

Kyphosis in *Barbus pergamonensis* (Cyprinidae-Actinopterygii) from Dalaman Stream Flowing to the Mediterranean Sea

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ABSTRACT

Skeletal deformities are relatively well described in both cultured and wild fish. These diseases are observed in many fish species and occur due to environmental and genetic factors. It negatively affects the biological performance and commercial value of fish. For this reason, this study attempts to quantify and identify kyphosis in the natural populations of *Barbus pergamonensis* Karaman, 1971 collected from Dalaman Stream (Göhlhisar/Burdur) and tends to find a possible relationship between these anomalies and several types of pollutants present in the environment. The current study found that the environmental pollutants may represent a potential risk to induce kyphosis in the natural populations of *B. pergamonensis*.

Keywords: Bergama barbel, pollution, skeletal deformities, pharmaceutical, pesticide

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INTRODUCTION

It is widely known that many anatomical diseases occur in fish due to the environmental and genetic factors (Jawad & Ibrahim, 2018). In recent decades, fishery biologists have become more interested in skeletal deformity diseases of wild and reared fish (Kužir et al., 2015). Many types of deformities (Afonso et al., 2000; Sato, 2006; Jawad et al., 2017; Jawad & Ibrahim, 2018) have been reported from the variety of fish species living in the different habitats (Iwasaki et al., 2018). It was reported that these deformities affect mostly the ribs, fins, cranium and vertebral column of fish (De La Cruz-Aguero & Perezgomez-Alvarez, 2001). These diseases have negative effects on the biological performance of fish as well as decreasing the commercial value (Raja et al., 2016; Majeed et al., 2018). In wild fish, skeletal deformities that occur in any life stage of fish can cause them difficulties such as defending their territory (Sato, 2006; Majeed et al., 2018), competing for a mate (Sato, 2006), and decreasing production performance (Noble et al., 2012). In farmed fish,

these injuries can affect the organism by reducing growth (Hansen et al., 2010), limiting their feeding ability (López-Olmeda et al., 2012; Okamura et al., 2018), increasing the infection (Janakiram et al., 2018) and mortality rate (Jara et al., 2017). Moreover, these negative effects of the skeletal deformities inevitably cause reasonable economic loss in the fish farms (Boglione, 2013a; Yıldırım et al., 2014).

There are a variety of reasons for the occurrence of the skeletal deformities in fish. These are mostly known as scoliosis, spondylolisthesis, lordosis (dorsal curvature, v shape), and kyphosis (ventral curvature, ^ shape (Yıldırım et al., 2014; Jawad et al., 2017). Although there are many reports of lordosis (Afonso et al., 2000; Fjellidal et al., 2009; Gorman et al., 2010), the rate of occurrence of kyphosis is less common than lordosis (Jawad et al., 2017). Like lordosis, kyphosis can also appear in both pre-haemal and haemal positions (Boglione et al., 2013a). All these deformities are linked to genetic and epigenetic factors (Nguyen et al., 2016). Some environmental parameters, such as salinity, sud-

den changes of water temperature and turbulence, oxygen content, organic compounds, radiation, heavy metals, industrial effluents, stress, food deficiency, parasitism (Dionísio et al., 2012; Fridman et al., 2012; Kolarevic et al., 2013) and pollution are among the most considerable epigenetic factors (Sfakianakis et al., 2006). In addition, pollution is considered as a useful index for evaluation of the frequency of deformities in fish (Boglione et al., 2013b).

Bergama barbel, *Barbus pergamonensis* Karaman, 1971, belonging the family Cyprinidae is a freshwater fish species distributed in the fresh waters of Turkey and Greece (Kottelat & Freyhof, 2007). The distribution areas of this fish in Turkey are reported to be from the Aegean drainages, the Bakır and Menderes Rivers and the drainages of Muğla (Kottelat & Freyhof, 2007). *Barbus pergamonensis* was reported as being heavily affected by agricultural activities, domestic and industrial pollution from the industry (Freyhof & Kottelat, 2018). Therefore, the aim of this study is to identify and quantify the skeletal deformities of *B. pergamonensis* collected from Dalaman Stream (Göhlisar/Burdur/Turkey) as well as finding a possible relationship between the deformities of this species and several types of pollutants that occur in this region.

