Histopathological and Immunohistochemical Evaluation of a Malignant Metastatic Iridophoroma in a Male Cambodian Siamese Fighting Fish (*Betta splendens*): Possibly Related to the Skin Nodule Syndrome?

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Simple Summary: There is a significant lack of knowledge of pathology of ornamental fishes. Ornamental fish diseases produce significant economic loss and also there is concern on animal welfare. Neoplastic diseases are multifactorial, although genetic defects are the ultimate reason for cancer development. Fishes are also common animal models of humans diseases, particularly cancer, and there is interest of numerous research groups to find reproducible and spontaneous tumours in animals that can be used for comparative pathology studies. Here we describe a case of malignant neoplasm (cancer) in an ornamental fish (Cambodian Siamese Fighting Fish) that seems to be very common in this species and we discuss the possible causes for the tumour development.

Abstract: Ornamental fish trade is a fundamental income source for south-east Asia region and worldwide. Pathological inherited or background lesions of inbreed stocks are scarcely studied and occasional reports lacking comprehensive and qualified pathological interpretation and the only source of disease surveys in these species. A recently "skin nodule syndrome' (SNS) has been described affecting Cambodian Siamese Fighting Fish (*Betta splendens*). Here we describe macroscopically, microscopically and immunohistochemically a malignant metastatic iridophoroma that resemble the lesions described for the SNS. The neoplasm was markedly infiltrative, affecting large areas of the musculature and with liver involvement. Immunohistochemical analysis yielded negative results for Melan A, PNLA-2, and S-100, likely due to species-specific reasons. A review of the literature suggests a likely familiar/genetic background mutation and/or predisposition of this species to develop this type of chromatophoroma, which produces profound economic loss. Further genetic analysis of large cohorts should be carried out to investigate iridophoroma tumourogenesis in the Cambodian Siamese Fighting Fish.

Keywords: *Betta splendens*; immunohistochemistry; iridophoroma; Siamese fighting fish

1. Introduction

Currently, ornamental fish trade is a multibillion dollar industry in more than 125 countries, and involves over 2,500 fish species, of which 60% are of freshwater origin (Dey 2016; Chan 2019; King 2019; Novák et al. 2020-in press). It has been estimated that about 30 freshwater fish species dominate the global market, among which is the Siamese fighting fish (*Betta splendens*) (Dey 2016).

This species inhabits intact marshlands in shallow zones in Thailand and other countries in south-east Asia, and currently is assessed as 'Vulnerable' in the IUCN Red List due to population decline (Vidthayanon 2013). Intensive breeding in Thailand was traditionally focused to game fighting because the males are very territorial, especially after isolation or during courtship (Monvises et al. 2009). Currently, breeding is mainly devoted to ornamental purposes, producing greater socio-economic benefits for the local population, as well as creating new varieties of fish with different color variations (Monvises 2009; Monticini 2010), among which is the very appreciated Cambodian *Betta splendens*, which has a pale body, ideally white or pale pink, with brightly red fins (Brammah 2015).

Several diseases of microbiological (Humphrey et al. 1986; Najiah et al. 2011; Sirimalaisuwan et al. 2017), parasitological (Ferguson and Moccia, 1980; Molnár et al. 2003; Senapin et al. 2014), and metabolic (Rahmati-Holasoo 2020) origin have been reported affecting this popular fish species. Recently, a preliminarily named 'skin nodule syndrome' (SNS) has been described affecting this fish species in central Thailand (Dong et al. 2018); histopathologically, authors reported severe necrosis and melanization in muscle layers, connective tissues, and internal organs such as kidney, liver, spleen, and

intestine, and microbiologically, 23 different bacterial species belonging to 14 genera were identified, remaining inconclusive the principal causative agent of SNS (Dong et al. 2018). Interestingly around the same time, and in two different countries, they were reported affecting this fish species a malignant iridophoroma (Rahmati-Holasoo et al. 2019), and a chromatophoroma of the iridophoroma or leucophoroma subtypes (Ciambrone et al. 2019). Particularly in the first case, the gross and histological lesions were very similar to those described in the SNS (Dong et al. 2018; Rahmati-Holasoo et al. 2019). In this article we describe the macroscopic, histopathological and immunohistochemical characteristics of a malignant metastatic iridophoroma in a male Cambodian Siamese fighting fish (*Betta splendens*), providing a comprehensive review of the literature in which a possible relationship with the SNS is discussed.

2. Materials and Methods

2.1. Ethics

Owner's informed consent on the postmortem procedures and the publication of the pictures and videos kindly provided by herself was obtained.

