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Study of Heavy Metals Pollution and Vitellogenin Levels in Brown Trout (*Salmo trutta trutta*) Wild Fish Populations

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Abstract: The objectives of this research were, first, to determine the concentrations of certain heavy metals in the edible tissue of wild brown trout (*Salmo trutta trutta*) from two different rivers located in the Abruzzi region (Italy), and then, to investigate the levels of variation in vitellogenin (VTG) associated with the presence of metalloestrogens. VTG is an effective indicator for endocrine disturbance, and an increase in the vitellogenin levels in male fish is widely employed as a biomarker of estrogenic contamination in the aquatic environment. The muscles of the trout were analyzed for As, Cd, Co, Cr, Ni, Pb, Al, and Zn using an inductively coupled plasma-mass spectrometer (ICP-MS), and Hg was measured using a direct mercury analyzer (DMA-80). The calculated values of the condition factor confirmed a healthy status for this species, indicating that the aquatic habit in both rivers is suitable for brown trout life. No significant difference in the concentrations of each metal were reported between the trout from the two rivers, and no significant difference for VTG levels were found between male and female fish. It is interesting to note the high concentrations of Al/Zn, while the Pb, Cd, and Hg concentrations in all of the samples were lower than those established by the European Commission.

Keywords: pollution; aquatic environment; metals toxicity

1. Introduction

Contamination from heavy metals has occurred in rivers worldwide, especially during the last decade, with river water and sediments being a major environmental focus [1]. Concerns over heavy metals are due to their profusion, persistence, and toxicity, as reported in several studies [2–4]. The pollution of aquatic environments is closely associated with human activities, and lakes and rivers can receive heavy metals as a result of discharges of untreated or inadequately treated wastewater from domestic, industrial, and agricultural sources, as well as by natural processes that can contribute to metal contamination.

Both natural and anthropogenic activities are largely liable for the heavy metal occurrence in the environment [5–8].

Heavy metals can be classified in two main groups, as essential and potentially toxic. Iron (Fe), manganese (Mn), cobalt (Co), nickel (Ni), copper (Cu), and zinc (Zn) are believed to be essential, as they form a cofactor for many enzymes, interact in metabolic activities, and are considered to be nutritionally essential for humans. The toxic heavy metals of arsenic (As), cadmium (Cd), lead (Pb), and mercury (Hg) do not show any nutritional or beneficial effects on human health and can have harmful effects, even at low concentrations when ingested over a long period [9]. In recent years, there has been an increase in articles showing that some heavy metals could influence the endocrine system, and Cd, Hg, As, Pb, Ni, Mn, and Zn have been classified as endocrine-disrupting chemicals (EDCs) or metalloestrogens [10,11]. These heavy metals exert estrogenic effects through the alteration of the gene expression and/or modifying the activity of estrogenic receptors. Their presence in terrestrial and aquatic environments is well documented [12–15].

Fish populations are repeatedly exposed to heavy metal contamination from different food sources and water conditions, and an understanding of toxicant uptake and responses in fish may therefore have a high ecological relevance. They are also an important link between the environment and human populations through fisheries and consumption by local and other markets [16].

Fish are frequently used in biomonitoring programs as they offer several specific advantages for describing the natural characteristics of aquatic systems and in assessing biological and biochemical responses to environmental contaminants [17,18]. Thus, they are recognized as bioindicators of chemical pollution [16]. Amongst the fish that are used for biomonitoring aquatic ecosystems, those of the genus Salmo, in particular various species of trout, are commonly employed to monitor loads of organic compounds and heavy metals [19–25].

Brown trout (*Salmo trutta trutta*) is a fish species native to rivers of the Abruzzi region and is the dominant species in most wild rivers, particularly in the Gran Sasso-Monti della Laga National Park. This park is one of the largest in Italy, and its biodiversity is among Europe's richest.

In order to assess exposure to or the effects of environmental pollutants on aquatic environments or ecosystems, several bioassays based on sensitive biomarkers have been developed and VTG has been demonstrated to be a consistent biomarker to evaluate the estrogenic pollution [17,26].

This study fits into a local biomonitoring plan program carried out from the National Park of Gran Sasso e Monti della Laga office to confirm the wild fish health status living in a protected area, apparently unpolluted. This investigation was conducted to measure the heavy metal contamination in wild brown trout from two different rivers located in the Gran Sasso-Monti della Laga National Park, Abruzzi region (Italy). Furthermore, to assess the potential adverse effects on the endocrine system from low-level environmental exposures to metals, we investigated the VTG pattern in fish. Finally, fish health was assessed using biometric indices, condition factor (K), and hepatosomatic index (HSI), representing integrative indicators of fish condition.