MATERIAL AND METHODS

Specimen collection

The individuals of *B. pergamonensis* ranging from 3.5 cm to 20 cm were collected from the Dalaman Stream from three different stations [1-) 37°13'36.53"N, 29°32'57.66"E; 2-) 37°14'39.39"N, 29°31'44.33"E; 3-) 37°15'10.14"N, 29°30'09.29"E, Göhlisar/Burdur/ Turkey] between June 2015 and June 2016. In total, 54 individuals were caught after seven sampling events carried out using electrofishing. All fish samples used in the current study were anaesthetized with MS-222. Fish samples were fixed in 4% formaldehyde solution and then deposited in 70% ethanol in the fish collection of the Ichthyology Laboratory at the Department of Biology, Burdur Mehmet Akif Ersoy University. Specimens were measured to the nearest 0.1 cm for standard length (SL) and weighed to the nearest 0.001 g for body weight.

X-ray treatment

In order to determine skeletal deformities of specimens, the fresh fish samples were individually x-rayed using a Fujifilm FCR Prima T®-Tokyo, Japan at Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Clinics. Specimens were x-rayed at the exposure time 100 kVp, 30 mAs, 3 second from the lateral positions of each sample to demonstrate the exact nature of the deformities.

Non-metallic inorganic parameters, dissolved metals, pharmaceutical and pesticide analysis in water samples

Many chemical parameters of Dalaman Stream were also analysed in order to determine the relationship between the pollution and kyphosis occurring in fish in the studied area. These includes non-metallic parameters, dissolved metals, pharmaceutical compounds from variety of drug groups and pesticides. Non-metallic inorganic parameters and dissolved metals shown in Table 1 were analysed in ALS Laboratory in Czechia. All meth-

ods used for the analysis of non-metallic inorganic parameters and dissolved metals/ major cations were shown in Table 1. Solid phase extraction (SPE) and liquid chromatography tandem mass spectrometry (LC-MS/MS) were used for the pesticide and pharmaceutical analyses.

For pharmaceutical analysis, 1 L surface water sample was filtered using glass fibre filters (GF/F, Whatman). After filtration, samples were adjusted to pH 2 with H₂SO₄ (98wt %). Then, solid phase extraction was conducted for pharmaceutical analysis using the method of Guzel et al. (2018). First, internal standard diazepam-d5 were added to the water samples. Then, Oasis HLB cartridges (500 mg, 6 cc) were conditioned with 5 ml dichloromethane (DCM), 5 ml methyl tert-butyl ether (MTBE), 5 ml methanol (MeOH) and 5 ml ultrapure water. Water samples were then added to the SPE cartridge at a flow rate of 15 ml/min and it was washed with 5 ml ultrapure water. Next, sample loading cartridges were dried under vacuum for 1 hour. Eluent was taken with 5 ml of MeOH and 5 ml 10:90 (v/v) MeOH/MTBE. The eluent was dried with nitrogen and then dissolved with 1 ml MeOH. Extracted samples were analysed by using LC/MS-MS.

Solid phase extraction for pesticide analysis was made by using modified method of Hladik et al. (2008). Internal standard dimethoate-d6 were added to the 1 L water samples. Oasis HLB cartridges (500 mg, 6 cc) were conditioned with 10 mL ethyl acetate, 10 mL MeOH and 5 mL of ultrapure water. Water samples added to the SPE cartridge at a flow rate of 10 ml/min. Then, samples were loaded to cartridges and it was dried under vacuum for 1 hour. Eluent was taken with 12 ml of ethyl acetate. The eluent was dried with nitrogen and then dissolved with 1 ml MeOH. Extracted samples were analysed by using LC/MS-MS.

