Pug-Headedness Anomaly in a Wild and Isolated Population of Native Mediterranean Trout *Salmo trutta* L., 1758 Complex (Osteichthyes: Salmonidae)

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Abstract: Skeletal anomalies are commonplace among farmed fish. The pug-headedness anomaly is an osteological condition that results in the deformation of the maxilla, pre-maxilla, and infraorbital bones. Here, we report the first record of pug-headedness in an isolated population of the critically endangered native Mediterranean trout *Salmo trutta* L., 1758 complex from Sardinia, Italy. Fin clips were collected for the molecular analyses (D-loop, *LDH-C1** locus. and 11 microsatellites). A jaw index (JI) was used to classify jaw deformities. Ratios between the values of morphometric measurements of the head and body length were calculated and plotted against values of body length to identify the ratios that best discriminated between malformed and normal trout. Haplotypes belonging to the AD lineage and the genotype *LDH-C1*100/100* were observed in all samples, suggesting high genetic integrity of the population. The analysis of 11 microsatellites revealed that observed heterozygosity was similar to the expected one, suggesting the absence of inbreeding or outbreeding depression. The frequency of occurrence of pug-headedness was 12.5% (two out of 16). One specimen had a strongly blunted forehead and an abnormally short upper jaw, while another had a slightly anomaly asymmetrical jaw. Although sample size was limited, variation in environmental factors during larval development seemed to be the most likely factors to trigger the deformities.

Keywords: small isolated population; Mediterranean native trout; morphological deformities

1. Introduction

Skeletal anomalies are commonplace characteristic in farmed fish all over the world [1–3]. In contrast, naturally originated malformations show a smaller incidence in wild fish populations due to the decreased viability of the abnormal fish in natural habitats [4,5]. The extent of the skeletal deformities in fish species has been reported to affect different anatomical body parts, such as the vertebral column, fins, and skull [1,6,7]. In particular, skull malformations involve mainly the splanchnocranium, hyoid arch, and gill cover [1,3].

Among these deformities, the pug-headedness anomaly is an osteological condition that results in the deformation of the maxilla, pre-maxilla, infraorbital bones, and ethmoid region. This condition determines bulging eyeballs, acutely steep foreheads, and incomplete closure of the mouth due to

projection of the lower jaw [8,9]. Pug-headedness can lead to starvation and rising mortality, due to the jaw deformity, especially during the larval stages [10]. Several causes have been suggested to explain the pug-headedness anomaly, including low genetic variability and epigenetic factors, such as embryonic development disorders and aberrations induced by environmental factors variation [11,12]. Among these, traumatic shock caused by daily variations in temperature, light cycles, salinity, and dissolved oxygen concentration during the early development seem to be the most important factors [2,9]. A higher incidence of pug-headedness has been found in polluted waters [12,13]. The pug-headedness deformity is widely documented in different captive fish species of both marine and freshwater habitats [14–18], but it is rare among adults in wild populations [19,20]. Among natural populations, jaw deformities are mostly restricted to different families of marine fish species, such as Moronidae [21], Pomacanthidae [22], Rachycentridae [23], Sparidae [24], Epinephelidae, and Cichlidae [20,25].

The pug-headedness malformation has also been reported in wild salmonid populations in only a few known cases [26]. To the best of our knowledge, this is the first documented occurrence of pug-headedness in a native Mediterranean trout population. Although the taxonomy of the Mediterranean trout has not yet been resolved [27], the species is listed with the name of *Salmo cettii* Rafinesque, 1810, and is considered to be critically endangered by the International Union for Conservation of Nature [28].

In particular, in this paper we report (1) a brief morphological characterisation of the head deformities, (2) the genetic characterisation of the population using nuclear (*LDH-C1** and 11 microsatellites) and mitochondrial (entire mtDNA control region, ~1 Kb) markers, and (3) a comparison of malformed and normal specimens.

