ActaFish. (2017) 2 (2): 19-24.
DOI 10.2312/ActaFish.2017.5.2.19-24

ARTIGO DE REVISÃO

Acta of Fisheries and Aquatic Resources

A review on occurrence of neoplasia in fish

Uma revisão sobre a ocorrência de neoplasias em peixes

Carlos Alberto Machado da Rocha[1*], Caroline Aquino Moreira-Nunes[2], Simone Machado da Rocha[1], Marcos Afonso Soares da Silva[3] & Rommel Rodríguez Burbano[2]

[1] Instituto Federal de Educação, Ciência e Tecnologia do Pará - IFPA
[2] Instituto de Ciências Biológicas, Universidade Federal do Pará - UFPA
[3] Sistema Educacional Sophos - Sophos

*Email: carlos.rocha@ifpa.edu.br

Recebido: 21 de setembro de 2016 / 7 de dezembro de 2016 / 1 de maio de 2017.

Abstract The study of spontaneous neoplasms in fish is supported, among other reasons, in the possibility of relating them to the water pollution; the fish in this case used as indicators of the presence of environmental carcinogens. The mechanisms of mutagenesis and carcinogenesis in fish are interconnected and influenced by environmental chemical or physical agents, or associated with infectious agents, especially retrovirus. In this regard, a summary of the findings of swim bladder leiomyosarcomas in association with retroviruses in Atlantic salmon from North America is presented.

Keywords: cancer in fish, leiomyosarcoma, papillomas, retrovirus.

Resumo O estudo das neoplasias espontâneas em peixes é apoiada, entre outras razões, na possibilidade de relacioná-las à poluição aquática; os peixes, neste caso, utilizados como bioindicadores da presença de carcinógenos ambientais. Os mecanismos de mutagênese e carcinogênese em peixes estão interligados e influenciados por agentes físicos ou químicos ambientais, ou, ainda, associados a agentes infecciosos, em especial os retrovírus. A este respeito, é apresentado um resumo dos achados sobre leiomiossarcomas de bexiga natatória em associação com retrovírus no salmão do Atlântico da América do Norte.

Palavras-chave: câncer em peixes, leiomiossarcoma, papilomas, retrovírus.

Introduction

The occurrence of malignancies is not a peculiarity of human tissues and is not even restricted to mammals. Possibly can affect the various animal groups. Assuming that the presence of a tumor in a tissue result of cell cycle control is lost, we can imagine that these accidents can happen in any living being that has tissue (Rocha, 2013). Carcinogenesis always starts with DNA damage. Generally, these damages are enhanced by chemical, physical or biological agents. One of the concerns about the causes of cancer in fish has been the carcinogenic environmental factors. Among these chemicals are those that expose the population to the risk of mutations that can later lead to cancer development.

One important aspect that supports the study of tumors in fish is their possible relationship with the pollution and the fact that they can be used as indicators of the existence of potentially dangerous environmental carcinogens to the higher vertebrates, including man (Sinderman, 1990). We also cannot forget that you cannot market the fish with visible tumors. In fish, skin tumors, being visible externally, are among the most frequently observed. However, as in higher vertebrates, neoplastic growth can occur in all organs, tissues and cell types. On the other hand, according to Groff (2004), a neoplasm in fish is often a benign condition, with relatively few exceptions malignant disease; the treatment is usually limited to surgery.

Methodology

ISSN: 2357-8068
Indexadores: Sumários (www.sumarios.org) - Diretórios: Diadorim (Diadorim.ibict.br) - Latindex (www.latindex.org)

There are numerous ways to reflect on the production of knowledge in an area. In this study, the choice was a literature review, carried out from a qualitative approach. Our choice is based on the understanding that the review of the literature is a vital part of the research process, as it involves locate, analyze, synthesize and interpret the preliminary investigation.

We use printed materials and also obtained databases publications on the Internet, as Bireme / VHL - Virtual Health Library (Virtual Health Library bireme.br www.) Scielo (www.scielo.br) and Medline/PubMed (http://www.ncbi.nlm.nih.gov/pubmed/). The production published electronically, in addition to enabling access to current discussions about cancer in fish, is the most easily accessed. Publications were selected in Portuguese and English, respectively the native language of the authors and the language that is most significant published works on all continents.

