

## SHORT COMMUNICATION

### Anthropogenic threats to fish of interest in aquaculture: gonad intersex in a wild population of thinlip grey mullet *Liza ramada* (Risso, 1827) from a polluted estuary in central Italy

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Mulletts (Osteichthyes, Mugilidae) are euryhaline marine fish, inhabiting coastal and inland brackish waters around the world. In the Mediterranean sea, six species, belonging to four genera, are present. *Oedalechilus labeo* (Cuvier, 1829) inhabits only marine coastal waters, while the other species [*Mugil cephalus* Linnaeus, 1758, *Liza ramada* (Risso, 1827), *Liza saliens* (Risso, 1810), *Liza aurata* (Risso, 1810), and *Chelon labrosus* (Risso, 1827)] usually migrate from the sea into inland transitional waters, such as rivers and coastal lagoons, where they represent a target for aquaculture and fisheries. In many Mediterranean countries, mullets represent the principal yield of lagoons fisheries and extensive aquaculture, e.g. the 'valliculture' of the Northern Italy and the 'howash' in Egypt (Ardizzone, Cataudella & Rossi 1988; Macfadyen, Nasr Allah, Reheem Kenawy, Ahmed, Hebicha, Diab, Hussein, Abouzied & El Naggar 2011). However, between 2006 and 2010, the mullet production in Mediterranean inland aquaculture decreased from 250 000 to 120 000 tons (FAO - General Fisheries Commission for the Mediterranean 2013).

Within Mediterranean mullets, the thinlip grey mullet *L. ramada* is one of the most abundant species in inland waters. This species, which shows a higher tolerance to low salinities with respect to

other mullet species (Cardona 2006), migrates as a juvenile in large schools from the sea into the estuarine zone of rivers, ascending far up water courses. Over there it finds more favourable trophic conditions, and hence it spends here most of the growing phase, before returning to sea for spawning (Pombo, Elliot & Rebelo 2005). The ovarian maturation is uniform and synchronous, mating occurs as a group event, and the spawning period ranges between September and January, depending on the geographical areas (Ben-Tuvia 1986). Thus, as for other inshore migratory fish species (Geeraerts & Belpaire 2010; Pujolar, Marino, Milan, Coppe, Maes, Capoccioni, Ciccotti, Bervoets, Covaci, Belpaire, Cramb, Patarnello, Bargelloni, Bortoluzzi & Zane 2012), the self-sustainability of local populations of thinlip grey mullet relies upon its annual reproductive success at sea, which in turn is influenced by the number and the physiological state (i.e. normal gonad maturation) of adults returning to the sea after having spent the rest of the year in inland waters.

Due to its capability to tolerate freshwaters, thinlip grey mullet is also one of the target species of fishery in large Mediterranean lentic ecosystems, such as natural lakes and reservoirs, where restocking of wild fingerlings from the estuarine

zone of rivers may be carried out (FAO - General Fisheries Commission for the Mediterranean 2013). The annual reproductive success of wild populations can be therefore considered as a major critical event both for conservation of local populations and the availability of juveniles for capture-based aquaculture.

Mugilids are gonochoristic species, and hermaphroditism and intersex are considered as uncommon among individuals living in unpolluted environments (McDonough, Roumillat & Wenner 2005). However, recent studies have reported the occurrence of gonadal disorders, such as intersex condition, within Mugilid species, such as flathead grey mullet *M. cephalus* (Ferreira, Antunes, Gil, Vale & Reis-Henriques 2004; Aoki, Nagae, Takao, Hara, Lee, Yeo, Lim, Park & Soyano 2010) and thicklip grey mullet *C. labrosus* (Puy-Azurmendi, Ortiz-Zarragoitia, Villagrasa, Kuster, Aragón, Atienza, Puchades, Maquieira, Domínguez, López de Alda, Fernandes, Porte, Bayona, Barceló & Cajaraville 2013; Bizarro, Ros, Vallejo, Prieto, Etxebarria, Cajaraville & Ortiz-Zarragoitia 2014), and have attributed the phenomenon to the exposure to chemical contaminants, in particular endocrine disrupting chemicals/compounds (EDCs) (Soyano, Aoki, Itashiki, Park, Nagae, Takao, Lee, Yeo & Zhong 2010; Puy-Azurmendi *et al.* 2013; Bizarro *et al.* 2014). Among the contaminants of human origin, EDCs have an effect analogous to sex steroids and can cause a wide variety of impairments in fish (WHO/IPCS, Damstra, Barlow, Bergman, Kavlock & Van der Kraak 2002), including reduction in gonad weight and volume, disorders in gonadal maturation, gonadic atresia and especially an increased occurrence of intersex (Scholz & Klüver 2009).