2. Materials and Methods

During the monitoring programme for the compilation of the official Fish Inventory of the Sardinia Region [29], among 116 native trout analysed in 10 rivers, two malformed specimens were found only in the Furittu Stream in June 2017 and June 2019 (39°26.248' N, 9°22.036' E) (Figure 1). The stream is located in the south-eastern sector of Sardinia, running for about 10 km to merge with the River S'Acqua Callenti to form the Flumendosa River. The Furittu stream is located in the mountainous area of Monte Genis, which is an area with very low anthropogenic pressure, is hard to reach and is not subjected to wind coming from polluted areas. The Furittu stream watershed area is 21 km^2, and the land use is largely natural and composed of forests, Mediterranean shrubland and very little pasture land. Bi-seasonal climatic features, with hot arid summers and a rainy autumn/winter season, determine a periodic of hydrographic isolation (up to four months per year) for the upper part of the stream. The stream is 0.5–4.5 m wide and 0.10–2.00 m deep with an average slope of 3.8% throughout its whole length. It has the typical geomorphologic characteristics of a Mediterranean mountain stream, with a complex and fragmented mesohabitat dominated by pools (72%), riffles (20%) and small cascades (8%). The streambed consists of rocks, boulders, gravel, rubble, and woody debris, with riparian vegetation composed of trees adjacent to the stream characterised by holm oak (*Quercus ilex*) and oleander (*Nerium oleander*).

A total of 16 trout were captured in the upper region of the stream using low-frequency, pulsed DC electrofishing and stored in cool, aerated water. All specimens were measured for total weight (TW, g) and total length (TL, cm), placed in a narrow transparent tank filled with water, and photographed from the left side. From each fish, a small fin clip was removed and conserved in 95% ethanol until DNA extraction. After processing, the fish were placed in large containers and released in the stream. Estimates of the total number of fish in a 100 m section of stream and trout estimated densities (N fish/m^2) were obtained using two-pass depletion method [30]. The Sardinian specimens were compared with another trout from Neia River (42°38.31' N, 13°14.96' E; Tronto basin of Italian Apennine area) that showed similar jaw anomalies.
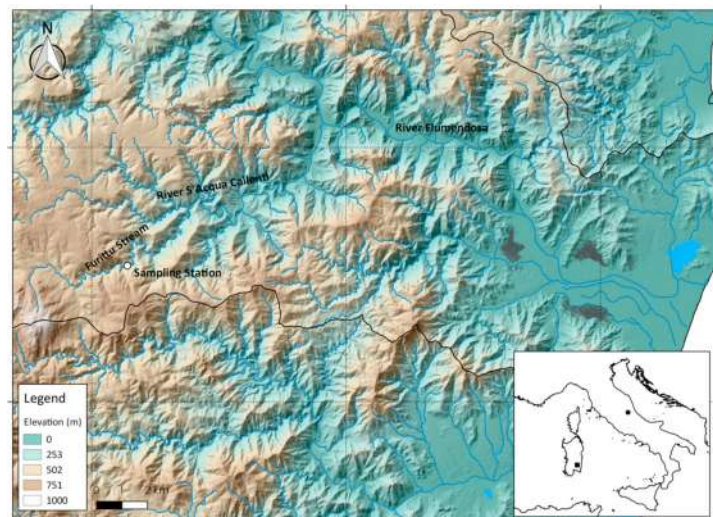
Figure 1. Geographic position of the sampling station in Furittu Stream, south-eastern Sardinia (Italy). Lower right panel: location of Furittu stream (■) and Neia River (●).

