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First evidence of intersex condition in Caspian kutum, *Rutilus kutum* (Kamenski, 1901) in the Southwest Caspian Sea

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ABSTRACT

The present study showed intersex conditions in the wild population of Caspian kutum in the Southwest Caspian Sea. Evidence and examples of morphological and structural changes have been found in recent years among Caspian kutum, *Rutilus kutum*. The present study included sampling and examining morphological and anatomical characteristics of 350 pieces of kutumspecimens (38.5 ± 1.87 cm, 755 ± 6.52 g, and +3yr) as per their gender. Out of these numbers, a single male (+3 years old and a length/weight of 32.25 cm/628.5 g respectively) showed anomalies comprising seminal and gonadal sacs with simultaneous presence of male and female gonads. Histological observations of the male gonad revealed the presence of ovotestis tissue containing oocytes and spermatozoa throughout the entire testis. Male gonad tissue exhibited somniferous tubular structures with spermatogonia, spermatocytes, and spermatids. The histological examination of the gonads also showed the predominance of oocytes at different developmental stages. The intersexuality might have been due to determining processes or a combination of natural malformation and chemical contamination.

Keywords: Kutum, Histological analysis, Intersex gonads, Oocytes.
Article type: Report.

INTRODUCTION

By a surface area of 373,300 km^2, the Caspian Sea is the largest landlocked water body in the world, wherein 120 species and subspecies of fish live (Pourkazemi & Razikazemi 2011). *Rutilus kutum*, known as Caspian kutum, is one of the most commercially important fish species in the Caspian Sea. The fish is found in the Azerbaijan Republic, Iranian coasts, and the northern shores of the Black Sea. (Berg 1964; Razavi-Sayyad 1990). Most of the kutum populations are distributed in the Southwestern Caspian Sea (Heidari *et al.* 2009; Pourkazemi & Razikazemi 2011). Kutum is a migratory anadromous teleost with a group-synchronous ovary, and the eggs are released once after ovulation (Sharyati 1993; Saeed 2010), on gravel and sandy substrates and aquatic weeds of rivers or lagoons (Abdoli 1999; Koohilai *et al.* 2016). The females and males reach sexual maturity between 2-3 and 3-4 years respectively (Afraei-Bandpeid *et al.* 2012). Kutum fish migrate to estuaries and coastal waters in mid-winter (Abdoli 1999). The fish migrate to the Iranian side of the Caspian Sea to spawn in the rivers that flow into the sea and in Anzali wetland from March to May (Fazli *et al.* 2013). However, if the kutum is caught in the delta, cannot spawn (Yousefian & Mosavi 2008). The biology of kutum reproduction in the Iranian side of the Caspian Sea has been studied mostly on subjects such as sex differentiation (Pourkazemi & Razikazemi 2011), oocyte development (Heidari *et al*. 2010), spawning (Dorafshan & Heyrati 2006; Heyrati *et al.* 2007; Yousefian & Mosavi 2008; Falahatkar *et al.* 2013; Koohilai*etal.* 2016), fertility (Farid-Pak 1968); Heidari *et al.* 2009;

