Spontaneous Melanotic Lesions in Axillary Seabream, *Pagellus acarne* (Risso)

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Short running title: Melanotic Lesions in Axillary Seabream

Abstract

In this paper, we describe spontaneous melanotic lesions in the skin of axillary seabream, *Pagellus acarne* (Risso) from a defined area of the Portuguese Coast, located in Cabo da Roca and Foz do Arelho. The lesions corresponded to black pigmentation spots on the skin of the head, fins, lips and conjunctiva and, additionally, black nodules on the skin of the head and lips. In some specimens, the nodular formations in the head changed their anatomical conformation. Histologically, there were melanophores scattered along the basement membrane or forming aggregates in the dermis, infiltrating the subcutaneous tissue but not invading the adjacent muscle tissue. The aim of this study was to characterize the macroscopic and microscopic features of the pigmented lesions. These fish show sessile hyperpigmented lesions (spots) that correspond to proliferative lesions of melanophores in the dermis and nodular lesions that correspond to neoplastic lesions, melanophoromas. The melanophores in such lesions showed high concentration of melanin in the cytoplasm, moderate pleomorphism and compact distribution throughout all of the dermis.

Keywords: Axillary seabream, melanophores, spontaneous melanotic lesions, hyperpigmented lesions, melanophoromas.

Introduction

The axillary seabream, *Pagellus acarne* (Risso) is a sparidae that, in terms of taxonomy, belongs to the Order of Perciformes. It is a species with high commercial value, native of the Portuguese Atlantic Coast, the Azores and Madeira.

The skin colour of the fish depends on chromatophores or pigment cells of the dermis. These cells contain pigments that absorb or reflect light. The chromatophores were classified as light absorbers, which correspond to melanophores (with brown or black pigment, the melanin), erythrophores, xanthophores and cyanophores (Fujii 2000) and reflectors of light, which includes leucophores and iridophores (Roberts 1978). Unlike birds and mammals, fish chromatophores are not constituents of the epidermis, but are located in the dermis (Takashima & Hibiya 1995). Fish, similarly to amphibians, have specialized cells, the melanophores, which contain multiple melanin granules, called melanosomes. The role of these cells is the concentration of pigments in the centre of the cell or its dispersion throughout the cytoplasm (Fujii 1969). The movement of these pigments allows colour changes, important for camouflage and social interactions (Roberts 1978). Mammalian melanocytes also produce melanosomes but, unlike melanophores, the pigment in these cells is transported to the cells periphery for subsequent exocytosis to the surrounding epithelial cells.

Chromatophoromas are common tumours in fish and outbreaks have been reported in both freshwater and marine fish throughout the world. Although most outbreaks by chromatophoromatosis have unknown aetiology, many studies suggest the potential exposure to chemical and radioactive carcinogens (Okihiro, Whipple, Groff, & Hinton 1993). However, other potential aetiological factors such as oncogenic viruses (Anders & Yoshimizu 1994), genetic predisposition or ultraviolet radiation (Vielkind & Vielkind 1982) have been studied. Fish melanomas (or melanophoromas) are tumours of the pigmented skin cells, the melanophores, which have the melanin pigment, black or dark brown. In fish, melanomas are common (Mawdesley-Thomas 1975, Freitas & Albuquerque 1982, Bruno & Poppe 1996, Munday, Su & Harshbarger 1998, Sakamoto & White 2002, Ramos & Peleteiro 2003) and outbreaks are described in marine fish such as perciformes, honnibe croaker, *Nibea mitsukurii* (Jordan & Snyder) in the Japanese Coast of the Pacific (Kimura, Taniguchi, Kumai, Tomita, Kinae, Yoshizaki, Ito & Ishikawa 1984), in millet butterflyfish, *Chaetodon miliaris* Quoy & Gaimard of the Hawaiian islands (Okihiro 1988), *Sebastes* spp. of Cordell Bank, off central California (Okihiro, Whipple, Groff & Hinton 1993) and beaked redfish, *Sebastes mentella* Travin of the North Atlantic (Bogovski & Bakai 1989). Disease outbreaks in freshwater fish have been described in perciformes of the Great Lakes, freshwater drum, *Aplodinotus grunniens* Rafinesque (cited in Okihiro *et al*. 1993) and also in cypriniforms, goldfish, *Carassius auratus auratus* (L.) (Mawdesley-Thomas 1975). Selective platyfish-swordtail hybrids (*Xiphophorus*) have been used as a model for studying the molecular and genetic mechanisms of the melanoma formation in animals and humans, due to their susceptibility to the development of such tumours (Kazianis, Khanolkar, Nairn, Rains, Trono, Garcia, Williams & Walter 2004). Such lesions have not been described previously in this species or in this region of the North Atlantic which is relatively free from major areas of industrial pollution.