As far as thinlip grey mullet is concerned, only Bayhan and Acarli (2006) described a single hermaphrodite specimen from a polluted lagoon in Turkey, on the basis of macroscopic gonad observation.

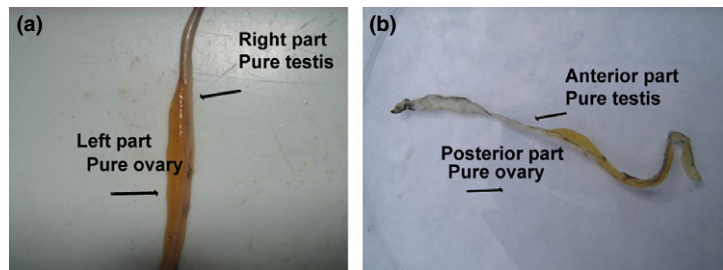
In this scenario, an investigation on the reproductive status and gonadal alterations in a natural population of thinlip grey mullet living in the lowest reaches of the Tiber River, between the southern area of the city of Rome and the estuarine zone (central Italy, Tyrrhenian Sea) was carried out during three sampling campaigns carried out in summer 2010, winter 2010/2011, and spring 2011. The sampling periods corresponded, respectively, to the periods of gonad maturation,

spawning, and recrudescence of the species. The river stretch (Lat: 41.812329°; Lon: 12.419330°–Lat: 41.752351°; Lon: 12.275301°, 225 km long) is characterized by the presence of the effluent discharges of a large urban sewage treatment plant and by high levels of contamination, due to civil and industrial drains (Minissi, Caccese, Passafiume, Grella, Ciccotti & Rizzoni 1998; Mancini, Caimi, Ciardullo, Zeiner, Bottoni, Tancioni, Cataudella & Caroli 2005a; Mancini, Formichetti, D'Angelo, Pierdominici, Sorace, Bottoni, Iaconelli, Ferrari, Tancioni & Rossi 2005b; Mattei, Cataudella, Mancini, Tancioni & Migliore 2006; Patrolecco, Capri, De Angelis, Pagnotta, Polesello & Valsecchi 2006).

Thinlip grey mullets were collected by gill nets (mesh size 40 mm). Adult fish with total length greater than 30 cm were randomly included in the study and immediately euthanized using an overdose of eugenol (330 ppm, Sigma-AldrichCo, LLC., Milano, Italy) to avoid any pain to fishes. Fish sampling and handling were done according to the Italian regulations and authorized by the Institutional Animal Care and Use Committee of the University of Rome 'Tor Vergata'.

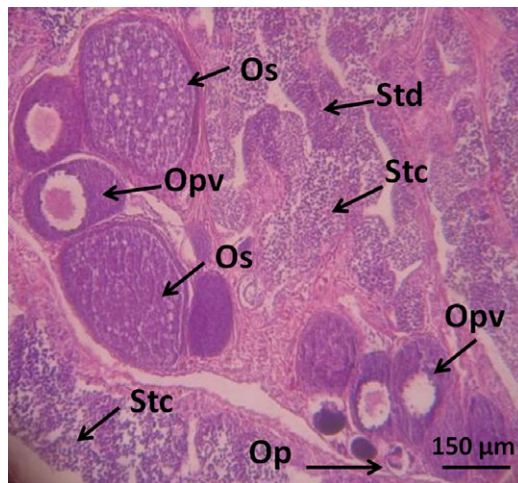
During the sex determination, carried out at anatomical level on a total of 156 thinlip grey mullets, as many as seven mullets showed compartmentalized gonads, as shown in Fig. 1. Histological analysis was carried out on 8–10 mm cross-sections of the central portion of each left gonad of all the 156 sampled mullets, after fixation for 24–48 h in Bouin's fluid, paraffin embedding and haematoxylin and eosin staining of 5 µm sections. Generally, five histological slides from each gonad were examined under a light microscope (Wild Leitz GMBH, Wetzlar, Germany). At the end of observation, as many as 24 mullets (15.4%, Table 1) showed intersex gonads, according to the classification of Hecker, Murphy, Coady, Villeneuve, Jones, Carr, Solomon, Smith, Van Der Kraak, Gross, Du Preez, Kendall and Giesy (2006). In the seven externally compartmentalized gonads, the male and female ovarian cells were concentrated in different and well-defined regions, while the other 17 intersex gonads were identified as testis containing a mixture of oocytes at primary and secondary development stages (testicular oocytes) randomly dispersed in the testicular tissue (Fig. 2). When apparently normal male and female gonads were examined, only poorly defined stages of testicular and ovary development were observed,