Masouleh *et al.* 2011; Samarin *et al.* 2011; Bandpei *etal.* 2011; Fazli *et al.* 2013), states of maturity (Sabet *et al.* 2009; Saeed 2010) and reproductive performance (Savadkouhi & Khara 2017). In addition, studies on reproductive impairments have been carried out on wild fish species in various parts of the world, such as male masculinization (Howell *et al.* 1980), interrupted or delayed testicular development (Lye *etal.* 1998), and abnormal reproductive behavior (Jones & Reynolds 1997). In wild populations of gonochoric fish, alterations may occur with the simultaneous presence of male and female reproductive stages in the same gonad at the same time which is not normal and is known as intersex (Nolan *et al.* 2001). In contrast, in protandry or protogynous fish species, the presence of the two types of gonads in the same fish is called hermaphroditism (Bahamonde *et al.* 2013) but in gonochoristic (fixed-sex) species intersex is not a normal reproduction mode or life cycle (Nolan *et al.* 2001; Tyler & Jobling 2008; Bahamonde *et al.* 2013). This means both testicular and ovarian cells appear in gonochoric fish gonads (Abdel Moneim *et al.* 2015). Widespread occurrences of intersex have been observed in freshwater and estuarine fish in various parts of the world (Jobling *et al.* 2002). There are several causes for intersex occurrences, among which temperature, pH, exogenous steroids, behavioral cues, parasites, and pollutants are considered to be involved in sex differentiation in fish (Minier *et al*. 2000; Devlin & Nagahama 2002; Hinck *et al.* 2009; Ichalal *et al.* 2016). The presence of intersex has been reported in the Cyprinidae, to which kutum belongs. The first findings reported were related to bream, *Abramis brama* in Netherlands (Sloof & Klootwijk-Vandijk 1982), Roach, *Rutilus rutilus* in British Isles (Jobling *et al.* 1998), chub, *Squalius cephalus* in England and France (Minier *et al.* 2000), barbel,*Barbus plebejus* in Italy (Viganò *et al.* 2001), common carp, *Cyprinus carpio* in Spain (Solé *et al*. 2002), spottail shiner, *Notropis hudsonius* in Canada (Aravindakshan *et al.* 2004), bighead carp, *Hypophthalmichthys nobilis* and silver carp, *H. molitrix* in USA (Papoulias *et al.* 2006). In general, intersexuality among wild fish is attributed to endocrine-disrupting substances (Nolan *et al.* 2001). However, there has been no earlier report on any cases of intersex gonads in Caspian kutum. This study aims to introduce the first gonadal intersex anomaly among Caspian kutum, *R. kutum* from the Southwest Caspian Sea.

MATERIALS AND METHODS

Sampling Area and Fish Collection

The fish specimens were captured using a beach seine net by local fishermen from the Sefidrud River estuary (37° 27'31.32"N; 49° 56'05.78" E) in April 2016. The river is approximately 670 km long and flows into the Caspian Sea (Asadollahfardi *et al.* 2018). Out of 350 fish specimens, only one intersex case was detected. Standard length (nearest 0.1 cm), body weight, and gonad weight (nearest 0.1 g) were measured. The entire gonad of intersex fish was removed, photographed, and fixed in 10% neutral buffered formalin for histological analyses.

Histological Analyses

Proximal, medium, and distal portions of the right gonads were fixed in Bouin's fixative solution, followed by tissue processing (consisting of dehydration, clearing, and paraffin embedding stages). Dehydration was conducted in ethanol 50, 70, 80, and 96% and 1-butanol, followed by clearing chloroform and paraffin embedding in a mixture of chloroform with purified paraffin at 56 °C. Then, the sample template was molded and assembled on a wooden base, sectioned longitudinally at 7 µm, mounted on slides coated with gelatin tissue (tissue to bind on the slide), and stained with hematoxylin and eosin (H&E). Next, the slides were studied through the light microscope (model Nikon E600, Japan) connected to a computer via Biocom program to study the tissues (Akhundov & Federov 1995; Hallajian 2009; Sharifpour *et al.* 2013), followed by photographing at 40X, 100X, and 200X magnifications.

RESULTS AND DISCUSSION

The mean standard length, body weight, and age of 350 pieces of Caspian kutum were 38.5 ± 1.87 cm,755 ± 6.52, and +3 years, respectively). Macroscopic examination of the collected fish showed characteristics of either male or female. However, in the macroscopic and histological analyses of the gonads, a single male fish (length, weight, and age 32.25 cm, 628.5 g, 3+, respectively) presented anomalies showing seminal or gonadal sac with simultaneous presence of male and female gonads. Thus, it was identified as an instance of intersex organism (length, weight and age 32.25 cm, 628.5 g, 3+ respectively), confirming the relatively low intersexuality occurrence rate (0.3%) in the Caspian kutum population, compared to many other fish species: 0.75% to 2.28% (Djoudad-Kadji *et al.* 2012; Ichalal *et al.* 2016; Hassel *et al.* 2018). The examined gonad comprised a testis developed in the proximal region, which occupied a small portion. However, the ovarian tissue occupied the most