Instrumental analysis was performed with Shimadzu CBM-20A ultra flow liquid chromatography, Shimadzu SIL-20A/HT auto sampler system and Shimadzu 8040 mass spectrometry. Pharmaceutical compounds were separated using pentafluorophenylpropyl (PFPP) column (Allure 50x2.150 mm, 5µm, Restek, Bellefonte, PA, USA) and pesticide compounds were separated by using Shim-pack Column, FC-ODS 150X2.0 mm, Kyoto, Japan. Pharmaceutical and pesticide compounds were detected with multiple reactions monitoring (MRM) mode. Total run time for pharmaceutical method and pesticide method were 18 and 20 minutes respectively.

RESULTS AND DISCUSSION

The skeletal deformities of the fish samples

The spinal curvature (kyphosis) was found in two (3.7%; A and B in Figures 1, 2) out of 54 adult fish sampled from three different stations in Dalaman Stream. The Standard length (SL) of these two samples were measured as 12.3 cm (A) and 12.1 cm (B) respectively (Fig. 1 and 2). In fish A, the vertebral anomaly was found between 17 and 47 vertebrae. Whereas the spinal anomaly was involved between 16 and 32 vertebrae in fish B. Although kyphosis in the caudal vertebrae of both samples were diagnosed, no external lesions were observed on these body of deformed specimens.

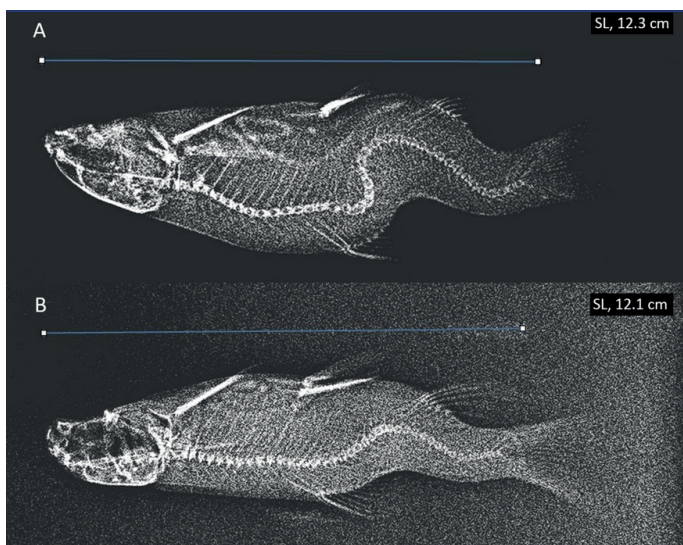


Figure 1. Radiological images of two *Barbus pergamonensis* samples diagnosed with kyphosis; (A) 12.3 cm SL; (B) 12.1 cm SL.



Figure 2. Photography images of two *Barbus pergamonensis* samples diagnosed with kyphosis; (A) 12.3 cm SL; (B) 12.1 cm SL.

Determining the chemicals, pharmaceutical and pesticide in water samples

Non-metallic inorganic parameters and dissolved metals are given in Table 1.

In the current study, 10 out of 14 non-metallic parameters and 11 out of 26 dissolved metals were found higher than normal values (see Table 1). 156 pharmaceutical compounds from variety of drug groups including painkillers (analgesics, anti-inflammatories and antipyretics), antibiotics, and antibacterials, cardiovascu-