The following descriptors in Portuguese and English were used in the search: câncer, neoplasia, papiloma, peixe, tumor, cancer, neoplasms, papilloma, fish, and tumour. Another strategy was the manual search in reference lists of identified and selected articles. Articles containing information on cancer in fish or contained information on its determinants were selected. This selection resulted in 20 articles, excluding duplications and articles without summary. Six more publications (four articles and two books) were obtained by manual search of selected references, totaling 26 documents listed in our list of references. After identification, the documents were grouped in chronological order, theme discussed and approach, facilitating their analysis and allowing know the prospects of interaction.

SPONTANEOUS NEOPLASMS IN FISHES

Although the number of publications is still not very relevant, the description of the occurrence of spontaneous tumors in fish is not a new activity, already being held for several decades. Table 1 summarizes some articles on a variety of tumors in different species of fish. Note that they are classified according to the classification of mammalian tumors.

One of the pioneering works was done by Brown et al. (1973), comparing the tumor frequencies in fish found in a polluted river basin in the Midwest region of the United States with those found in unpolluted Canadian waters. Grizzle, Schwedler & Scott (1981) identified papillomas in the oral mucosa and lips of the black bullhead *Ictalurus melas* collected in a chlorinated sewage lagoon. Another interesting example, in that same decade, is in Meyers & Hendricks (1983) with histopathology descriptions of four cancers (lymphoma, cutaneous fibrosarcoma, renal cystadenoma and dermal hemangioma) in three species of fish of the family Salmonidae.

Munday, Su & Harshbarger (1998) performed a histological study in a large sample of *Salmo salar* Southeast Tasmania. Besides low prevalence of parasitic protists (*Kudoa thyrsites* and *Pleistophora* sp.), tests also identified abscesses and neoplastic lesions of lymphoma, melanoma and thyroid tumor.

Lima, Souza, Mesquita, Souza & Chinelli (2002) conducted a radiological and pathological study of bone tumors in various swordfish *Trichiurus lepturus* specimens from Brazilian coast, finding two basic types of osteoma (solid, spongy). Another valuable contribution is the publication of Ramos & Peleteiro (2003), which presents three spontaneous neoplasms in fish Portugal: a leiomyoma originating from the smooth muscle of the intestinal wall of sardine (*Sardina pilchardus*) captured from the wild; fibropapilloma of skin in the upper right commissure of the lid of a player bass *Dicentrarchus labrax* species; multiple skin melanomas of an aquarium carp (*Cyprinus japonicus*). In none of the cases described in this study showed the presence of metastases.

Knüsel, Brandes, Lechleiter & Schmidt-Posthaus (2007) reported two independent cases of spontaneous occurrence of gills tumors in carp *Cyprinus carpio*. Histologically the tumors were composed of blast cells, as they differentiated into cartilage and gill lamellae embedded in abundant.
fibrous connective tissue. Based on these results, it was diagnosed branchial blastoma. In northwestern Iran, Rahmati-holasoo, Hobbenaghi, Tukmechi & Morvaridi (2010) analyzed tumor masses in Oscar *Astronotus ocellatus* jaw. The autopsies showed no masses or internal organ damage. The analysis by light microscopy revealed multiple invasive cell masses in the dermis with neoplastic changes including polymorphism in the core. Specific staining techniques complemented the diagnosis of squamous cell carcinoma.

Table 1 Studies on occurrence of spontaneous tumors in fish species.

Fishes	Types of Cancer	Reference	Publication year
Ictalurus melas	Papillomas.	Grizzle Schwedler & Scott	1981
Oncorhynchus nerka	Dermal fibrosarcoma and thymic lymphoma.	Meyers & Hendricks.	1983
Oncorhynchus tshawytscha	Renal papillary cystadenoma		
Salmo gairdneri	Capillary hemangioma		
Ameiurus nebulosus	Papillomas and hepatic neoplasia.	Spitsbergen & Wolfe.	1995
Salmo salar	Lymphoma, melanoma and thyroid tumor.	Munday, Su & Harshbarger	1998
Platichthys flesus	Liver cancer.	Cachot, Cherel, Galgani & Vincent	2000
Rineloricaria strigilata	Bony plates neoplasms.	Flores-Lopes, Malabarba, Pereira & Silva	2001
Trichiurus lepturus	Osteomas.	Lima, Souza, Mesquita, Souza & Chinelli	2002
Platichthys flesus	Liver cancer.	Koehler & Van Noorden.	2003
Dicentrarchus labrax	Skin fibropapilloma	Ramos & Peleteiro.	2003
Sardina pilchardus	Leiomyoma.		
Cyprinus japonicus	Melanoma.		
Cyprinus carpio	Branchial blastomas.	Knüsel, Brandes, Lechleiter & Schmidt-Posthaus	2007
Astronotus ocellatus	Squamous cell carcinoma.	Rahmati-holasoo, Hobbenaghi, Tukmechi & Morvaridi	2010
Plectropomus leopardus	Melanoma.	Sweet et al.	2012