2.2. Animal and case history

The fish had been acquired from a local pet store in February 2018 and housed alone in a 20-liter aquarium equipped with a filter, led lights (regulated by a clock to provide 12 hours of light per day) and a submersible heater to keep the water temperature at 26 ºC. Water quality was weekly maintained by partial water changes using bottled mineral water. The aquarium also had natural plants such as…. and….. (¿Elodea densa, la Lenteja de agua, la Espada del amazonas o el Bambú??), and no ornamental rocks were inside the aquarium to avoid tears in the fins. The fish was fed daily using commercial

betta fish pellets composed of salmon, herring, halibut, shrimp, wheat flour, wheat gluten, and wheat germ, and it was sporadically supplemented with live brine shrimp.

Seven months after its acquisition, effacing the right flank under the dorsal fin and occupying the entire trunk up to the ventral fins, a 10 x 15 x 3 mm, irregularly round, white, apparently firm mass was observed. The colour was largely similar to that of the rest of the body of the fish although slightly brighter (Figure 1A). Even with the lesion, the fish initially showed a normal, active behavior, eating normally. However, as the mass grew, the fish presented difficulties in swimming, with an impossibility of maintaining balance, defeating itself towards the right side, and remaining most of the time on the bottom, even to access food (Figure 1B) and Supplementary Materials Video S1). The fish stayed alive 10 more months, during which the owner did not apply any treatment, and, especially during its last week of life, the fish was lethargic, immobile at the bottom of the aquarium, and even refused the food that was offered. The owner also observed rapid and severe deterioration of all the fins of the fish, with shortening and irregular edges. Finally, the fish died in July 2019, and it was submitted to the Veterinary Faculty, University of Las Palmas de Gran Canaria, for post-mortem examination.

2.3. Post-mortem examination and histopathology

Necropsy was performed using procedures previously described (Fisher and Myers 2000). The carcass and tissue samples from all major organs were preserved in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 µm for light microscopy and stained with hematoxylin and eosin (HE). Special stains performed on selected sections included Gram-stain for bacteria, Ziehl Neelsen (ZN) for acid-fast organisms, periodic acid-Schiff (PAS) and Gomori Methenamine Silver (GMS) for

protozoa and fungal hyphae and Masson's Trichrome for connective tissue and muscle evaluation (Bancroft and Stevens, 1996).

2.4. Immunohistochemistry

For immunohistochemical analysis 4-mm-thick sections from the parafine blocks were mounted onto positively charged slides (SuperFrost Plus; Menzel Gläser, Braunscheig, Germany). Antigen retrieval, labeling, and counterstaining were performed on a Bond-Max Autostainer (Leica Biosystems, Newcastle-upon-Tyne, UK) using the Bond polymer refine detection system (Leica Biosystems). Primary antisera were specific for Melan-A (A103 clone, monoclonal [Leica Microscopy, Milton Keynes, UK]; 1 in 100 dilution; antigen retrieval in buffer pH 9.0 buffer [ER2, Leica Biosystems] for 20 minutes), PNL-2 (sc-59306, monoclonal [Santa Cruz, Heidelberg, Germany]; 1 in 300 dilution; antigen retrieval in pH 6.0 buffer [ER1, Leica Biosystems] for 20 minutes) and S-100 (NCL-L-S100p, polyclonal [Leica Microscopy, Milton Keynes, UK]; 1:2000 dilution; antigen retrieval in pH 6.0 buffer [ER1, Leica Biosystems] for 10 minutes). Sections from a canine oral melanocytic neoplasm, known to express Melan-A and PNL-2, were used as a positive control.

3. Results

Macroscopically, in addition to the previously described lesion on the right side of the trunk, multifocal nodules measuring 5 x 15 x 1 mm and 1 x 4 x 1 mm were observed on the left side of the trunk under the dorsal fin up to the ventral fins and on the left side rostral to the eye in proximity to the mouth, respectively. Transverse cut sections exhibited a markedly infiltrative mass occupying a large percentage of the right side

musculature. The mas extended into the median plane and multifocally coalesded with dorsal ventral and left side musculature. The mass was firm, and the cut surface was gritty and chalky, multinodular with poorly defined margins. The mass occupied approximately 70% of the total area of the transverse section performed through the right mass.(Figure 2A and 2B). There was no evidence of other macroscopic lesions.