Material and methods

The fish in the study were caught in the hook fishing, with gill nets and trawls in the area of Cabo da Roca and Foz do Arelho (figure 1).

The first case was recorded in April 2007 and, until April 2008, 61 axillary seabream with altered skin pigmentation were observed and studied. All specimens were measured, weighed and photographed. The average length and weight (± SD) of the 61 axillary seabream observed was 30.2 ± 2.14cm and 403 ± 0.09g, respectively. The biggest and smallest specimens had 35cm and 700g; 27cm and 250g, respectively. Axillary seabream skin with and without colour changes were studied macro-and microscopically. The location, size and colour of the lesions observed in different specimens were recorded and full necropsy of all specimens was carried out.

The species caught along with the axillary seabream, such as atlantic horse mackerel, *Trachurus trachurus* (L.); blue jack mackerel, *Trachurus picturatus* (Bowdich); black seabream, *Spondyliosoma cantharus* (L.); european conger, *Conger conger* (L.); blackbelly rosefish, *Helicolenus dactylopterus* (Delaroche); atlantic mackerel, *Scomber scombrus* L.; chub mackerel, *Scomber japonicus* Houttuyn and blue whiting, *Micromesistius poutassou* (Risso) were observed macroscopically in their outer surface.

For the histopathological study, 17 specimens of axillary seabream with altered pigmentation and two healthy specimens were processed.

The axillary seabream with altered pigmentation were fixed whole in 10% buffered neutral formalin (diluted in 10% salt water). The samples collected from different organs and tissues with altered pigmentation were processed by routine laboratory methods, sectioned at 3 µm and stained with hematoxylin and eosin (H&E). The pigmented lesions were stained with Masson Fontana (Churukian 2002) and bleaching. Samples of spleen and liver were stained with Ziehl-Neelsen.

Results

Healthy axillary seabream

Normal pigmentation

The body surface of the normal axillary seabream was a pale-gray pink colour, and the head was darker especially in the inter-orbital space. The fins exhibited a pale pink colour, although the paired fins revealed a black spot in the insertion area.

Microscopic examination

Skin samples made at the inter-orbital space allowed identifying the presence of melanophores in two different intra-dermal locations: in the dermal-epidermal junction (next to the basement membrane) and between cells of the subcutaneous adipose tissue and muscle. Sometimes, melanin granules appeared between the cells of the adipose tissue. The melanophores present at the dermal-epidermal junction were isolated, forming a single, irregular, discontinuous row, and full of brown or black colour pigments. In areas with scales, melanophores were in the stratum spongiosum of the dermis, adjacent to these structures.

Axillary seabream with altered skin pigmentation

Macroscopic examination

Axillary seabream with altered skin pigmentation had dark spots on the head, in several locations, from the upper lip, inter-orbital space and the region above the eye (figure 2). The spots were flat, compact, with irregular contours, not encapsulated, with variable size and shape, with location at times unilateral, or projecting into the trunk (figure 3), along the pectoral and caudal fins, lips and conjunctiva. In other specimens, the spots covered the entire head and operculum up to the lips, giving a metallic colouring.

Some axillary seabream showed black nodular lesions in the inter-orbital space, projecting to the surface of the skin and causing for uneven surface and abnormal conformation of the head (figures 4 and 5), as well as sessile nodular formations of varying size on the lips (figure 6).

The pigment spots were not associated with any other type of lesion on the skin surface.

The internal organs showed no macroscopic changes.

Microscopic examination

The histopathological examination of pigmented lesions allowed the identification of melanotic lesions resulting from the proliferation of melanophores. In most cases, the epidermis was absent. Flat or sessile lesions were characterized by marked hyperplasia of dermal melanophores (figure 7), with abundant intracytoplasmic melanosomes (figure 8). In these lesions, melanophores isolated and scattered along the basement membrane or scattered in the dermis were also observed (figures 9 and 10). Some of these melanophores had dendritic morphology.

In two specimens, the presence of granulomas in the dermis was observed.

The nodular lesions had exuberant hyperplasia of melanophores in the dermis, intensely pigmented, forming multiple layers arranged in bundles (figure 11) or in spiral, forming a whirling (figures 11, 12A, B), surrounded by a stroma rich in fibroblastic cells and highly vascularized. These nodular lesions revealed a population of polymorphic cells consisting of melanophores with intracytoplasmic melanosomes and macromelanophore (figure 13). These melanophores did not invade the adjoining subcutaneous tissue.