CHEMICAL POLLUTION AND CANCER

Several publications indicate that water pollution induced pathological changes in fish (Grizzle, Schwedler & Scott, 1981; Cachot, Cherel, Galgani & Vincent, 2000; Flores-Lopes, Malabarba, Pereira & Silva, 2001; Koehler & Van Noorden, 2003; Brown et al., 1973; Bernet, Schmidt, Meier, Burkhardt-Holm & Wahli, 1999; Rochman, Hoh, Kurobe & Teh, 2013). According to Bernet, Schmidt, Meier, Burkhardt-Holm & Wahli (1999), the direct observation of the health of aquatic organisms, the detection of harmful effects on the environment and aquatic organisms, not detected by traditional chemical monitoring; the fish often exposed to contaminants, especially in waters where the dilution rate waste is low and neoplasms are specific lesions, commonly found in polluted areas of bottom fish, revealing an association between the injury and exposure to irritants.

Brown et al. (1973) presented data on the incidence of tumors found in Canada's fish (1.03%) compared with tumors in fish examined from the river basin Fox (4.38%). Differences in the incidence of tumors were more pronounced in the rough fish (*Ictalurus*, Ameiuridae, *Cyprinus*), which are found more often in waters with higher pollution. In this study, the Lake of the Woods, Ontario, Canada, was used as a control region. Conversely, the Fox River water system in Northern Illinois (USA) had already been polluted for many years and were found between the pollutant mercury, lead, arsenic, toluene, crude oil, petrol, benzanthracene, chlorinated hydrocarbons, phosphates and sulfates.

Flores-Lopes, Malabarba, Pereira & Silva (2001) described histological changes in the bony plates of the armored fish *Rineloricaria strigilata*, from Guaiba Lake in southern Brazil. Because of the greater urban and industrial concentration in the area, Guaiba Lake receives pollutant contributions, mainly from industrial and domestic waste dumps. The incidence of neoplasms in bony plates was 8.7%. According to the authors, the occurrence of cancer, with an identical pattern in bony plates of various individuals of *R. strigilala*, is evidence of the presence of pollutants and / or degraded habitats in the Guaiba Lake, and the analysis of spatial and

temporal variations of your frequency of occurrence in this environment is an important source of evaluation and monitoring of this source water quality.

In flounder from *Platichthys flesus* species there is a curious fact. In polluted environments from North Sea, the females of this species have a three times higher incidence of macroscopic hepatic nodules that progress to hepatic carcinoma, compared to males of the same age. Koehler & Van Noorden (2003) demonstrated that sex differences in the induction of detoxification and biotransformation enzymes such as NADPH-CYP450 reductase contribute to the increased incidence of liver cancer induced pollution on *Platichthys flesus* females.

In a recent publication, Rochman, Hoh, Kurobe & Teh (2013) warn about the risks associated with ingestion of plastic debris in aquatic ecosystems, which include their own physical components of the material, its chemical ingredients and environmental chemicals adsorbed. In the experiment, *Oryzias latipes* (japanese medaka) fishes exposed to a mixture of polyethylene with chemical pollutants adsorbed in a marine environment, bioaccumulated these pollutants and suffered from toxicity and liver disease; fish fed with virgin polyethylene fragments also showed signs of stress, though less serious than the first group. In addition, a focus eosinophilic cell change (a tumor precursor) was seen at a fish treatment with virgin plastic and a tumor (hepatocellular adenoma comprising 25% of the liver), was observed in a fish from the plastic-marine treatment.