2. Materials and Methods

2.1. Sampling and Samples Preparation

Adult brown trout (n = 34) were collected via angling in two different rivers, the Ruzzo and Mavone, located in the Gran Sasso-Monti della Laga National Park (Figure 1), Abruzzi region (Italy), during April 2018, after the breeding season. All of the fish were killed by a concussion blow to the head, and the total length and total weight were measured. Blood (1–1.5 mL) was immediately taken from the tail veins of each trout and was stored in commercially available no anticoagulant tubes. Fish were kept at refrigeration temperature and brought to the laboratory, where a standard necropsy was performed. The sex of all of the fish was recorded, as well as the state of the health and the presence of any alteration of normal secondary sex characteristics that could be an indicator of exposure to EDs. The livers and kidneys were removed, and the livers were weighed to determine the HSI. The HSI was calculated as the ratio of liver mass to body mass. The K was calculated as K = 100 * W/L 3, where W = body weight (g) and L = length (cm) [17].

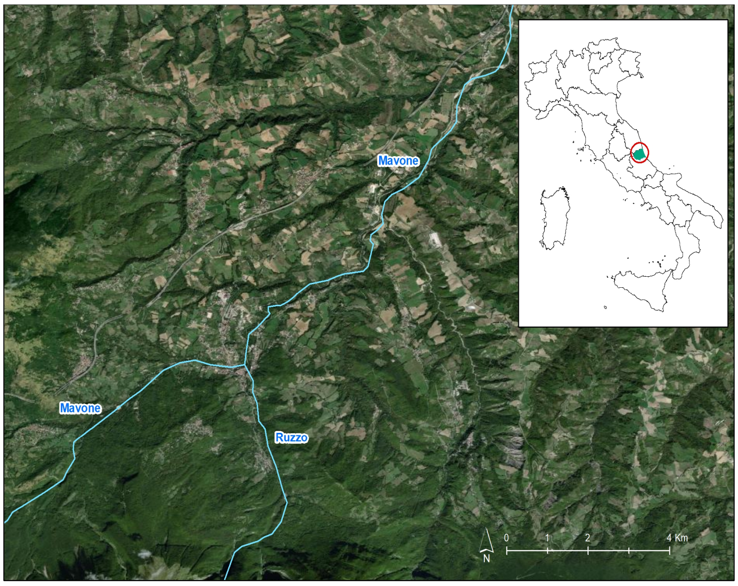

Figure 1. Rivers and locations of the sampling sites.

2.2. Vitellogenin Analysis

The serum of each trout was stored at −80 °C before determination of the total serum VTG concentrations. The VTG levels were measured by species-specific Enzyme Linked Immuno Sorbent Assays (ELISA) developed for trout (My BioSource; San Diego, CA, USA), as previously described by Zezza et al., 2020 [17].

2.3. Chemical Analysis

All of the samples were carefully dissected using clean equipment to separate the edible portion, a fillet of the dorsal muscle, from the bone, and were homogenized and stored at −20 °C before analysis. The first step of the heavy metal (As, Cd, Co, Cr, Hg, Al, Ni, Pb, and Zn) content determination was a microwave digestion process, carried out in a microwave digestion lab station (Ethos 1, Milestone, Shelton, CT, USA). In a teflon vessel, 7 mL of HNO_3 (70% v/v) and 1.5 mL of H_2O_2 (30% v/v) were added to each aliquote of sample (1.0–1.5 g). Secondly, an inductively coupled plasma-mass spectrometer (ICP-MS Xseries II, Thermo Scientific, Bremen, Germany) was used to determine the levels of trace metals, as previously described [27].

Determination of the total mercury was carried out using a DMA-80. Through the steps of thermal decomposition, catalytic reduction, amalgamation, desorption, and atomic absorption spectroscopy, this system ensured a direct analysis of Hg with no pre-treatment required and no waste production [28]. Furthermore, during each analytical session Certified Reference Materials (Oyster Tissue, 1566b, from the National Institute of Standard and Technology), blank reagents and standard solutions were processed to verify the performances of the methods. Recovered concentrations of the certified material were within 10% of the certified values, and the limit of quantification (LOQ) for both techniques was 0.001 mg/Kg.

2.4. Statistical Analyses

All comparisons of the vitellogenin concentrations in male and female fish from the two rivers, as well as the heavy metal concentrations, were performed through a non-parametric Mann–Whitney test.

3. Results and Discussion

3.1. Biometric Parameters and VTG Concentrations

The weight, length, HSI, K, and concentrations of vitellogenin in the trout coming from the Mavone and Ruzzo rivers are shown in Table 1.