significant portion of the gonad (Fig. 1), similar to the finding in *Trachurus trachurus* (Ichalal *et al.* 2016), however contrary to that found in *Coryphaena hippurus* (Retheesh *et al.* 2017). The testis was seen to be whitish, whilethe ovary was pinkish-brown in normal gonads. The presence of oocytes in the female lobe was detected in the distal portion (Fig 1). The macroscopic and histological analyses of gonads revealed the presence of both ovarian and testicular tissues. Since the intersex specimen was obtained from the wild catch in the Sefid-Rud estuary, it was impossible to determine whether the fish was reproductively functional. Therefore, it was impossible to determine if it was an undifferentiated or differentiated gonochoristic species (Yamamoto 1969; Jafri & Ensor 1979).

The mature ovotestis was subjected to histological analysis by microscope using proximal (Fig. 2), middle (Fig. 3), and distal (Fig. 4) regions of gonads. This showed variation in the composition of male and female cells and their arrangement within the gonads. The gonads presented oocytes at various stages of development. Intersex cells in this particular kutum (testis and ovary) were identified based on shape, size, cytology criteria, and characteristics of the nucleus (Tyler & Sumpter 1996; Nolan *et al.* 2001). The tissues were distinct from each other since the gonad was predominantly ovarian, and the testicular tissue was always white. The male tissue was restricted to the proximal region and the gonad surrounding the ovarian sinus, called the accessory reproductive structure. In most of the histological sections, oocytes and sperm were found to exhibitnormal development. Macroscopic and microscopic examinations of the ovarian portion showed the presence of oocytes at different developmental stages and the testicular portion with spermatozoa mass (Fig. 2), confirming the rare occurrence of intersex in Caspian kutum. In the testis, seminiferous tubules were evident, and cells showed in all stages of spermatogenesis (spermatozoa, spermatid, spermatocytes, and spermatogonia).

Samples with hydrated oocytes and testicular tissue containing sperm were recorded simultaneously in both regions of the gonad. A predominance of spermatogonia was found compared to spermatids and spermatocytes, with several primary and secondary stages of oocytes in testicular tissue. Seminiferous tubules containing cells in different stages of spermatogenesis were observed in the testicular portion. Oocytes at different developmental stages (1-4) were detected in the middle gonad region of intersex male. In the present study, we detected only one specimen with characteristics of intersex after examining 350 specimens, suggesting a low occurrence rate of such abnormality. Such a low occurrence of intersex in Caspian kutum may be due to the current climatic condition and/or natural anthropogenic pollution as has been described for other species (Retheesh *et al*. 2017). It has been found that various natural and synthetic pollutants can alter sexual differentiation in wild populations of gonochoric species by inducing sexual inversion and/or intersexuality (Colborn and Clement 1992) and also by endocrine disruption caused by water contamination (Sadovy de Mitcheson & Liu 2008). Kutum fish are known to spend part of their life cycle in some regions of the South Caspian Sea, wherein water and sediment are affected by contaminants (De Mora *et al.* 2004; Saeidi & Jamshidi 2010; Gharedaashi *et al.* 2013; Zahedi *et al.* 2014; Hoseini *et al.* 2015). The combination of heavy metals with detergents has a considerable effect on the mortality of Caspian kutum (Gholami *et al.* 2010). Insecticides such as fipronil applied in rice cultivation have also been found to affect alterations in the organ-somatic index and DNA structure in Caspian kutum (Ardeshir *et al.* 2018). It has been confirmed that kutumexposure to the butachlor (a herbicide used as weed control in rice fields in the study area) resulted in a decreased number and volume of sperm as well as an increased rate (28.6%) of abnormal spermatozoa (Lasheidani *et al.* 2008).