The histochemical reactions of black granules were positive with the specific coloration of Masson Fontana.

After bleaching of these lesions, the melanophores described above showed moderate pleomorphism, confirming the spindle, star-shape morphology, or, conversely, round, large cells, with centrally positioned nucleus and very pale, slightly granular cytoplasm. There were no mitotic figures or vascular invasion by these cells.

These lesions were somewhat similar to those observed in domestic animals (Goldschmidt, Dunstan, Stannard, von Tscharner, Walder & Yager 1998), the presence of melanotic benign neoplastic lesions of the skin, that is, melanoma or melanophoroma.

The histopathological examination of internal organs, liver, spleen and anterior kidney demonstrated an increase in the number and area occupied by the melanomacrophage centres (MMC), compared to healthy fish sampled in the same geographic area, as well as the presence of extensive granulomatous lesions, sometimes multifocal.

The granulomas had a necrotic, amorphous, eosinophilic centre, surrounded by mononuclear inflammatory cells. There was no evidence of the presence of bacteria Ziehl-Neelsen positive, particularly *Mycobacterium* spp.

Discussion

Melanotic lesions observed in axillary seabream as described in this work cannot be mistaken for physiological changes, such as colour changes observed during courtship or spawning season resulting from hormonal action on the pigment cells (Kaleta 2009).

In these axillary seabream, as was also observed with yellowtail rockfish, *Sebastes flavidus* (Ayres) with altered skin pigmentation, the most affected area is the dorsal-anterior one (Okihiro, Whipple, Groff & Hinton 1993). However, in *S. flavidus*, pigmentation in the gingiva, tongue, urogenital papilla, conjunctiva and cornea was also observed. In *Sebastes mentella*, spots on the skin of the trunk, head and fins were observed (Bogovski & Bakai 1989). In axillary seabream, the inter-ocular, dorsal and anterior prevalence and distribution of pigmentation spots is probably due to the normal number and location of melanophores, as suggested by Okihiro *et al*. (1993) in *S. flavidus*. In the studied species, it was not possible to observe the epidermis in detail due to damage at capture.

It would seem that in this study the melanotic pigmentation spots observed in axillary seabream correspond to hyperplastic lesions of melanophores, whereas nodules correspond to melanophoromas.

The classification scheme for *Xiphophorus* melanomas proposed by Gimenez-Conti, Woodhead, Harshbarger, Kazianis, Setlow, Nairn & Walter (2001), based on histopathologic features, classifies as "Melanotic hyperpigmentation", when there is moderate accumulation of pigmented cells restricted to the dermis and the basal layer of the skin. This corresponds with the more pronounced pigmentation spots observed in axillary seabream. The pigmented nodular lesions observed in axillary seabream correspond to the stage of "pre-malignant lesions", in which there is an increase in the number of melanophores, but the cells are confined to the dermis, contrary to the stage of "melanoma", in which there is, according to the same authors, invasion and destruction of the muscle tissue.

The classification in domestic mammals distinguishes, on the one hand, benign melanocytomas or melanomas (dermal melanomas) and, on other hand, malignant melanomas. The classification criteria are based on the location exclusively dermal of melanocytes, on the absence of invasion of the epidermis, subcutaneous tissue and adjoining muscle and on the absence of vascular or lymphatic invasion (melanocytomas). The phenotypic and mitotic activity of neoplastic cells, especially after bleaching are also criteria considered for diagnosis (Goldschmidt *et al*. 1998).

The axillary seabream targeted in this study would probably be a closed population, and the observed melanotic lesions may correspond to different stages of development.

In the internal organs of axillary seabream with altered pigmentation, metastases were not observed. Okihiro *et al*. (1993) reported the presence of multiple nodular masses in the kidney, spleen, mesentery and liver observed in *S. flavidus* and gills of olive rockfish, *Sebastes serranoides* (Eigenmann & Eigenmann). However, the axillary seabream observed showed extensive granulomatous lesions, multifocal, centred on the MMC of the spleen, as observed by Couillard, Williams, Courtney & Rawn (1999) in atlantic tomcod, *Microgadus tomcod* (Walbaum), which aetiologic process was associated with effluents from the pulp industry and other environmental contaminants. The increased risk of cancer was associated with chronic infections caused by bacteria of the type

Mycobacterium spp., namely *M. marinum* in japanese rice fish, *Oryzias latipes* (Temminck & Schlegel), under the influence of a mutagen factor (benzo[a]pyrene) (Broussard, Norris, Schwindt, Fournier, Winn, Kent & Ennis 2009). Considering that no Ziehl-Neelsen positive bacteria were identified, the presence of granulomatous lesions in axillary seabream cannot be assigned, without further study of another nature, to exposure to contaminants.