**Figure 1** Intersex gonads in thinlip grey mullet from the lowest stretch of Tiber river (central Italy): note the well defined left–right (a) and rostral–caudal (b) gonad partitioning in the ovarian and testicular regions.



**Table 1** Number of male, female and intersex thinlip grey mullets (*Liza ramada*) collected from the lowest stretch of Tiber river (central Italy), by sampling season

Sampling season	Number (%) of thinlip grey mullets examined			
	Normal Males	Normal Females	Males with intersex signs	Females with intersex signs
Summer 2010	21 (42.8)	19 (38.8)	9 (18.4)	0 (0)
Winter 2010/2011	26 (40.0)	24 (36.9)	15 (23.1)	0 (0)
Spring 2011	23 (54.8)	19 (45.2)	0	0
Total	70 (44.9)	62 (39.7)	24 (15.4)	0 (0)



**Figure 2** Photomicrographs of some testicular oocytes observed in this study. Different developmental stages of ovarian and testicular elements are present: spermatocytes (Stc); spermatids (Std); primary (Op), previtellogenic (Opv) and secondary (Os) oocytes. Haematoxylin and eosin staining.

irrespective of the sampling season (data not shown).

As expected, the highest intersex rate (23.1%) was observed in winter, in correspondence of the reproductive period of this species. Intersex was also observed in 18.4% of the fish collected in summer, the period of gonad maturation, and

were absent in spring, during the recrudescence phase.

Similar results have been reported for small-mouth bass, where the highest prevalence of intersex was observed during the prespawn season, and a decrease in its incidence in correspondence of postspawn (Blazer, Iwanowicz, Iwanowicz, Smith, Young, Hedrick, Foster & Reeser 2007).

This study represents the first report of a high rate of intersex gonads in a wild population of *L. ramada*, with a winter peak reaching the 23.1% of the examined fish. Similar rates of intersex gonads have been observed in other mullet species sampled from polluted environments: 21% in flathead grey mullets from the Douro estuary, northern Portugal (Ferreira *et al.* 2004), and up to 50% in thicklip grey mullets from the Bay of Biscay, northern Spain (Puy-Azurmendi *et al.* 2013; Bizarro *et al.* 2014). Partially in agreement with our findings on thinlip grey mullet, the intersex gonads reported in those studies consisted of testis–ova gonads, characterized by the presence of oocytes in the testis and with previtellogenic oocytes surrounded by testis tissue.

In *C. labrosus*, the intersex condition has been associated with increased transcription levels of well-known biomarkers of xenoestrogenicity, such as the *vitellogenin* and *cyp19 aromatases* genes (Puy-Azurmendi *et al.* 2013; Bizarro *et al.* 2014). Further works are required to evaluate the same

biomarkers in *L. ramada* populations living in sites with different levels of pollution.

It has been reported that intersex fish show limited reproductive performance (Harris, Hamilton, Runnalls, Vinciotti, Henshaw, Hodgson, Coe, Jobling, Tyler & Sumpter 2011), and therefore the occurrence of gonadal alterations, such as those observed in this study, may represent a threat for the conservation of thinlip grey mullet stocks and a possible limit for the lagoon fisheries and extensive aquaculture of this species. In the Mediterranean sea, there has been a decline in fry availability of some mullet species due to pollution and overfishing of parent stocks, as it is the case of flathead grey mullet, that showed a decrease in number (Crosetti & Cataudella 1995). The same authors described *L. ramada* as still abundant, despite massive fishing, and more tolerant to coastal organic pollution and eutrophication. The findings herein reported suggest the presence of some threat also for this mullet species.

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