Histologically, arising from the dermal pigment layer, there was a poorly-demarcated, densely cellular, markedly infiltrative pleomorphic neoplasm. Neoplastic cells formed haphazardly oriented bundles and streams that infiltrated, effaced and replaced large areas of the musculature. The neoplasm extended deeply and destroyed vertebral bodies entrapping the spinal cord. Infiltration within the vertebral canal was otherwise not visible in the plane of section examined. (Figure 3A). Neoplastic cells were round to spindloid, with moderate amounts of cytoplasm that contained abundant acicular, colorless crystals (Figure 3B). Cells had one round, paracentral nucleus with finely stippled chromatin. Anisokaryosis and anisocytosis were mild to moderate and mitoses were not observed in ten high-power fields (400x, equivalent to 2.73mm^2). There was occasional locally extensive epidermal erosion and ulceration. The underlying dermis was mildly expanded by oedema and mild to moderate numbers of lymphocytes and histiocytes. Further changes affecting the muscle were consistent with atrophy, degeneration and necrosis depicted by shrunken myofibres with angular borders, fragmentation and hypereosinophilia. Under polarized light the crystals had a yellow-orange to green-apple colour (Figure 3C and 3D). Sections from liver exhibited multifocal metastatic foci. Additional serial sections were stained with Gram, ZN, PAS and GMS and bacteria or fungal organisms were not observed.

Immunohistochemical analysis with Melan-A, PNLA-2 and S-100 did not label any of the neoplastic cells. Positive controls reacted appropriately.

4. Discussion

The pigment cells of the skin of lower vertebrates such as fishes, amphibians, and reptiles, are collectively referred to as chromatophores (Bagnara et al., 1968; Rahmati-Holasoo et al., 2019). Chromatophores are derived from the neural crest during embryonic development, and three different groups of are recognized: light absorbing (melanophores, xantophores, erythrophores, and cyanophores), light reflecting (iridophores), and light scattering (leucophores) (Amiri and Shaheen, 2012; Ciambrone et al., 2019). Ultrastructural characteristics of iridophores have been described in detail in the blue variant of the Siamese fighting fish (Amiri and Shaheen, 2012). Iridophores are rather round in shape and contain stacks of guanine platelets, which they use to reflect light and produce iridescence (when arrangements of the platelets are precisely organized) or look white when they are less organized (Fujii, 2000; Schartl et al., 2016; Ciambrone et al., 2019).

Pigment cell tumors are more common in fish compared with those of mammals (Masahito et al., 1989; Okihiro et al., 1992), and may occur in combination with hereditary, carcinogenic, and aging factors; melanomas in platyfish/swordtail hybrids (*Xiphophorus maculatus* crossed with *X. helleri*) have been shown to be determined genetically (Anders, 1967); Hyodo-Taguchi and Matsudaira (1984) were able to induce melanomas in fish with chemical carcinogens; Setlow et al. (1989) induced melanomas in fish by exposure to filtered radiation from sunlamps; erythrophoromas have been reported especially in goldfish more than three years old (Etoh et al. 1983).

Iridophoromas have been reported in both marine [croaker (*Nibea mitsukurii*) (Kimura et al., 1984), brown-barred butterflyfish (*Chaetodon multicinctus*) (Okihiro,

1988), Hawaiian goldring surgeonfish (*Ctenochaetus strigosus*) (Work and Aeby, 2014), Indian oil sardine (*Sardinella longiceps*) (Singaravel et al., 2016), and Indian mackerel (*Rastrelliger kanagurta*) (Singaravel et al., 2017)] and freshwater fish species [freshwater drum (*Aplodinotus grunniens*) (Masahito et al., 1989), European grayling (*Thymallus thymallus*) (Schmidt-Posthaus et al., 2005), and Siamese fighting fish (Rahmati-Holasoo et al., 2019)]. Grossly, iridophoromas are whitish or metallic silver in color (Okihiro, 1988; Singaravel et al., 2016; Singaravel et al., 2017; Rahmati-Holasoo et al., 2019). Histologically, our case is very similar to that recently observed in a Siamese fighting fish in Iran, although in that case metastases were observed, in addition to the liver, in kidney, spleen and intestine (Rahmati-Holasoo et al., 2019).

None of the antibodies used in our study cross-reacted immunohistochemically with the fish antigens. Immunohistochemistry has been rarely used to diagnose chromatophoromas in fish. S-100, PNL-2, Melan-A, and SOX10 are commonly used melanocytic immunohistochemical markers in humans and other species, and have been used to identify chromatophoromas in amphibians and reptiles (Muñoz-Gutiérrez et al., 2016); however, the specificity of these antibodies in fish has not been fully investigated. Immunohistochemical staining of a chromatophoroma of the xantophoroma/erythrophoroma subtype in a crevice kelpfish (*Gibbonsia montereyensis*) was strongly positive with a murine monoclonal antibody for Melan-A (Camus et al., 2011). When immunohistochemistry was applied for studying chromatophoromas in two cyprinid species, PNL-2 was useful to distinguish tumors of chromatophore origin, Melan-A appeared to be less sensitive, and SOX10 was not a useful marker for these tumors (Siniard et al., 2019); unfortunately, the authors did not specify what type of chromatophoromas were included in the study. Recently, Muñoz-Gutiérrez et al. (2016) reported the immunohistochemical staining profiles of two iridophoromas in captive