Genetic analyses were performed by using both mitochondrial (D-loop) and nuclear markers (*LDH-C1** and 11 microsatellite loci). The entire mitochondrial control region (D-loop) was PCR amplified and sequenced using the primers 28RIBa [31] and HN20 [32]. PCR amplifications were performed in 20 μL of reaction mixtures (approximately 80 ng of template DNA, 0.15 units MyTaq polymerase (Bioline GmbH, Luckenwalde, Germany), 1X MyTaq Buffer (Bioline GmbH, Luckenwalde, Germany) and 5 pmol of each primer) for 30 cycles (95 °C, 45 s; 53 °C, 30 s; 72 °C 90 s). Cycling was preceded by a 3 min denaturing step at 95 °C and followed by a 7 min final extension at 72 °C. The mtDNA sequences were aligned using the computer program ClustalW [33] and compared with reference *S. trutta* CR sequences from GenBank using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The specimens were genotyped at the *LDH-C1** gene, coding the LDH enzyme [34].

A 440 bp segment of the *LDH-C1** nuclear locus was PCR-amplified with the primers Ldhxon3F and Ldhxon4R [34] and digested with a BseLI restriction enzyme. *LDH-C1** allows the discrimination of north-western European populations, characterised by the *90 allele, from native Mediterranean population (*100 allele), and hybrids that present both alleles [34]. PCR amplifications were performed in 25 μL of reaction mixtures (approximately 200 ng of template DNA, 0.15 units MyTaq polymerase (Bioline GmbH, Luckenwalde, Germany), 1× MyTaq Buffer (Bioline GmbH, Luckenwalde, Germany)and 5 pmol of each primer) for 30 cycles (95 °C, 60 s; 64 °C, 60 s; 72 °C 60 s). Cycling was preceded by a 5-min denaturation step at 95 °C and followed by a 10-min final extension at 72 °C. Amplicon digestion was performed using BseLI restriction enzyme (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturers' protocol and visualised on 2% agarose gel. Following [35], eleven non-coding microsatellite loci were labelled with fluorescent dye and multiplexed in two separate reactions. PCR amplifications were performed in 15 μL of reaction mixtures containing approximately 80 ng of template DNA, 0.4 unit MyTaq polymerase (Bioline GmbH, Luckenwalde, Germany), 1X MyTaq Buffer (Bioline GmbH, Luckenwalde, Germany), and 2.5 pmol of each primer. A Touchdown protocol was used to optimise the amplification. PCR amplicons were electrophoresed using an ABI-PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) [36]. ARLEQUIN 3.5.1.3 [37] was used to calculate the observed (Ho) and expected (He) heterozygosity and deviations from the Hardy–Weinberg equilibrium, while the inbreeding coefficient (F_{IS}) was estimated in FSTAT [38]. The software COLONY software [39] was used to determine family structure in the Furittu stream population (length of run = 3, level of precision likelihood = high). The pair likelihood score (PLS) /full likelihood (FL) combined (= FPLS) algorithm was selected to establish only full-sibs listings. Finally, individual admixture coefficient (*q*) were estimated for Furittu population using the software STRUCTURE [40] A domestic sample from two hatcheries was used for comparison. The domestic

ancestry in Furittu population was calculated performing a run assuming a K = 2 (i.e., domestic vs. native) adopting a burn-in of 1,000,000 iterations, followed by 500,000 iterations leaving the other parameters as default.