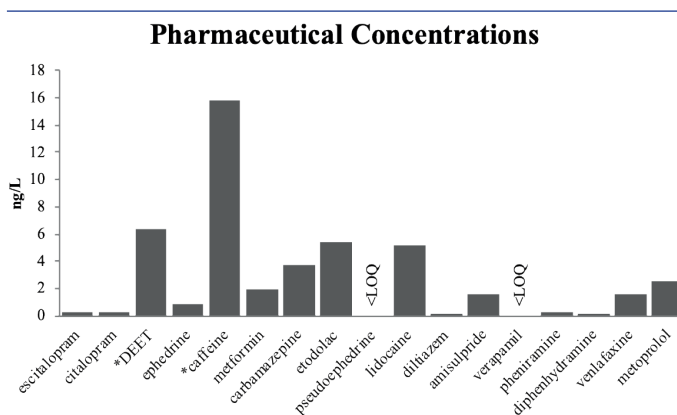


Figure 3. Pharmaceutical concentrations of water samples (DEET and caffeine concentrations reduced 10 times, <LOQ: Lower than quantitation limit).

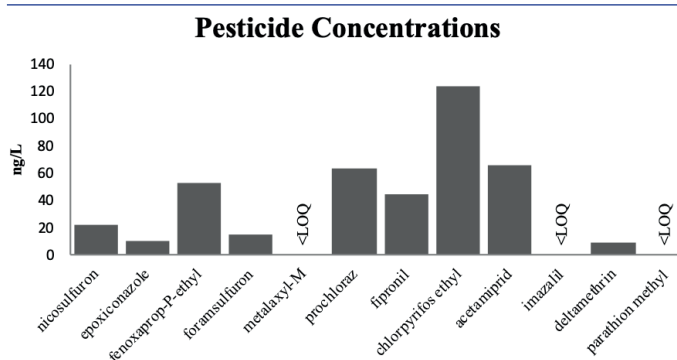


Figure 4. Pesticide concentrations of water samples (<LOQ: Lower than quantitation limit).

lar drugs (β - blockers), hipolipidemics, central nervous system drugs, stimulants and illicit drugs were investigated in water samples. 17 of these pharmaceuticals (escitalopram, citalopram, DEET, ephedrine, caffeine, metformin, carbamazepine, etodolac, pseudoephedrine, lidocaine, diltiazem, amisulpride, verapamil, pheniramine, diphenhydramine, venlafaxine, metoprolol) were detected in ng/L values (Fig. 3).

In addition, 144 pesticides that include herbicides, insecticides, acaricides, fungicides were analysed and 12 of them (nicosulfuron, epoxiconazole, fenoxaprop-P-ethyl, foramsulfuron, metalaxyl-M, prochloraz, fipronil, chlorpyrifos ethyl, acetamiprid, imazalil, deltamethrin, parathion methyl) were detected in the water samples (Fig. 4).

The results of non-metallic inorganic parameters were given in Table 1. Ammonia and ammonium ions as N, ammonia and ammonium ions as NH_4 and nitrite as N were found lower than method detection limits. According to Turkey Surface Water Quality Regulations, nitrates and total kjeldahl nitrogen as N concentrations were found as 2nd grade water quality (less