snakes: both tumors were positive for S-100, only one was positive when using PNL2, and both were negative for Melan-A and HMB45. A relevant difficulty for the immunhistochemical study of this type of tumors is that while melanocytes have been studied in depth due to their importance in the human species and domestic mammals, knowledge of other pigment cell types lags behind (Schartl et al., 2016). The small number of studies, as well as the variability of the results, shows the need to validate the reagents created for mammalian species in fish species and/or to develop specific antibodies for immunohistochemical diagnosis in fish.

Because chromatophoromas are usually locally aggressive, surgical excision is the most common and effective option for treatment. Recently, liquid nitrogen cryosurgery was successfully used to remove a chromatophoroma in a captive largemouth bass (*Micropterus salmoides*) (Yaw et al., 2016). Chemotherapy and radiation therapy have not been reported in fish. In our case, the owner had not requested any treatment for her fish, but given the extent of the lesions, we believe that surgical excision would not have been possible.

The recently reported 'skin nodule syndrome' (SNS) affecting extensively farmed betta fish in central Thailand is characterized by the presence of cutaneous nodules in which severe muscular necrosis and "melanization" are observed histologically; severe necrosis and "melanization" may also be present in kidney, liver, spleen, intestine, and abdominal connective tissue (Dong et al., 2018). Authors reported that the disease occurs in approximately 1% of the fish commercial farms, resulting in diminished saleability (Dong et al., 2018). Authors identified using 16S rRNA 23 bacterial species belonging to 14 genera from four fish suffering from SNS; although the causative agent remains unclear, authors suggested involvement of multiple bacterial infections (Dong et al., 2018).

The description, almost at the same time in the same fish species, of a malignant iridophoroma (Rahmati-Holasoo et al. 2019) and a chromatophoroma of the iridophoroma or leucophoroma subtypes (Ciambrone et al. 2019), together with our case, leads us to analyze whether they could be related to SSN, suggesting therefore the possibility of establishing another etiology for this syndrome, consisting of a tumor process involving neoplastic chromatophores rather than an infectious origin.

The histological lesions reported by Dong et al. (2018) are very similar especially to the malignant iridophoroma reported by Rahmati-Holasoo et al. (2019) and to the lesions described in the present case. When Okihiro (1988) studied the prevalence of iridophoromas in brown-barred butterflyfish, he initially misinterpreted the olive-green pigment as being melanin and nonsignificant, establishing an incorrect diagnosis of benign fibromas, misleaded also due to the large amounts of collagenous stroma he observed. Although Dong et al. (2018) suggested involvement of multiple bacterial infections in the SSN including four species of *Mycobacterium*, no inflammatory lesions were reported, nor were they in the tumors that we are discussing (Ciambrone et al. 2019; Rahmati-Holasoo et al. 2019) or in the present case.

Although in our case no microbiological cultures were performed, special stains for bacteria resulted in no observable microorganisms; Ciambrone et al. (2019) also did not observe acid-fast bacteria associated to the chromatophoroma of the iridophoroma or leucophoroma subtype, and aerobic and anaerobic cultures from the iridophoroma reported by Rahmati-Holasoo et al. (2019) resulted in no growth. On the other hand, the mere isolation of a microorganism from a lesion does not imply that it is the causative agent; immunohistochemistry is a more appropriate technique to establish a direct relation between a microorganism and the lesions, thus providing evidence to suggest pathogenicity (Orós et al., 1996). And finally, several species of *Mycobacterium*,

including *M. marinum* and *M. chelonae* have been identified in clinically asymptomatic Siamese fighting fish (Sirimalaisuwan et al., 2017).