To verify jaw deformities, we used a modified version of the jaw index (JI) [41] (Figure 2a). We categorised the specimens to different degrees of upper jaw index (UJI) as follow: values above 1 were categorised as normal jaw deformity (NJ), from 0.99 to 0.90 as mildly jaw-deformed (MJD) and below 0.89 severely jaw-deformed (SJD).
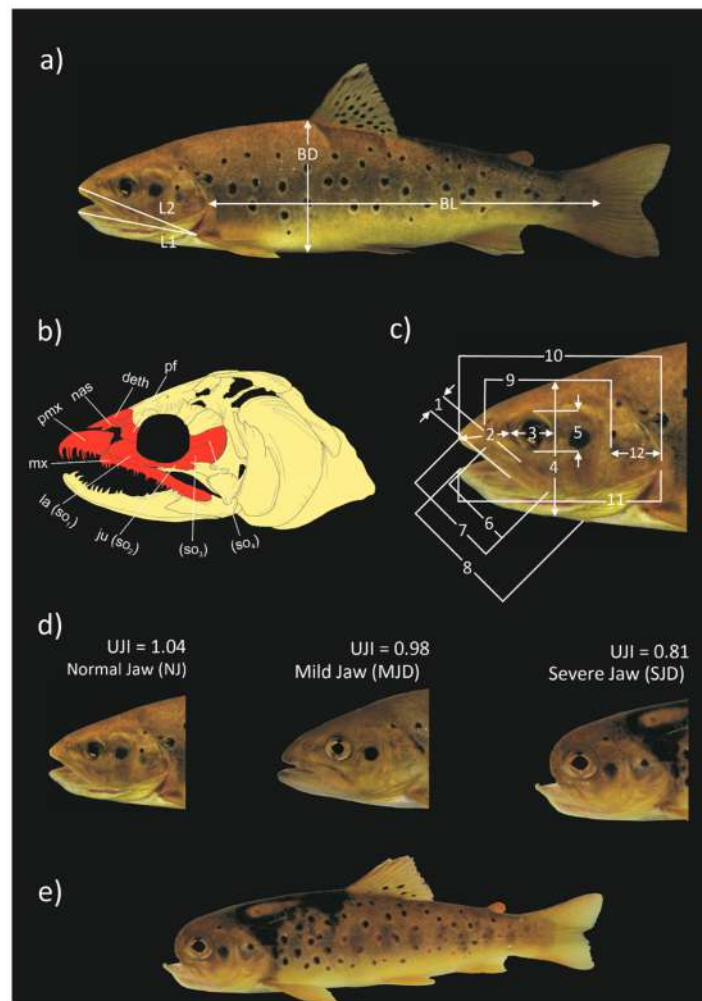
Figure 2. Specimens from Furittu stream. (**a**) Locations of L1 and L2 measurements used to calculate the upper jaw index (UJI), body length (BL) and body depth (BD), (**b**) skull of trout and in red bones involved in the pughead deformity (pmx = premaxilla; mx = maxilla; deth = dermethmoid; nas = nasal; pf = prefrontal; la = lacrimal (suborbital 1); ju = jugal (suborbital 2); so3 = suborbital 3; so4 = suborbital 4), (**c**) measurements taken for morphometric comparison (Table S1, Supplementary Materials), (**d**) examples of normal jaw (NJ), middle jaw deformity (MJD) and severe jaw deformity (SJD). Pughead specimen of Mediterranean trout (**e**).

Since the affected species often show other skull bones deformities (Figure 2b), morphological variables of the head [42] (Figure 2c) were collected from each trout after setting the scale factor using TPSDIG2 v2.31 [43]. Ratios between values of morphometric measurements of the head and body length (BL) were calculated and plotted against values of BL, to identify the ratios that best discriminated between the malformed (MJD, SJD) and normal trout (NJ). To estimate the changes in nutritional conditions among normal and deformed specimens, we used the residuals analysis in linear regression between LT and TW after logarithmic transformation of the data [17].

3. Results

In the Furittu stream, a total of 16 adult trout were examined (12.5–25.5 cm and 24.9–183.1 g) (Table S1, Supplementary Materials). The estimates of the total number of fish in the 100 m of section varied between 15 ± 4 and 18 ± 0.5 in June 2017 and 2018, respectively. Estimated densities were relatively low in both campaigns and ranged from 0.06 ind/m^2 to 0.09 ind/m^2.