Furthermore, very low levels of Diazinon (an organophosphate pesticide) depressed spermatogenesis in testes and sperm quality of kutum (Masouleh *et al.* 2011). In line with the earlier findings, the impact of water pollution on the reproductive biology of kutum and the emergence of intersex cases among such species might be related to the high concentration of trace elements detected in the kutum gonads caught in the study area as compared to kutum caught from the eastern part of the South Caspian Sea (Sattari *et al.* 2019). In conclusion, the study showed the rare occurrence of intersex in Caspian kutum, indicating the simultaneous presence of the gonad and ovary tissue system in male kutum, as evidenced by macroscopic and histological observations. However, further research is necessary to shed more light on this subject since our study relies on the record of a single specimen.

Suggestion for further research

The causes of intersexuality might be due to determining processes or a combination of natural malformation and chemical contamination. Therefore, continuous monitoring of kutum populations in the Southwest Caspian Sea is required.

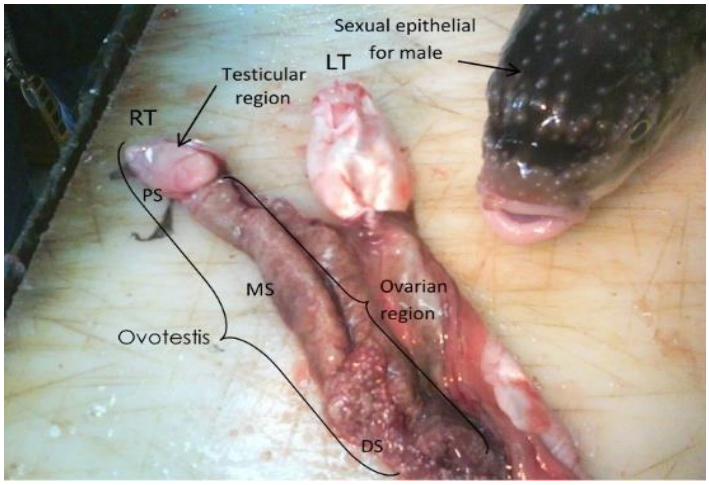

Fig. 1. Macroscopic appearance of intersex specimen. PS: proximal; MS: middle; DS: distal section: RT: right testis; LT: left testis.

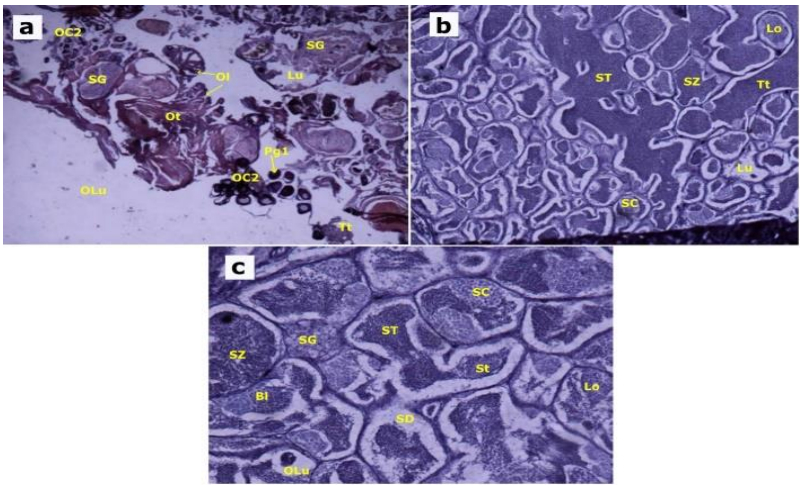

Fig. 2. Histology of right side gonad from intersex Caspian kutum, *Rutilus kutum* captured in the Southwest Caspian Sea: proximal section; a) H & E 40X; b) HE 100X; c) HE 200X. Basal lamina (Bl); structures showing the lobules (Lo); lumen (Lu); Secondary oocyte (OC2); Ovarian lamellae (Ol); ovarian lumen (OLu); ovary tissue (Ot); early primary growth oocyte (Pg1); spermatocyte (SC); sperm duct (SD); spermatogonia (SG); spermatozoa (ST); spermatid (St); sperm sinus and sperm duct filled with spermatozoa (SZ); testicular tissue (Tt).