The number of fish that, as described in Thailand (Dong et al., 2018), suffer from this syndrome, could invalidate our hypothesis. However, large chromatophoroma epizootics have been reported affecting fish species. Marine species involved with chromatophoroma epizootics include croakers (*Nibea mitsukurii*) in Japan (Kimura et al., 1984), butterflyfish (*Chaetodon multicinctus* and *C. miliaris*) in Hawaii (Okihiro, 1988), deepwater redfish (*Sebastes mentella*) in the North Atlantic (Bogovski and Bakai, 1989), Pacific rockfish (*Sebastes* sp.) in California (Okihiro et al., 1993), coral trout (*Plectropomus leopardus*) in Australia (Sweet et al. 2012), and Hawaiian goldring surgeonfish (*Ctenochaetus strigosus*) (Work and Aeby, 2014). Freshwater epizootics have involved drum (*Aplodinotus grunniens*) from the Great Lakes (Okihiro et al., 1993), and domestic goldfish (*Carassius auratus*) (Ishikawa et al., 1978; Etoh et al., 1983). Even on some occasions, the prevalence described has been very high, such as that reported for iridophoromas in brown-barred butterflyfish in Hawaii (22-50%) (Okihiro, 1988).

Several causes have been suggested to explain the development of chromatophoromas in fish populations. Potential exposure to chemical and/or radioactive carcinogens was suggested for iridophoromas in Hawaiian brown-barred butterflyfish (Okihiro, 1988), and for chromatophoromas in Pacific rockfish (*Sebastes* spp.) (Okihiro et al., 1993). Environmental exposure to UV radiation was suggested to explain the high prevalence rates (15%) of melanomas in coral trout in the Great Barrier Reef (Sweet et al. 2012). The capacity of platyfish/swordtail hybrids to develop melanomas was believed to be either dependent on the dilution of modifier genes responsible for the regulation of melanophore proliferation (Anders 1967) or on the amplification of oncogene-related segments of the genome (Vielkind and Vielkind, 1982; Vielkind and Dippel 1984). On

other occasions, the causative agent remained unknown (Work and Aeby, 2014) or a combination of environmental factors including chemical contaminants and oncogenic viruses (Anders and Yoshimizu, 1994), was suggested (Singaravel et al., 2016). Neither in our case nor in the two previous descriptions in Siamese fighting fish (Ciambrone et al. 2019; Rahmati-Holasoo et al. 2019) has the etiological agent been investigated.

Given that the number of animals studied to characterize the SNS was only four, further studies are necessary in order to (i) characterize the lesions in a larger number of individuals checking for neoplastic pigment cells proliferations, (ii) determine using immunohistological techniques and experimental inoculations if the origin of the SNS is bacterial, and (iii) examine whether commercial breeding of Siamese fighting fish in large numbers by repeated artificial mating over many years has determined a possible genetic origin for this syndrome.

5. Conclusions

The description of this new case of iridophoroma in the Cambodian variant of the Siamese fighting fish, and the similarity of the lesions observed with those described in the recently described SSN, suggests the possibility of discussing another etiology for this syndrome, consisting of a tumor process involving neoplastic chromatophores rather than an infectious origin. In addition, the immunohistochemical study highlights the need to develop specific antibodies for immunohistochemical diagnosis in lower vertebrates, thus contributing to significant advances in comparative pathology.

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References

Amiri, M.H.; Shaheen, H.M. Chromatophores and color revelation in the blue variant of the Siamese fighting fish (*Betta splendens*). Micron **2012**, *43*, 159-169. https://dx.doi.org/10.1016/j.micron.2011.07.002

PubMed ID: 21803590

Anders, F. Tumor formation in platyfish-swordtail hybrids as a problem of gene regulation. Experientia **1967**, *23*, 1-10. https://dx.doi.org/10.1007/BF02142235

PubMed ID: 6067751

Anders, K.; Yoshimizu, M. Role of viruses in the induction of skin tumours and tumour-like proliferations of fish. Dis. Aquat. Org. **1994**, *19*, 215-232. https://dx.doi.org/10.3354/dao019215

Bagnara, J.T.; Taylor, J.D.; Hadley, M.E. The dermal chromatophore unit. J. Cell Biol. **1968**, *38*, 67-79. https://dx.doi.org/10.1083/jcb.38.1.67

PubMed ID: 5691979

Bancroft, J.D., Stevens, A. *Theory and Practice of Histological Techniques*, 4th ed.; Churchill Livingstone: New York, USA, 1996; pp. 1-766.

Bogovski, S.P.; Bakai, Y.I. Chromatoblastomas and related pigmented lesions in deepwater redfish, *Sebastes mentella* (Travin), from North Atlantic areas, especially the Irminger Sea. J. Fish Dis. **1989**, *12*, 1-13. https://dx.doi.org/10.1111/j.1365-2761.1989.tb01234.x

Brammah, M. *The Betta Bible: The Art and Science of Keeping Bettas*, 1st ed.; CreateSpace Independent Publishing Platform: Scotts Valley, CA, USA, 2015; pp. 1-318.