The incidence rate of jaw deformities of Furittu stream trout was 12.5% (2 of 16 native trout, 95% confidence interval [CI] = 1.6–38.6%). One specimen, which showed a strongly blunted forehead and an abnormally short upper jaw (UJI = 0.81) was categorised as SJD, while the another one manifested a slightly asymmetrical jaw (UJI = 0.98, categorised as MJD). The remaining specimens showed values of UJI above 1 and were considered normal (NJ) (Figure 2d). The Neia trout with an UJI of 0.95 was categorised as MJD (Table S1, Supplementary Materials).

The mtDNA analysis showed that all Sardinian trout shared the same haplotype (AD-Tyrr7) belonging to the AD lineage (Adriatic) (sensu [44]), which was observed for the first time in this population. The new sequence was deposited in GenBank under accession number MT503201. In all samples, we observed the genotype *LDH-C1*100/100* fixed in the Mediterranean native population. The trout population from the Neia River (central Apennine) showed high frequency of the non-native allele *LDH-C1* 90* (0.77) and the presence of AT non-native mitochondrial lineage with a frequency of 0.23. The 11 microsatellites showed higher levels of observed heterozygosity in the Neia population (0.80) compared to the Furittu stream population (0.50) (Table 1). In Furittu stream, the mean *q* values were close to 1 with a very narrow CI (mean = 0.99, CI= 0.97–1).

Table 1. Mitochondrial lineage and *LDH-C1** observed frequencies, observed (H$_O$) and expected (H$_E$) heterozygosity and inbreeding coefficient (F$_{IS}$) estimated from 11 microsatellite loci in the Furittu and Neia populations. AD (Adriatic), ME (Mediterranean) and AT (Atlantic) lineages of *Salmo trutta*. Standard deviation (s.d.).

River	Sample Size	MtDNA			LDH-C1*		Microsatellite		F$_{IS}$
		AD	ME	AT	*90	*100	H$_O$ (s.d.)	H$_E$ (s.d.)	
Furittu	16	1.00				1.00	0.50 (0.26)	0.46 (0.14)	−0.089
Neia	22	0.45	0.32	0.23	0.77	0.23	0.80 (0.18)	0.83 (0.20)	0.051

No significant departure from the Hardy–Weinberg equilibrium was found in the studied populations. Analysis of family structure suggested that the Furittu stream population was composed of eight families of generally one or two individuals (Table S2, Supplementary Materials). The two abnormal specimens were found to be unrelated.