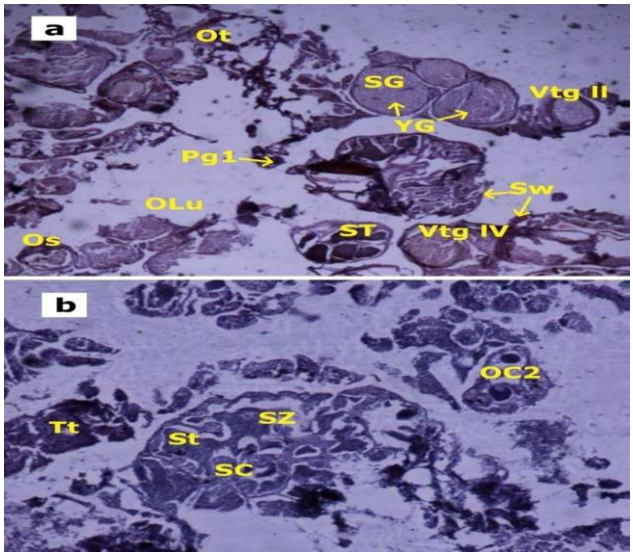

Fig. 3. Histology of right side gonad from intersex Caspian kutum, *Rutilus kutum* captured in the Southwest Caspian Sea medium section. a) H & E, 40X; b) H&E 100X; secondary oocyte (OC2); ovarian lumen (OLu); ovary sinus (Os); ovary tissue (Ot); early primary growth oocyte (Pg1); spermatocyte (SC); spermatogonia (SG); spermatozoa (ST); spermatid (St); sinus wall (Sw); sperm sinus and sperm duct filled with spermatozoa (SZ); testicular tissue (Tt); primary and secondary vitellogenesis stages (Vtgll); quaternary vitellogenic stage (VtglV); yolk granules (YG).

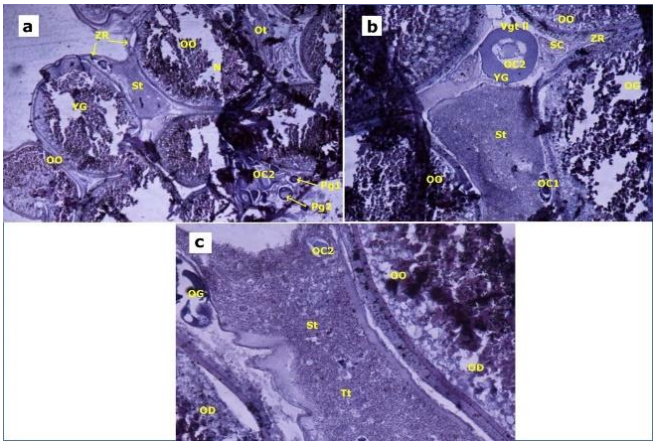

Fig. 4. Histology of right side gonad from intersex Caspan kutum, *Rutilus kutum* captured in the Southwest Caspian Sea: distal section. a) H & E, 40X; b) H&E 100X; c) H & E 200X. Disintegration of the nucleus (N); Primary Oocyte (OC1); Secondary Oocyte (OC2); Oil droplets (OD); Oil globules (OG); Oocyte or Ovum (OO); Ovary tissue (Ot); Early primary growth oocyte (Pg1); later primary growth stage (Pg2); Spermatocyte (SC); Testicular tissue (Tt); Primary and secondary vitellogenesis stages (Vtgll); Yolk granules (YG); Zona radiata (ZR).

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