Camus, M.S.; Hyatt, M.W.; Clauss, T.M.; Berliner, A.L.; Camus, A.C. Chromatophoroma in a crevice kelpfish (*Gibbonsia montereyensis*). Vet. Clin. Pathol. **2011**, *40*, 549-552. https://dx.doi.org/10.1111/j.1939-165X.2011.00365.x

Chan, F.T.; Beatty, S.J.; Gilles, A.S.Jr.; Hill, J.E.; Kozic, S.; Luo, D.; Morgan, D.L.; Pavia, R.T.B.Jr.; Therriault, T.W.; Verreycken, H.; Vilizzi, L.; Wei, H.; Yeo, D.C.J.; Zeng, Y.; Zięba, G.; Copp, G.H. Leaving the fish bowl: the ornamental trade as a global vector for freshwater fish invasions. Aquat. Ecosyst. Health Manag. **2019**, *22*, 417-439. https://dx.doi.org/10.1080/14634988.2019.1685849

Ciambrone, C.; Chen, L.R.; Tokarz, D.A.; Lewbart, G.A. Chromatophoroma in a Siamese fighting fish (*Betta splendens*). Vet Rec. Case Rep. **2019**, *7*, e000840. https://dx.doi.org/10.1136/vetreccr-2019-000840

Dey, V.K. The global trade in ornamental fish. InfoFish Int. **2016**, *4*, 52-55.

Dong, H.T.; Senapin, S.; Phiwsaiya, K.; Techatanakitarnan, C.; Dokladda, K.; Ruenwongsa, P.; Panijpan, B. Histopathology and culturable bacteria associated with "big belly" and "skin nodule" syndromes in ornamental Siamese fighting fish, *Betta splendens*. Microb. Pathog. **2018**, *122*, 46-52. https://doi.org/10.1016/j.micpath.2018.06.005
PubMed ID: 29864533

Etoh, H.; Hyodo-Taguchi, Y.; Aoki, K.; Murata, M.; Matsudaira, H. Incidence of chromatoblastomas in aging goldfish (*Carassius auratus*). J. Natl. Cancer Inst. **1983**, *70*, 523-528. PubMed ID: 6572741

Ferguson, H.W.; Moccia, R.D. Disseminated hexamitiasis in Siamese fighting fish. J. Am. Vet. Med. Assoc. **1980**, *177*, 854-857. PubMed ID: 7451326

Fisher, J.P., Myers, M.S. Fish necropsy. In *The Handbook of Experimental Animals: The Laboratory Fish*, 1st ed., Ostrander, G.K., Ed.; Academic Press: London, UK, 2000; pp. 543-556. https://dx.doi.org/10.1016/B978-012529650-2/50049-4

Fujii, R. The regulation of motile activity in fish chromatophores. Pigment Cell Res. **2000**, *13*, 300-319. https://dx.doi.org/10.1034/j.1600-0749.2000.130502.x

PubMed ID: 11041206

Humphrey, J.D.; Lancaster, C.; Gudkovs, N.; McDonald, W. Exotic bacterial pathogens *Edwardsiella tarda* and *Edwardsiella ictaluri* from imported ornamental fish *Betta splendens* and *Puntius conchonius*, respectively: isolation and quarantine. Aust. Vet. J. **1986**, *63*, 369-371. https://dx.doi.org/10.1111/j.1751-0813.1986.tb02900.x

PubMed ID: 3827773

Hyodo-Taguchi, Y.; Matsudaira, H. Induction of transplantable melanoma by treatment with N-methyl-N'-nitro-N-nitrosoguanidine in an inbred strain of the teleost *Oryzias latipes*. J. Natl. Cancer Inst. **1984**, *73*, 1219-1227. https://dx.doi.org/10.1093/jnci/73.5.1219

PubMed ID: 6593492

Ishikawa, T.; Masahito, P.; Matsumoto, J.; Takayama, S. Morphologic and biochemical characterization of erythrophoromas in goldfish (*Carassius auratus*). J. Natl. Cancer Inst. **1978**, *61*, 1461-1470. https://dx.doi.org/10.1093/jnci/61.6.1461

PubMed ID: 281553

Kimura, I.; Taniguchi, N.; Kumai, H.; Tomita, I.; Kinae, N.; Yoshizaki, K.; Ito, M.; Ishikawa, T. Correlation of epizootiological observations with experimental data: chemical induction of chromatophoromas in the croaker, *Nibea mitsukurii*. In *Use of*

Small Fish Species in Carcinogenicity Testing, Hoover, K.L., Ed.; Natl. Cancer Inst. Monogr. **1984**, 65, 139-154. PubMed ID: 6431288