Several incidents where sentinel species have responded to the effects of chronic exposure to environmental levels of contaminants are discussed in a review LeBlanc & Bain (1997), including the development of tumors in fish, immunosuppression in marine mammals, pseudohermaphroditism in invertebrates, amphibians teratogenicity and aberrations in the sexual development of fish and reptiles. However, there are intriguing situations in which tumors are observed in fish that inhabit reservoirs, ponds and rivers relatively protected, where no evidence of elevated levels of anthropogenic environmental contaminants. This type of occurrence was reported by Spitsbergen & Wolfe (1995) in numerous specimens of brown bullhead catfish (*Ameiurus nebulosus*) from various aquatic environments relatively unpolluted in the State of New York (USA).

ULTRAVIOLET RADIATION AND CANCER

Ultraviolet (UV) radiation, particularly at wavelengths around 260 nm, is strongly absorbed by nitrogenated bases of DNA and can cause a photochemical fusing adjacent pyrimidine bases to form thymine dimers. The DNA of the skin exposed to the sun can acquire hundreds of these dimers during the day that are normally repaired. The repair mechanism, however, can also fail. Thus, failure of repair enzyme that removes those thymine dimers result in abnormal occurrence of cellular death and increased incidence of skin cancer (Rocha, 2013). It has previously been reported that UV radiation in aquatic systems promotes harmful effects on marine and freshwater organisms, being able to penetrate to about 60 m deep in the sea (Haeder, Helbling, Williamson & Worrest, 2011).

Sweet et al. (2012) reported type melanoma skin cancer in wild populations *Plectropomus leopardus* (coral trout or leopard coral trout) inhabiting the region of the Great Barrier Reef, between Australia and Papua New Guinea, directly below the larger hole in the layer of ozone. Of the 136 analyzed fish, approximately 15% of these lesions presented in the form of black spots on the leather (melanosis), some covering almost the whole body of the animal. In the absence of microbial pathogens and due to the great similarities with UV radiation-induced melanomas in mammals, the authors concluded that the most likely cause was environmental exposure to UV radiation.

INFECTIOUS AGENTS AND CANCER

Several types of cancer tend to become more frequent in populations exposed to risk factors, such as exposure to certain infectious agents, chemicals and carcinogenic physical agents in the environment. It is estimated that infectious agents are responsible for a significant percentage of cases of malignancies in the world. Among the biological agents, bacteria and viruses associated with chronic infections have been implicated as well cancer-inducing agents in animals and man (Rocha, 2013).

The literature contains numerous publications in which members of the virus family Herpesviridae, Papillomaviridae, and Retroviridae are associated with tumor development in fish (Coffee, Casey & Bowser, 2013). An interesting example of herpesvirus found associated with tumor development in fish was presented by Hedrick, Groff, Okihiro & McDowell (1990). In this case, the association was with epidermal hyperplasia in mirror carp *Cyprinus carpio*, in captivity in northern California (USA). Papillomas were found mainly in fish caudal regions, including the fins. The characteristics of papillomatous growth and morphology of the

virus-infected cells were found in consistence with the descriptions *Herpesvirus cyprini*, previously known in japanese carp populations.

Peters and Watermann (1979) described the occurrence of three skin papillomas in flatfishes, being structurally distinct injuries and apparently due to different causative agents: papillomas of the dab *Limanda limanda* from the German Bight; papillomas on flatfishes after several years in captivity aquaria; the complex type of papilloma in North Pacific flatfishes. The authors attribute etiology or some viral etiology contribution only in this third papilloma, in North Pacific. This disease is a virus-induced hypertrophy of individual dermal connective tissue cells, which leads to the formation of a raspberry-shaped tumor like swelling in the skin. The structure and the distribution of these complexes papillomas indicate that a papillomavirus is probably involved.

In the review of Coffee, Casey & Bowser (2013), the authors present epidemiological and morphological characteristics of various proliferative diseases in fish that have been associated with one or more retroviruses, such as angelfish Lip fibroma, fibroma Hooknose Cutaneous / Fibrosarcoma, Epidermal Papilloma White Sucker, Muskellunge and Northern Pike Smooth-Type Epidermal Hyperplasia, Atlantic Salmon Epidermal Papillomatosis, Muskellunge and Northern Pike Lymphosarcoma, Chinook Salmon plasmacytoid Leukemia, Yellow Perch Discrete Epidermal Hyperplasia, Atlantic Salmon Swim Bladder Leiomyosarcoma, Walleye Discrete Epidermal Hyperplasia, Walleye Dermal Sarcoma. These proliferative diseases are typically seasonal occurrence, affecting populations of wild fish and farming, and most lesions disappear spontaneously.