Table 1. Mean values and standard deviation of weight, length, hepatosomatic index (HSI), condition factor (K), and variation in vitellogenin (VTG) concentration in male and female trout.

River	Sex	Weight (g)	Length (cm)	HSI	K	VTG (ng/mL)
Mavone	F	162.73 ± 99.33	24.59 ± 3.31	1.06 ± 0.35	1.24 ± 0.14	92.63 ± 58.99
	M	258.44 ± 362.04	26.00 ± 8.86	0.87 ± 0.31	1.23 ± 0.10	84.49 ± 64.21
Ruzzo	F	182.13 ± 122.83	25.38 ± 4.98	0.98 ± 0.19	1.12 ± 0.19	88.23 ± 72.43
	M	149.00 ± 44.69	24.00 ± 2.00	1.01 ± 0.14	1.17 ± 0.07	59.93 ± 38.73

Comparing both the weight and length, the biometric parameters showed no significant differences ($p > 0.05$) between the male and female trout from both rivers. Positive correlations were also found for trout length and weight in both rivers (Tables 2 and 3).

Table 2. Correlations among different parameters in the trout from Mavone river.

	Weight	Total Length	HSI	K
Rank-Order Correlation	**correlations**	**correlations**	**correlations**	**correlations**
Weight	1.000			
Total length	0.980 *	1.000		
HIS	0.068	0.073	1.000	
K	−0.354	−0.449 *	0.008	1.000

* = $p < 0.05$.

Table 3. Correlations among different parameters in the trout from Ruzzo river.

	Weight	Total Length	HSI	K
Rank-Order Correlation	**Correlations**	**correlations**	**correlations**	**correlations**
Weight	1.000			
Total length	0.935 *	1.000		
HSI	0.042	0.110	1.000	
K	0.482	0.236	0.038	1.000

* = $p < 0.05$.

The trout sampled in this study all reported a K > 1, except for one sample that reported a value below 1. The K of fish species is a very important parameter for understanding fish biology and pathology. Many factors affect the growth condition of fish, including reproductive cycles and availability of food, as well as habitat and environmental factors [29], and K is a parameter that can be used as a good indicator of water quality or the general health of fish populations. In the present study, the K values suggest a state of wellbeing for this species, indicating that both rivers are a nutrient-rich environment with favorable conditions for brown trout.

The results of the VTG levels are reported in Table 1. No significant differences in VTG levels ($p > 0.05$) were found when comparing male and female trout from the Mavone and Ruzzo rivers. Of particular interest are the VTG levels found in males from both rivers. Only one male reported a minimum value of 5.6 ng/mL, while the others showed concentrations from 26.1 ng/mL to 187.6 ng/mL (mean value of 74.67 ng/mL). These values could be interpreted as a sign of an estrogen-induced effect, and in this case, the VTG represents a biomarker of EDs exposure. The VTG levels in females ranged from 5 ng/mL to a maximum value of 202.6 ng/mL (mean value of 90.77 ng/mL), but the presence of high levels of VTG in females is a normal condition, and the highest levels could be an effective index of reproductive development. The VTG levels were not correlated with heavy metal concentrations, suggesting additional sources of contamination with estrogenic endocrine-disrupting chemicals.

3.2. Heavy Metals Concentrations

The results of the current study confirmed the presence of all of the heavy metals being investigated in the trout from the two rivers. The descriptive statistics (range, mean, and standard deviation) of the nine metals measured in the trout during the sampling period and from the two rivers are shown in Table 4. During the study period, the mean concentrations of each of the heavy metals did not show any significant variation between the two rivers ($p > 0.05$).

Table 4. Mean values and standard deviation (SD) of heavy metal concentrations in trout from the Mavone and Ruzzo rivers. All of the concentrations are expressed as mg/kg wet weight.

Rivers	Al	Cr	As	Cd	Pb	Hg	Co	Ni	Zn
Mavone	0.39 ± 0.32	0.03 ± 0.02	0.09 ± 0.02	0.01 ± 0	0.01 ± 0	0.03 ± 0.04	0.04 ± 0.03	0.04 ± 0.02	16.26 ± 5.67
Ruzzo	1.72 ± 3.4	0.02 ± 0.01	0.11 ± 0.04	0.01 ± 0	0.01 ± 0	0.05 ± 0.02	0.03 ± 0.02	0.04 ± 0.02	12.40 ± 7.61

Heavy metal concentrations in the trout, independent of the sample's origin, followed the order Zn > Al > As > Ni > Co > Hg > Cr > Cd > Pb.