King, T.A. Wild caught ornamental fish: a perspective from the UK ornamental aquatic industry on the sustainability of aquatic organisms and livelihoods. J. Fish Biol. **2019**, *94*, 925-936. https://dx.doi.org/10.1111/jfb.13900
PubMed ID: 30671948

Masahito, P.; Ishikawa, T.; Sugano, H. Pigment cells and pigment cell tumors in fish. J. Invest. Dermatol. **1989**, *92*, 266S-270S. https://dx.doi.org/10.1038/jid.1989.79
PubMed ID: 2654300

Molnár, K.; Shaharom-Harrison, F.; Székely, C. A survey of coccidian infections of freshwater fishes of Peninsular Malaysia, with descriptions of three species of *Goussia* Labbé, 1896 (Apicomplexa: Eimeriidae). Syst. Parasitol. **2003**, *55*, 11-18. https://dx.doi.org/10.1023/A:1023929107411
PubMed ID: 12815211

Monticini, P. The ornamental fish trade. Production and commerce of ornamental fish: technical-managerial and legislative aspects. Globefish Res. Program. **2010**, *102*, 1-134.

Monvises, A.; Nuangsaeng, B.; Sriwattanarothai, N.; Panijpan, B. The Siamese fighting fish: well-known generally but little-known scientifically. Sci. Asia **2009**, *35*, 8-16. https://dx.doi.org/10.2306/scienceasia1513-1874.2009.35.008

Muñoz-Gutiérrez, J.F.; Garner, M.M.; Kiupel, M. Cutaneous chromatophoromas in captive snakes. Vet. Pathol. **2016**, *53*, 1213-1219. https://dx.doi.org/10.1177/0300985816644302

PubMed ID: 27121299

Najiah, M.; Lee, K.L.; Noorasikin, H.; Nadirah, M.; Lee, S.W. Phenotypic and genotypic characteristics of *Mycobacterium* isolates from fighting fish *Betta* spp. in Malaysia. Res. Vet. Sci. **2011**, *91*, 342-345. https://dx.doi.org/10.1016/j.rvsc.2010.09.010

PubMed ID: 20971487

Novák, J.; Kalous, L.; Patoka, J. Modern ornamental aquaculture in Europe: early history of freshwater fish imports. Rev. Aquac. **2020**, *12*, 2042-2060. https://dx.doi.org/10.1111/raq.12421

Okihiro, M.S. Chromatophoromas in two species of Hawaiian butterflyfish, *Chaetodon multicinctus* and *C. miliaris*. Vet. Pathol. **1988**, *25*, 422-431. https://dx.doi.org/10.1177/030098588802500604

PubMed ID: 3212887

Okihiro, M.S.; Whipple, J.A.; Groff, J.M.; Hinton, D.E. Chromatophoromas and related hyperplastic lesions in Pacific rockfish (*Sebastes* spp.). Mar. Environ. Res. **1992**, *34*, 53-57. https://dx.doi.org/10.1016/0141-1136(92)90082-W

Okihiro, M.S.; Whipple, J.A.; Groff, J.M.; Hinton, D.E. Chromatophoromas and chromatophore hyperplasia in Pacific rockfish (*Sebastes* spp.). Cancer Res. **1993**, *53*, 1761-1769. PubMed ID: 8467494

Orós, J.; Rodríguez, J.L.; Herráez, P.; Santana, P.; Fernández, A. Respiratory and digestive lesions caused by *Salmonella arizonae* in two snakes. J. Comp. Pathol. **1996**, *115*, 185-189. https://dx.doi.org/10.1016/S0021-9975(96)80040-5
PubMed ID: 8910746

Rahmati-Holasoo, H.; Pedram, M.S.; Ebrahimzadeh Mousavi, H.A.; Shokrpoor, S.; Lewbart, G.A.; Azizi, A.; Mohammadian Rasanani, A. Malignant iridophoroma in a male Siamese fighting fish (*Betta splendens* Regan): a clinical, surgical and histopathological study. Bull. Eur. Assoc. Fish Pathol. **2019**, *39*, 106-113.

Rahmati-Holasoo, H.; Shahbazi, M.; Ebrahimzadeh Mousavi, H.A.; Shokrpoor, S.; Pourmortazavi Bahambari, M.; Azizi, A.; Mokhatari, A. Polycystic kidney disease in discus (*Symphysodon aequifasciatus*) and Siamese fighting fish (*Betta splendens*): a histopathological study. Bull. Eur. Assoc. Fish Pathol. **2020**, *40*, 38-44.