Table 1. Non-metallic and dissolved metals inorganic parameters

No	Parameter	Limit of reporting (mg/l) (LOQ)	Method	Result (mg/l)
Nonmetallic Inorganic Parameters				
1	Ammonia and ammonium ions as N	0.040	W-NH4-SPC	<LOQ
2	Ammonia and ammonium ions as NH ₄	0.050	W-NH4-SPC	<LOQ
3	Chloride	1.00	W-CL-IC	568
4	Inorganic Nitrogen as N	0.500	W-NING-SPC	0.876
5	Nitrates	0.27	W-NO3-SPC	3.88
6	Nitrite + Nitrate as N	0.060	W-NNO-SPC	0.876
7	Nitrites	0.0050	W-NO2-SPC	<LOQ
8	Organic Nitrogen as N	0.50	W-NORG-SPC	0.70
9	Orthophosphate	0.040	W-PO4O-SPC	0.049
10	Total Kjeldahl Nitrogen as N	0.50	W-NKJ-PHO	0.70
11	Total Nitrogen as N	1.0	W-NTOT-CC	1.6
12	Nitrate as N	0.060	W-NO3-SPC	0.876
13	Nitrite as N	0.0020	W-NO2-SPC	<LOQ
14	Orthophosphate as P	0.010	W-PO4O-SPC	0.016
Dissolved Metals / Major Cations				
1	Aluminium	0.010	W-METAXFL1	<LOQ
2	Antimony	0.010	W-METAXFL1	<LOQ
3	Arsenic	0.0050	W-METAXFL1	<LOQ
4	Barium	0.00050	W-METAXFL1	0.0243
5	Beryllium	0.00020	W-METAXFL1	<LOQ
6	Boron	0.010	W-METAXFL1	0.016
7	Cadmium	0.00040	W-METAXFL1	<LOQ
8	Calcium	0.0050	W-METAXFL1	42.6
9	Chromium	0.0010	W-METAXFL1	0.0019
10	Cobalt	0.0020	W-METAXFL1	<LOQ
11	Copper	0.0010	W-METAXFL1	<LOQ
12	Iron	0.0020	W-METAXFL1	<LOQ
13	Lead	0.0050	W-METAXFL1	<LOQ
14	Lithium	0.0010	W-METAXFL1	0.0044
15	Magnesium	0.0030	W-METAXFL1	31.9
16	Manganese	0.00050	W-METAXFL1	<LOQ
17	Molybdenum	0.0020	W-METAXFL1	<LOQ
18	Nickel	0.0020	W-METAXFL1	0.0039
19	Phosphorus	0.010	W-METAXFL1	0.025
20	Potassium	0.015	W-METAXFL1	1.43
21	Selenium	0.010	W-METAXFL1	<LOQ
22	Silver	0.0010	W-METAXFL1	<LOQ
23	Sodium	0.030	W-METAXFL1	6.66
24	Thallium	0.010	W-METAXFL1	<LOQ
25	Vanadium	0.0010	W-METAXFL1	0.0022
26	Zinc	0.0020	W-METAXFL1	<LOQ

contaminated water) (Anonymous, 2016). And total nitrogen as N and orthophosphate as P concentrations were found as 1st grade water quality (high quality water). But for the ECE (Economic Commission for Europe) Standard Statistical Classification of Surface Freshwater Quality for the Maintenance of

Aquatic Life total nitrogen as N was found as class IV (UNECE, 1994)

Twenty-six metals and major cations were analysed in water samples (Table 1). Most of the detected metals and major cations

were lower than the quantification limits. According to ECE Standard Statistical Classification of Surface Freshwater Quality for the Maintenance of Aquatic Life only chromium was classified as class II, the other quantified metals were classified as class I.

To the best of our knowledge, a few biological studies (Gaygusuz et al., 2013; Erk'akan et al., 2014) have been conducted on this species (Freyhof & Kottelat, 2018). Kyphosis which is a skeletal deformity of fish was studied by many researchers (Hansen et al., 2010; Boglione et al., 2013a; Hayes et al., 2013; Jawad & Ibrahim, 2018). In addition, there are some reports on kyphosis in cyprinid species from different regions (Bogutskaya et al., 2011). The adverse effects of skeletal deformities on the fish health and economy were also reported in detail in the previous studies (Yildirim et al., 2014; Nguyen et al., 2016). In recent years, although the number of studies on wild and cultured fish health have been increasing in Turkey, there are limited studies on the skeletal deformities of fish such as cultured sharpnose seabream *Diplodus puntazzo* (Yildirim et al., 2014) and silverside *Atherina boyeri* (Jawad et al., 2017). In the current study, kyphosis was detected in the *Barbus pergamonensis* population of Dalaman Stream. It is the first study that kyphosis was detected in the wild population of this fish. In addition, there is no kyphosis report from other fish species from Dalaman Stream.