In the Furittu stream, one trout showed anomalies in skull measurements typical of pug-headedness osteological malformation (SJD), with a shortened neurocranium and upper jaw (Figure 2e), while another specimen (MJD) had slightly shorter upper jaw and greater head. In particular, the pug-headed trout (SJD) showed a greater depth of the head (DH) (Figure 3a), while the specimen with the middle jaw deformity (MJD) had the longest head (LHL) (Figure 3b). Surprisingly, the SJD individual also showed a longer LHL compared to normal specimens (NJ) (Figure 3b). The longer LHL found in the specimen affected by the pug-headedness (SJD) was thought to be a consequence of a slightly greater operculum length (LO) (Figure 3c). In the SJD specimens, the snout was almost absent due to the curving of the ethmoid region (deth, nas), and maxillary bones (mx) (Figure 2b). These malformations were confirmed by the smallest snout (LS) in SJD, while the MJD trout showed the longest LS (Figure 3d). The anomalies of infraorbital bones (pf, la, ju, so3, so4) in SJD trout determined larger and bulbed eyeballs compared to MJD and NJ specimens (Figure 3e,f) despite the fact that the ratio between HO and VO didn't show differences from the regular circular shape of infraorbital bones. As shown in the MJD specimen, the deformed trout from the Neia River exhibited a slightly prominent lower jaw in comparison to normal trout from Sardinia (Figure 3b).
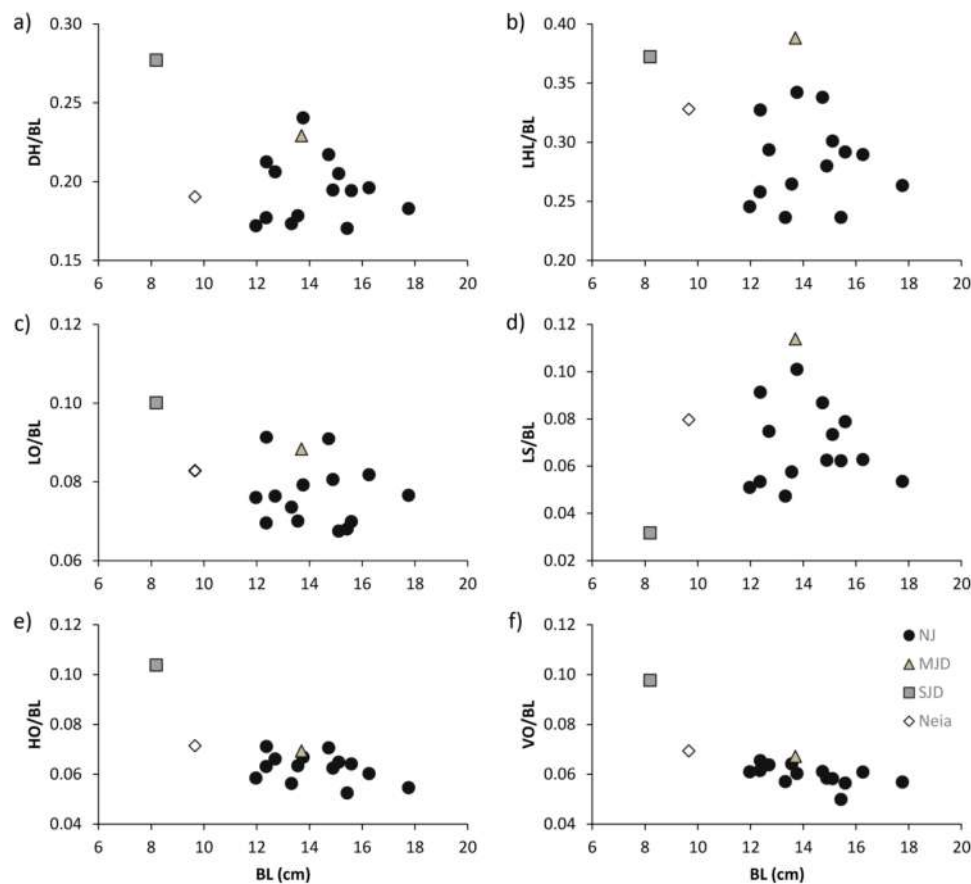
Figure 3. Relationship between body length (BL) and the ratios among values of head depth (DH) (**a**), lower head length (LHL) (**b**), operculum length (LO) (**c**), snout length (SL) (**d**), horizontal orbital length (HO) (**e**) and vertical orbital length (VO) (BL) for specimens with normal jaw deformity (NJ), middle jaw deformity (MJD) and severe jaw deformity (SJD) from Furittu stream and one specimen from Neia river (**f**).

Despite this deformity, the residuals generated by linear regression (LT/TW) revealed no relevant differences between normal and deformed specimens, indicating that the deformed trout were robust and healthy (Table S1, Supplementary Materials).

4. Discussion

The pug-headedness deformity in adults wild brown trout was first described in 1929 by the American ichthyologist Eugene Wills Gudgers [26]. Here, we present the first scientific report of head skeletal deformities in a wild population of Mediterranean native trout and one of the few cases reported for adult specimens of the genera *Salmo*.

In the Furittu stream, the head malformations were observed in two specimens out of 16 Mediterranean trout (12.5% of occurrence) captured during two sampling campaigns conducted in June 2017 and June 2018. One specimen (SJD) showed the typical malformation of pug-headness, with the antero-posterior compression of the ethmoid region and upper jaw, while the other specimen (MJD) had a slightly shorter upper jaw and a longer head. The Neia trout (MJD) also exhibited an asymmetry between the lower and upper jaw.

