technique (Table 1). The number of leucocytes was significantly higher in meagre from the sarcoma group than the control group, using counts from Giemsa-stained smears ($p > 0.05$). Lysozyme activity tested in undiluted meagre plasma, from both the sarcoma and control groups, was low and extremely variable, even negative sometimes. As a control, undiluted Senegalese sole *Solea senegalensis* Kaup, 1858 plasma was analysed and showed 8.1 ± 0.7 U $ml^{-1}$ of lysozyme activity.

The diploid number of 2n = 48 and the karyotype, which comprised 9 metacentric (Pairs 1 to 9), 7 submetacentric (Pairs 10 to 16), 4 subtelocentric (Pairs 17 to 20) and 4 telocentric chromosome pairs (Pairs 21 to 24), were also established for the first time (Fig. 4). A percentage of hypodiploid metaphases was observed in chromosomal preparations of the sarcoma; this contrasts with preparations of the kidney and spleen, in which all observed metaphases were diploid. From analyses of 6 aneuploid karyotypes, 4 out of the 24 chromosome pairs seemed to be more prone to chromosome loss (Pairs 1, 9, 10 and 24) (Fig. 5).

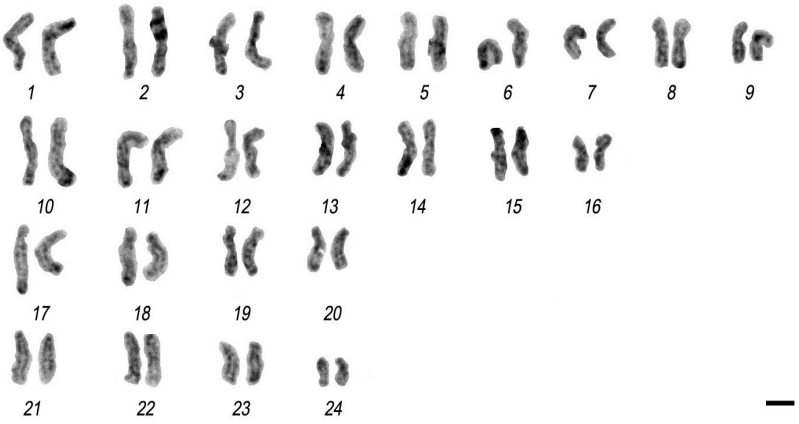


Fig. 4. *Argyrosomus regius*. Diploid karyotype of meagre (control) with 2n = 48 chromosomes (9 metacentric pairs, 1 to 9; 7 submetacentric pairs, 10 to 16; 4 subtelocentric pairs, 17 to 20; and 4 telocentric pairs, 21 to 24). Scale bar = 2 µm

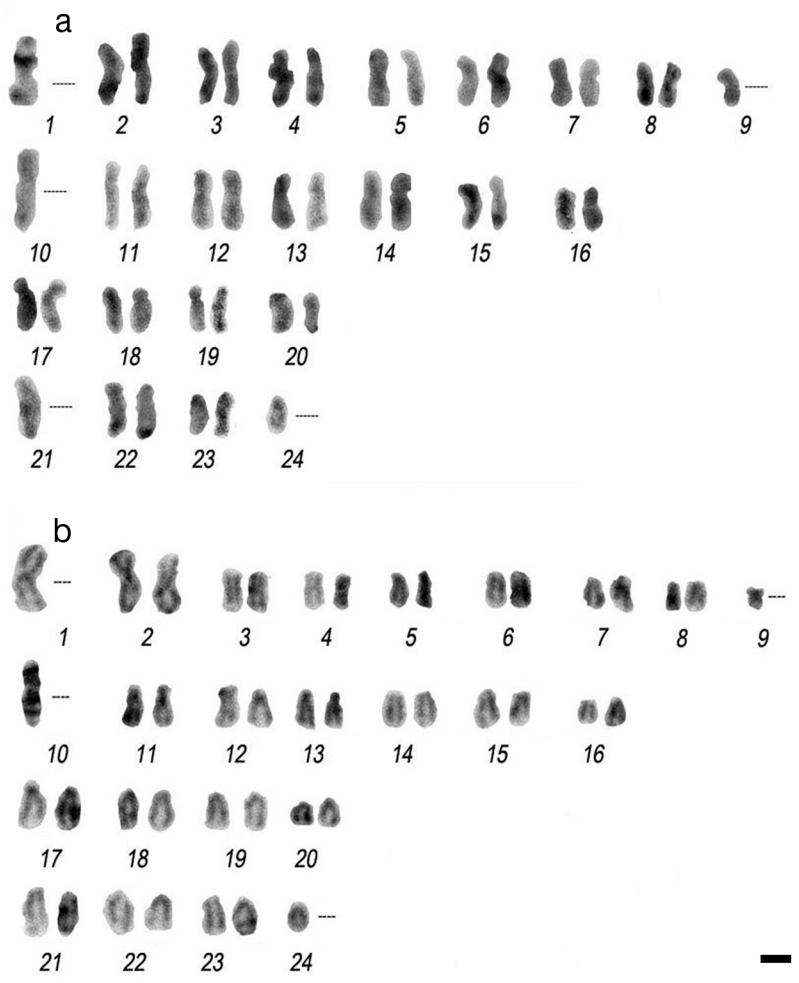


Fig. 5. *Argyrosomus regius*. Aneuploid karyotypes of meagre. (a) Chromosome loss in Pairs 1, 9, 10, 21 and 24. (b) Chromosome loss in Pairs 1, 9, 10 and 24. Scale bar = 2 µm