Schartl, M.; Larue, L.; Goda, M.; Bosenberg, M.W.; Hashimoto, H.; Kelsh, R.N. What is a vertebrate pigment cell? Pigment Cell Melanoma Res. **2016**, *29*, 8-14. https://dx.doi.org/10.1111/pcmr.12409
PubMed ID: 26247887

Schmidt-Posthaus, H.; Pugovkin, D.; Wahli, T. Iridophoroma in a European grayling (*Thymallus thymallus*) in Switzerland. Bull. Eur. Assoc. Fish Pathol. **2005**, *25*, 36-39.

Senapin, S.; Phiwsaiya, K.; Laosinchai, P.; Kowasupat, C.; Ruenwongsa, P.; Panijpan, B. Phylogenetic analysis of parasitic trematodes of the genus *Euclinostomum* found in *Trichopsis* and *Betta* fish. J. Parasitol. **2014**, *100*, 368-371. https://dx.doi.org/10.1645/13-285.1

PubMed ID: 24490744

Setlow, R.B.; Woodhead, A.D.; Grist, E. Animal model for ultraviolet radiation-induced melanoma: Platyfish-swordtail hybrid. Proc. Natl. Acad. Sci. U.S.A. **1989**, *86*, 8922–8926. https://dx.doi.org/10.1073/pnas.86.22.8922

PubMed ID: 2813430

Singaravel, V.; Gopalakrishnan, A.; Raja, K. Iridophoroma in Indian mackerel, *Rastrelliger kanagurta* (Cuvier, 1816). J. Appl. Ichthyol. **2017**, *33*, 116-118. https://dx.doi.org/10.1111/jai.13226

Singaravel, V.; Gopalakrishnan, A.; Vijayakumar, R.; Raja, K.; Asrafuzzaman, S. Occurrence of chromatophoroma or chromatophoromatosis in *Sardinella longiceps* (Valenciennes, 1847) from Tamilnadu, southeast coast of India. J. Appl. Ichthyol. **2016**, *32*, 712-717. https://dx.doi.org/10.1111/jai.13084

Siniard, W.C.; Sheley, M.F.; Stevens, B.N.; Parker-Graham, C.A.; Roy, M.A.; Sinnott, D.M.; Watson, K.D.; Marinkovich, M.J.; Robertson, J.A.; Frei, S.; Soto, E.

Immunohistochemical analysis of pigment cell tumors in two cyprinid species. J. Vet. Diagn. Invest. **2019**, *31*, 788-791. https://dx.doi.org/10.1177/1040638719864380
PubMed ID: 31328699

Sirimalaisuwan, A.; Teeraruk, P.; Kanjanapitakchai, P.; Kaewsakhorn, T.; Potibut, P.; Pikulkaew, S. Detection of *Mycobacterium marinum* in clinically asymptomatic Siamese fighting fish (*Betta splendens*) from ornamental fish shops in Chiang Mai Province, Thailand. Asian Pacific J. Trop. Dis. **2017**, *7*, 344-346. https://dx.doi.org/10.12980/apjtd.7.2017D6-446

Sweet M.; Kirkham, N.; Bendall, M.; Currey, L.; Bythell, J.; Heupel, M. Evidence of melanoma in wild marine fish populations. PLOS ONE **2012**, *7*, e41989. https://dx.doi.org/10.1371/journal.pone.0041989
PubMed ID: 22870273

Vidthayanon, C. *Betta splendens*. The IUCN Red List of Threatened Species 2013, e.T180889A7653828.https://dx.doi.org/10.2305/IUCN.UK.2011-1.RLTS.T180889A7653828.en

Vielkind, J.R.; Dippel, E. Oncogene-related sequences in xiphophorin fish prone to hereditary melanoma formation. Can. J. Genet. Cytol. **1984**, *26*, 607-614. https://dx.doi.org/10.1139/g84-094

Vielkind, J.; Vielkind, U. Melanoma formation in fish of the genus *Xiphophorus*: a genetically-based disorder in the determination and differentiation of a specific pigment cell. Can. J. Genet. Cytol. **1982**, *24*, 133-149. https://dx.doi.org/10.1139/g82-013
PubMed ID: 6751505

Work, T.M.; Aeby, G.S. Skin pathology in Hawaiian goldring surgeonfish, *Ctenochaetus strigosus* (Bennett). J. Fish Dis. **2014**, *37*, 357-362. https://dx.doi.org/10.1111/jfd.12112
PubMed ID: 23617760

Yaw, T.J.; Michaels, B.; Reavill, D.; Zaffarano, B.A. Liquid nitrogen cryosurgery for a chromatophoroma in a captive largemouth bass (*Micropterus salmoides*). J. Exot. Pet Med. **2016**, *25*, 305-310. https://dx.doi.org/10.1053/j.jepm.2016.07.001