The study of swim bladder leiomyosarcoma in Atlantic salmon (*Salmo salar*) presents an interesting example of neoplasm occurring demonstrates is associated with viral infection. An outbreak of neoplastic disease in the swim bladder of *Salmo salar*, with suspected viral etiology was first reported in a Scottish commercial farm marine fish in 1975 (McNight, 1978). In that work, the cancer was investigated, the tumor was classified as leiomyosarcoma and had a prevalence of 4.6% of a population of 500 fish in the second year of life. Tumors were grown to such a degree of persistence, a low mortality was observed. Affected fish showed signs of lethargy, lack of appetite and bleeding on the fins and body surfaces.

Three decades later, Paul et. al. (2006) published an article in the Journal of Virology, presenting new piscine retroviruses in association with an identified leiomyosarcoma surge swim bladder of Atlantic salmon. These researchers reported the cloning, the complete nucleotide sequence and the transcriptional profile of the Atlantic salmon swim bladder sarcoma virus (SSSV).

Bowser et al. (2012) disclosed the occurrence of retroviruses in association with leiomyosarcomas in Atlantic salmon from North America. The finding occurred in the swim bladder of adult salmon, older age (3-4 years) kept in a quarantine facility of North Attleboro National Fish Hatchery (NANFH), Massachusetts, USA. It was pale tan masses, coalescing, smooth, firm, with up to 2 cm in diameter, with expansion on the wall of the swim bladder and reaching the luminal space. Despite its presence in cancer fish, the precise role of retroviruses in the disease process is still not fully elucidated.

Final Considerations

Fish Neoplasms such as those of higher vertebrates can arise in every organ, tissue and cell types. The mutagenesis and carcinogenesis mechanisms appear to be intrinsically linked and often influenced by chemical or physical agents in the environment - environmental carcinogens. On the other hand, a large number of tumors present in fish association with infectious agents, most of these related to retroviruses.

The tumors of the skin, especially the papillomas, are the most commonly affect the fish and the study of these tumors is interesting because it can be associated with water pollution and the presence of carcinogens that presents a danger to all higher vertebrates, and can be used in comparative oncology, serving as a model to help understand changes similar in man.

Referências

Bernet, D.H., Schmidt, H., Meier, M., Burkhardt-Holm, P. & Wahli, T. (1999). Histopatology in fish: proposal for a protocol to assess aquatic pollution. *J. Fish Dis.* 22: 25-34.

Bowser, P.R. , Casey, J.W., Casey, R.N., Quackenbush, S.L. Lofton, L. Coll, J.A. & Cipriano, R.C. (2012). Swimbladder Leiomyosarcoma in Atlantic Salmon (*Salmo salar*) in North America. *J. Wildl. Dis.* 48(3): 795–798.

Brown, E.R., Hazdra, J.J., Keith, L., Greenspan, I., Kwapinski, J.B.G. & Beamer, P. (1973). Frequency of Fish Tumors Found in a Polluted Watershed as Compared to Nonpolluted Canadian Waters. *Cancer Res.* 33: 189-198.

Cachot, J., Cherel, Y., Galgani, F. & Vincent, F. (2000). Evidence of p53 mutation in an early stage of liver cancer in European flounder, *Platichthys flesus* (L.). *Mutat. Res. – Genet. Toxicol. Environ. Mutagen.* 464: 279-287.

Coffee, L.L., Casey, J.W. & Bowser, P.R. (2013). Pathology of Tumors in Fish Associated with Retroviruses: A Review. *Vet. Pathol.* 50(3): 390-402.

Flores-Lopes, F., Malabarba, L.R., Pereira, E.H.L. & Silva, J.F.P. (2001). Alterações histopatológicas em placas ósseas do peixe cascudo *Rineloricaria strigilata* (Hensel) (Teleostei, Loricariidae) e sua frequência no lago Guaíba, Rio Grande do Sul, Brasil. *Rev. Bras. Zool.* 18(3): 699 -709.