## DISCUSSION

The presence of a variety of neoplastic lesions has frequently been reported in the thymus of wild and farmed fish (Roald & Hastein 1979, Bernstein 1984, Warr et al. 1984, Bruno & Smail 1998, Hayashi et al. 2008). Apart from a preliminary account by the authors (Soares et al. 2011), thymic neoplasia has never been reported in meagre *Argyrosomus regius* or any other marine species. The presence of sarcomas was linked to reduced overall condition in meagre, since a lower condition factor ($K$) (and hence, reduced body robustness) and a higher number of leucocytes were observed in the sarcoma group, suggesting a stimulation of the innate immune system, despite the negligible values for lysozyme.

In this study, multifocal areas of necrosis were evident within the neoplastic tissue, a feature consistent with a sarcomatous proliferation, associated with a secondary chronic inflammatory response. The reddish colour of the thymus observed in this study may be indicative of an infection, as in similar cases previously observed by Powell (2000), in which a pale white thymus exhibited a red haemorrhagic surface when affected by severe systemic infections. However, in this study, the levels of bacteria detected in the sarcomas were insufficient to result in an haemorrhage. Although the isolated bacteria, *Vibrio alginolyticus*, were normally associated with acute septicaemias and chronic focal lesions in marine fish, they did not induce significant infection in the meagre examined. The pronounced vascularization observed histologically in meagre thymic sarcomas was clearly responsible for the reddish colouration in the lesions seen at necropsy. Still, the lower haematocrit values and elevated numbers of leucocytes in meagre exhibiting sarcomas suggested the presence of inflammation and impaired health.

Normally in teleosts, only a thin layer of mucosal epithelial cells separates the thymus from the external environment (O'Neil 1989). However, in the current case, the epithelial layer was hyperplastic and apparently intact, thereby providing additional protection to the underlying tissue. In the current study, as well as in that undertaken by Bruno & Smail (1998), there was no evidence of septicaemia and no bacteria were isolated in other haematopoietic organs. Moreover, no mortality was associated with thymic sarcomas, and affected fish displayed normal swimming and eating behaviour (Soares et al. 2011).

Elevated lysozyme activity has been recorded in marine fish species, following infection, environmental, or nutritional stress (Saurabh & Sahoo 2008, Estévez et al. 2011). In the present study, lysozyme activity was almost absent in the plasma of meagre from both sarcoma and control groups. A similar response was observed for cod *Gadus morhua* when exposed to stressors (Magnadóttir et al. 1999). However, lysozyme activities were observed in the serum of meagre juveniles exposed to nutritional stress (Estévez et al. 2011). The different responses of lysozyme activity in both studies of meagre suggest differences in the activity of the biological fluids used or different mechanisms of response towards different stressors.

NBT reduction is a simple assay widely used to demonstrate the production of superoxide anion from phagocytes. The superoxide anion and the hydroxyl free radical are reactive oxygen species, which are highly bactericidal (Ellis 1999). The fact that bacteria were only isolated in some individuals from the sarcoma group, increasing the variability within the group, likely explains the absence of significant differences in activated leucocytes between the meagre sarcoma and control groups. These findings suggest that only non-specific activation of immune defences occurred in sarcoma-affected meagre.

Poorly differentiated sarcomas represent a significant challenge to pathologists; consequently, descriptions of characteristic chromosomal aberrations are especially useful in these cases (Bridge 2008). The cytogenetic characterisation of sarcoma cells in this study revealed a considerable percentage of aneuploid metaphases (not observed in the other studied tissues).

In addition, in karyotypes, some of these metaphases showed that some chromosomal pairs appear to be more prone to loss than others. Such non-random chromosome losses in aneuploid situations have previously been observed in humans and plants (Cheng & Murata 2002, Takeuchi et al. 2009). Indeed, certain chromosome pairs are lost more often than expected under the assumption of non-random segregation. Moreover, chromosome changes in human cancer cells appear to evolve by non-random losses and/or gains of particular homologues or groups (e.g. Cigudosa et al. 1998).

However, in order to verify whether the chromosomal loss observed in this study does indeed reflect differential chromosomal susceptibility and to check for structural chromosomal aberrations, the application of differential chromosomal banding techniques to aneuploid karyotypes of sarcoma-affected *Argyrosomus regius* is essential. The precise identification of characteristic structural and numeric cytogenetic alterations can also guide future molecular studies into the nature of the genes involved in the neoplastic transformation of meagre. Such information would be of value in understanding the pathogenesis of similar tumours in other animals, including man.

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