In unpolluted natural habitats, the occurence of pug-headedness in other genera is generally less than 1% [45]. The occurrence of head deformities in the Furittu Stream exceeded the rates observed in the polluted habitats (from 0.5% to 3%) [13], while it was comparable with that of hatchery-reared fish

larvae, which have a much greater frequency of skeletal skull abnormalities, including pug-headedness, compared to their wild populations [1,3,46].

The periodic hydrographic isolation that occurs in the downstream part of the river could represent an impassable barrier that prevents the connectivity with domestic trout that have been stocked in the Flumendosa River since the beginning of the 1960s. In fact, the trout population of the Furittu stream is immune by genetic introgression with non-native traits, as detected by the genetic analysis. Before this analysis, in Sardinia, ancestral trout populations (AD lineage, *sensu* [44]) were found in two watersheds of southern Sardinia (Cixerri and Pula basins) [47–50]. Although these populations exist in small headwater habitats isolated by artificial or natural barriers, morphological analysis revealed no presence of trout with head deformations [49]. Furthermore, in a comparative study in morphological characterisation of Corsican and Sardinian trout, it was highlighted that relatively larger heads are present in native trout populations compared to Atlantic and *S. macrostigma* specimens, while no head skeletal aberrations were found [51].

Even though many studies have reported a wide range of factors that trigger such deformities, the exact cause may be difficult to determine. Possible sources of such an aberration could include a wide range of epigenetic factors, such as temperature and oxygen fluctuation during egg incubation [9,52], prenatal stress of mature females induced by environmental changes [53], influences of diet composition on larval phases [54,55], and environmental pollution [13,14]. However, endogamy in small populations is indicated as the most likely factor to trigger the deformity [17]. Low genetic variability, as a result of inbreeding depression, is known to occur in fragmented and small salmonid populations [56,57]. Salmonid populations with morphological deformities, due to the loss of genetic variation and inbreeding depression, show a lower survival rate compared to the normal conspecific populations [58,59]. In this context, similar morphological deformities were detected in two watersheds showing significant differences in levels of genetic introgression with non-native traits and genetic diversity. High levels of non-native variants and genetic variability ($H_O = 0.80$) characterise the Neia River population. Contrarily, lower genetic variability ($H_O = 0.44$) was observed in the Furittu stream population, similarly to what has been observed in other isolated populations on Sardinia [49] and elsewhere (e.g., [35,36]). Additional analysis performed with the colony seems to suggest that the abnormal fish are unrelated. Moreover, in both populations, the observed heterozygosity (H_O; Furittu = 0.49; Neia = 0.80) was similar to the expected heterozygosity (H_E; Furittu = 0.45; Neia = 0.83), suggesting the absence of inbreeding or outbreeding depression. Ultimately, though the small number of markers used does not allow us to exclude that the pugheaded malformation may have a genetic basis, our preliminary analyses suggest inbreeding or outbreeding depression is not likely to be the cause of the observed deformities.

However, we can also exclude the presence of pollutants and pathogens as possible causative factors of this malformation. In fact, the Furittu stream is a well-preserved river with very low anthropogenic pressure. Although sample size was limited, unfavourable abiotic conditions, such as variations in environmental factors such as hypoxia, solar radiation, and temperature during larval development seem to be the most likely factors to trigger the observed deformities. In this context, the Mediterranean streams are often subject to prolonged droughts, which reduce and fragment the trout habitat, with a possible increase in water temperature. The results of this study indicate a need for investigation into the causes and control of head malformation in Mediterranean trout population. Practical conservation management measures should include long-term monitoring programmes in order to estimate the population size and abundance, ecological requirements, and protection of stream habitats. The dissemination of information regarding native trout conservation status and the involvement and education of local people and regional authorities are also crucial for conservation of the species.

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