Grizzle, J.M., Schwedler, T.E. & Scott, A.L. (1981). Papillomas of black bullheads, *Ictalurus melas* (Rafinesque), living in a chlorinated sewage pond. *J. Fish Dis.* 4(4): 345-351.

Groff, J.M. (2004). Neoplasia in fishes. *Vet. Clin. North. Am. Exot. Anim. Pract.* 7(3): 705-756.

Haeder, D.P., Helbling, E.W., Williamson, C.E. & Worrest, R.C. (2011). Effects of UV radiation on aquatic ecosystems and interactions with climate change. *Photochem. Photobiol. Sci.* 10: 242–260.

Hedrick, R.P., Groff, J.M., Okihiro, M.S. & McDowell, T.S. (1990). Herpesviruses detected in papillomatous skin growths of koi carp (*Cyprinus carpio*). *J. Wildl. Dis.* 26: 578–581.

Knüsel, R., Brandes, K., Lechleiter, S. & Schmidt-Posthaus, H. (2007). Two independent cases of spontaneously occurring branchioblastomas in koi carp (*Cyprinus carpio*).*Vet. Pathol.* 44: 237−239.

Koehler, A. & Van Noorden, C.J.F. (2003). Reduced nicotinamide adenine dinucleotide phosphate and the higher incidence of pollution-induced liver cancer in female flounder. *Environ. Toxicol. Chem.* 22(11): 2703–2710.

LeBlanc, G.A. & Bain, L.J. (1997). Chonic toxicity of environmental contaminants: sentinels and biomarkers. *Environ. Health Perspect.* 105: 65–80.

Lima, F.C., Souza, A.P.M., Mesquita, E.F.M., Souza, G.N. & Chinelli, V.C.J. (2002). Osteomas in cutlass fish, *Trichiurus lepturus* L., from Niteroi, Rio de Janeiro state, Brazil. *J. Fish. Dis.* 25(1): 57-61.

McNight, I.J. (1978). Sarcoma of the swim bladder of Atlantic salmon (*Salmo salar* L.). *Aquaculture*. 13: 55–60.

Meyers, T.R. & Hendricks, J.D. (1983). Histopathology of four spontaneous neoplasms in three species of salmonid fishes. *J. Fish Dis.* 6(6): 481-499.

Munday, B.L., Su, X-Q & Harshbarger, J.C. (1998). A survey of product defects in Tasmanian Atlantic salmon (Salmo salar). *Aquaculture.* 169: 297-302.

Paul, T.A., Quackenbush, S.L., Sutton, C., Casey, R.N. Bowser, P.R. & Casey, J.W. (2006). Identification and characterization of an exogenous retrovirus from Atlantic salmon swim bladder sarcoma. *J. Virol.* 80: 2941–2948.

Peters, N. & Watermann, B. (1979). Three types of skin papillomas of flatfishes and their causes. *Mar. Ecol. Prog. Ser.* 1: 269-276.

Rahmati-holasoo, H., Hobbenaghi, R,, Tukmechi, A. & Morvaridi, A. (2010). The case report on squamous cell carcinoma in Oscar (*Astronotus ocellatus*). *Comp. Clin. Pathol.* 19(4): 421-424.

Ramos, P. & Peleteiro, M.C. (2003). Três casos de neoplasias espontâneas em peixes. *Rev. Port. Ciênc. Vet.* 98 (546): 77-80.

Rocha, C.A.M. (2013). *As Pernas do Caranguejo: Cancer crura*. Belém: Carlos Rocha (Editor).

Rochman, C.M., Hoh, E., Kurobe, T. & Teh, S.J. (2013). Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress. *Nat. Sci. Rep.* 3, Article 3263.

Sinderman, C.J. (1990). Neoplastic Diseases, pp. 173-199. In: *Principal Diseases of Marine Fish and Shellfish*. New York: Academic Press, Inc.

Spitsbergen, J.M. & Wolfe, M.J. (1995). The Riddle of Hepatic Neoplasia in Brown Bullheads from Relatively Unpolluted Waters in New York State. *Toxicol. Pathol.* 23: 716-725.

Sweet, M., Kirkham, N., Bendall, M., Currey, L. Bythell, J. & Heupel, M. (2012). Evidence of Melanoma in Wild Marine Fish Populations. *PLoS ONE* 7(8): e41989.