Considering that the maximum levels established by the European Commission (EC) for Cd, Pb, and Hg in fish for human consumption are 0.05, 0.3, and 0.5 mg/kg of wet weight, respectively, the results for all of samples were lower than the legislated limits [30].

Among the investigated heavy metals, Zn and Al had the highest concentrations in the trout samples, whereas the Cd and Pb concentrations were much lower. Zn is an essential metal for animals and humans because of its important role in biological systems, as it is necessary for the function of many metalloenzymes [31]. The average Zn levels reported in the trout muscle in the present study are higher than those found in farmed rainbow trout from several countries [31–33]. Atmospheric fallout is considered to be the main source of Zn pollution in the environment. However, upstream industrial and municipal wastewater discharges and the use of galvanized metals and metal surfaces also contribute to this pollution. Amongst the non-essential metals, Al was the most abundant metal in the analyzed samples. The presence of Zn and Al could have several origins, namely: the industrial plants present along both rivers that produce aluminum frames, the runoff water coming from the roofing drainage systems that are often made of metallic materials, or guardrails and construction materials such as plumbing and stormwater drainpipes. Furthermore, it should be considered that galvanized metals have a variety of applications in the modern community, but they have also a large potential for contributing heavy metals to runoff during their life cycle. To facilitate stormwater drainage, metal culverts were previously installed along the Mavone and Ruzzo rivers, and, although it is known that corrosion affects the internal surface of these metal culverts, it is difficult to determine whether this corrosion caused the significant elevation in Zn/Al concentrations reported in this study.

Al is considered, along with Cd, to be one of the most potent metalloestrogens. It interferes with most physical and cellular processes. It can interfere with cell growth regulatory processes through many pathways, including altering gene expression [34]. Fish exposure to Al causes a pathological alteration in cardiovascular, respiratory ion-regulatory, changes in hemoglobin concentration, and reduction in fish growth besides endocrine disruption [35–37].

As was the second non-essential metal broadly present in the fish, but at lower levels compared with those reported in other studies [31–33]. For As, no limits have been set, but the US Environmental Protection Agency has considered an upper limit of 1.3 mg/kg of wet weight [38]. There is suggestive evidence from in vivo studies of its estrogenic activity, but we have also to consider that the proportion of inorganic As in fish is very low, below 1%, and the presence of organic As in fish poses less health problems than inorganic arsenic.

Cd is well recognized as an ED, and its interactions with estrogen receptors are the best characterized of all of the heavy metals, and it is implicated in the development of several types of cancer. The Cd concentrations found in the trout in this study were very low and would not cause concern. The same situation occurred for Pb levels. In the United States, as well in European countries, due to legislative restrictions, the large reduction in Pb emissions from transportation sources have resulted in a reduction of Pb levels in terrestrial and aquatic ecosystems. The data reported in the present study support the trend of decreasing Pb levels in aquatic organisms.

Among the essential metals, Co and Ni were present in all of the trout samples, but at very low concentrations. Both metals are considered essential trace elements for animals, but exposure to excess levels of Ni and Co may result in adverse effects, causing neurotoxicity, hepatotoxicity, nephrotoxicity, gene toxicity, reproductive toxicity, and increased risk of cancer [39,40].

Cr, which is considered an essential trace element, was also detected in all of the samples at low concentrations, but can also show aquatic toxicity depending on the oxidation state. Two forms of Cr may be present in the water environment; the trivalent which is a more stable state in oxidation, and hexavalent, which is more toxic and can induce alterations at biochemical, histologic, genetic, and immunologic levels [41].

Hg does not play any metabolic function but can be harmful for humans, even at low concentrations, when ingested over a long time period. The analyzed samples for trout in this study showed lower concentrations compared with the minimum values reported in the same species [42] in Norway, and ten times lower than the maximum levels established by the European Commission, indicating that Hg can be considered to be not harmful in the rivers considered in this study.

4. Conclusions

This study provides information on the VTG levels and the concentrations of heavy metals and trace elements present in wild brown trout living in the Gran Sasso-Monti della Laga National Park, a reserve of natural lands and a source of biodiversity. A positive correlation was reported for the trout length and weight in both rivers, while no difference was found with reference to the biometric parameters.

The concentrations of Cd, Pb, and Hg found in the muscles of the trout did not exceed the legal limits for fish consumption, suggesting that the rivers inside the park are not subjected to a specific source of contamination for these heavy metals. More interesting and troubling is the presence of higher concentrations of Al and Zn, whose levels should be investigated more accurately with further analyses, because, at present, no data exist on their presence in water, sediments, and potential trout prey, and this could be the first step to discover the suspected sources of contamination.

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