Melanomacrophage centres (MMC) are focal accumulations of pigmented macrophages, and may contain melanin pigments, and they are, therefore, considered a component of the reticuloendothelial system and, hence, part of the fish's defence system (Roberts 1975). These structures serve as material deposits, such as melanin, which are metabolically inert or necessary for recycling such as iron pigments or lipofucsin. The proliferation of MMC was associated with various natural factors such as age (Blazer, Wolke, Brown & Powell 1987), malnutrition and infectious diseases (Wolke 1992), and, also, in response to environmental stress (Fournier, Summers, Courtney & Engle 2001). Given that melanin has the ability to link to aromatic and cyclic compounds and cations, it can selectively take up harmful compounds (Roberts 1975). Notwithstanding the fact that the presence of extra-cutaneous melanin was highly variable between different species of fish (Zuasti, Ferrer, Aroca & Solano 1990), there was high concentration of melanin in the spleen, liver and kidney of axillary seabream with altered skin pigmentation. The changes observed in the internal organs may reflect a close relationship between the appearance of the skin and the overall health of axillary seabream. The sporadic nature of the occurrence of pigmented lesions may point towards the existence of specific conditions in that geographical area, whether concerning the fish *per se,* or concerning the environment.

Literature refers, as potential aetiologic agents in the development of melanotic lesions, oncogenic viruses, genetic predisposition, increasing age, exposure to UV radiation and xenobiotic compounds (Okihiro 1988, Okihiro *et al*. 1993). In this study, no diagnostic laboratory tests were performed to search for viral particles, so the viral aetiology cannot be definitively ruled out.

Melanomas were induced experimentally in platyfish-swordtail hybrids, southern platyfish, *Xiphophorus maculatus* (Günther) crossed with green swordtail, *Xiphophorus hellerii* Heckel (Gimenez-Conti *et al*. 2001) and swordtail fish using UV rays (Setlow, Woodhead & Grist, 1989; Sokkar, Mahmoud & Mahrous 2001). Bogovski & Bakai (1989) indicate mutagenic events as the cause of the emergence of pigmented lesions in *S.*

mentella. It would be necessary to study other batches of axillary seabream from geographically distant locations, in order to assess whether the changes could have a genetic origin.

Age was associated with increased prevalence of pigmented cells, and it has been documented in goldfish, in *Xiphophorus* and *S. flavidus* hybrids (Okihiro *et al*. 1993). The maximum size of the axillary seabream is referenced by 36 cm (Froese & Pauly 2011). In fish with altered pigmentation, the average length was 30.2 cm ± 2.11 cm. It might be questioned whether the presence of axillary seabream with altered pigmentation, with this length, in this geographical area, is due simply to the fact that only specimens with this size were available.

In *Xiphophorus* hybrids, tumours may be selectively induced by exposure to UV radiation (Setlow *et al*. 1989; Wang, Setlow, Berwich, Polsky, Marghoob, Kopf & Bart. 2001). However, in wild populations, the penetration of UV radiation through the water is minimal, so it is unlikely that this benthic-pelagic species of common distribution between 40 and 100 meters (Froese & Pauly 2011) is subject to significant radiation.

Numerous studies report outbreaks of skin tumours in wild populations of fish from freshwater, seawater and estuaries, associated with contaminants (Baumann 1998), including polyaromatic hydrocarbons (PAHs). The correlation between environmental chemicals and neoplasia of the pigmented cells has been suggested in some species (Kimura, Taniguchi, Kumai, Tomita, Kinae, Yoshizaki, Ito & Ishikawa 1984; Okihiro *et al*. 1993) and induced by chemical agents such as N-nitroso-N-methylurea in *Xiphophorus* hybrid fish (Kazianis, Gimenez-Conti, Trono, Pedroza, Chovanec, Morizot, Nairn & Walter 2001).

The possibility that chemical agents, which may play an aetiologic role in the development of melanotic lesions in axillary seabream, may be present in the environment would lead to the assumption that these substances could cause altered pigmentation or tumours in other fish that inhabit the same geographical catching area. However, among the different species observed, only the axillary seabream showed altered pigmentation.

Although the aetiology of pigmentation changes in axillary seabream has not been determined, from all the fish caught, only in axillary seabream do cells seem to have lost the ability to regulate the mechanisms of colour adaptation. Currently, the prevalence of axillary seabream with altered pigmentation has been underestimated, as fishermen remove the affected fish, which would be rejected at the auction centre. The economic losses associated with these lesions are due to the fact that this fish are rejected as unfit for public consumption.

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