Previous reports discussed the reasons of skeletal deformities which are genetic and environmental factors (Quigley, 1995; Divanach et al., 1996; Divanach et al., 1997; Sfakianakis et al. 2006; Harris et al., 2014). Micropollutants in the aquatic habitats is one of the major issues worldwide (Eggen et al., 2014). These micropollutants have based on the anthropogenic effects, such as pharmaceuticals, pesticides, personal care products and industrial chemicals (Luo et al., 2014). It is known that all these micropollutants have toxic effects on aquatic species (Morasch et al., 2010; Pochodylo & Helbling, 2015; Minguez et al., 2016). Aquatic communities can be dramatically affected by the cocktail of low concentrations of micropollutants (Morash et al., 2010). The effects of cocktail micropollutants can be much higher than the effect of a single micropollutant.

In the current study, the relationship between *B. pergamonensis* and anthropogenic factors occurred in the habitat of this species were emphasised. The pollution effecting the aquatic environment has also been increasing in Turkey with the rising industrialisation and other reasons (Deniz Innal Pers. Obs.). In the study area of the current research, these reasons can be referred for many reasons. First, the studied area has been used as a cultivated area for the production of carrot, sugar beet, maize, green beans and wheat. In order to maintain the agricultural activities in the region, the local people and industries are using variety of chemicals (Deniz Innal Pers. Obs.). Another reason for the pollution can be the treatment facilities in the area. It was observed that the discharge of a treatment facility has been performing via Dalaman Stream. Furthermore, it was detected that the waste from the marble factory and other mining facilities have been carried to this aquatic system. The road construction on the highway between Gölhisar and Çavdır changed the structure of Dalaman Stream. Moreover, the construction of a reservoir on the system for the aim of irrigation has also adverse effect on this prob-

lem. The last two reasons caused the decrease of water flow of Dalaman Stream.

In this study, pollution parameters in the studied area have been detected in detail. In total, 17 pharmaceutical and 12 pesticide concentrations were found over the threshold values. Caffeine as a pharmaceutical concentration was detected at the highest concentration (158.51 ng / L). Caffeine concentration is newly used as an anthropogenic effect indicator. Because caffeine is used only by humans (Sauvé et al., 2012). Similarly, in some studies, the concentration of caffeine was found to be in the highest concentrations (Yu & Cao, 2016; Guzel et al., 2018). When considered the caffeine level found in the current study, it can be supposed that the anthropogenic effect plays a main role for this reason. Whereas the highest concentrated pesticide was detected as chlorpyrifos ethyl (123.40 ng/L). One of the most frequently detected compounds was the specific metabolite of chlorpyrifos in wastewaters which indirectly and/or directly reaching to surface waters (Gracia-Lor et al., 2017). These pesticides are sold from different companies which is mainly used to eradicate several pests including worms and insects. Pesticide usage is highly toxic for the non-target organisms such as fish (Ogunfowokan, et al., 2012; Qu, et al., 2011). Ecotoxicological studies show that such pollutants can cause undesirable effects on fish even at very low concentrations (Faggiano et al., 2010; Ogunfowokan et al., 2012). These kind of detrimental effects of kyphosis can result in death of fish species through impaired metabolism. In the study, the detected pharmaceutical, pesticide and inorganic compound concentrations were low but there may be negative effects on the living organisms when all the substances are present at the same time in the water.

CONCLUSIONS

Environmental pollutants may represent a potential risk to induce spinal deformities in natural populations of *B. pergamonensis*. As a result, the increasing pollution and the detrimental chemical contents releasing from the different systems to Dalaman Stream should be firmly decreased in order to prevent the health problems of the aquatic organism living in the region. Furthermore, the emergent skeletal deformities of *B. pergamonensis* and potentially other fish species living in the region seem to be a serious problem for the future of aquatic organisms in Dalaman Stream. Consequently, the further studies should focus on the prevention of these deformities and the main reasons of these health problems in detail.

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Ethics Committee Approval: This study was carried out in accordance with animal welfare and the ethics of trial. All procedures were performed in accordance Law on Veterinary and Medical Activities and National Animal Welfare Act. Therefore ethical approval was not required.

Conflict of Interests: Authors declare that there is no conflict of interest.

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