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An evidence of intersex males in Jajroud River Loach, *Oxynoemacheilus bergianus* (Derjavin, 1934)

Amir Pourshabanan[1, 2], Alireza Sari [1]*, Sudabe Parsa[1,3], Bahman Zeynali[1] and Masoumeh Malek[1]

[1] *School of Biology and Center of Excellence in Phylogeny of Living Organisms, College of Science, University of Tehran, Tehran, Iran, postcode: 14155-64155, Tehran, Iran*

[2] *Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran*

[3] *Faulty of Biological Sciences, Kharazmi University, Karaj, Iran*

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Abstract

In the present study, Jajroud River loach, *Oxynoemacheilus bergianus* (Derjavin, 1934), was examined monthly for a previous observation of oocyte presence within testes tissues. Accordingly, the gonad development in *O. bergianus* is examined histologically. Although our data show typical fish gonad development and differentiation in female and some male individuals, some males show intersexuality. Gonad histology and development of females' ovaries were carried out to provide comparative data on oocyte staging in testes. Intersex males were observed throughout the year except in March and July, especially in individuals larger than 5.3 cm total length. Occurrence of intersex males seems to be associated with sex ratios and their deviation from 1:1 and fish age at different times of the year. As no real ovotestes were observed, histological changes were considered to be a transient stage in the testicular tissue with no evidence of sex change in fish. In the absence of testes fully transformed to ovaries, this type of change was considered as intersex.

Keywords: Endocrine disruptors; Gonad histology; Intersex; Sex ratio

Introduction

The Jajroud River loach, *Oxynoemacheilus bergianus* (Derjavin, 1934) is a widely distributed species in Asia, where it occurs in Iran and East Mediterranean countries such as Turkey, Syria, and Lebanon to Jordan (1). Currently, there is only one study on reproduction of this species (2), but there is no published data describing gonad histology. Previous histological observations of a few testes (3) showed that fish demonstrated gonadal change characterized by the presence of oocytes within normal testes tissue with no evidence of ovotestes. This has also been reported in whitefish (4) and other species.

Among the vertebrates, bony fishes show diversity in sexual reproduction strategies and sex determination modes compared with birds and mammals (5-7). Classically, the sex determination mechanisms are

* **Corresponding author:** sari@ut.ac.ir

controled by two factors: genetic and environ-mental ones, or both (8-10). According to Stelkens and Wedekind (11), fish sex is determined by genetic and environmental factors, but in some genetically sex-determined fishes, sex reversal is induced environmentally or by a combination of both. There are high genetic diversities regarding the genes and their interactions involved in sex determination. Five master genes have been found in fishes and in most species one gene is located on one chromosome, or several genes are located on different chromosomes involved in sex determination. The *dmY* and *sdY* genes located on Y chromosome are involved in male testes development and sex determination, while *gsdf*, *amhr2* and *amhy* are involved in sex determination of either male or female depending on fish species. The latter group of genes produces the steroid hormones of opposite sexes (androgen or oestrogen) under the endocrine disrupting chemicals in some fishes, which results in intersexuality of fish (10-17). The environmental factors involved in fish sex change act at gene level, cell and ultimately on sex organs (17). These factors with different mechanisms result in sex change including effects on meiosis in ovary (18) and also increase or decrease in number of germ cells (19-20). In most but not all fishes, higher frequency of germ cells is essential in sex determination towards female. In the condition with reduced frequency of germ cells in ovary, at higher temperature, some somatic cells undergo mitosis and therefore, they form spermatogonia. In later stage spematogonia, cells go through meiosis and produce spermatozoa and fish sex will change to male (20-24). In contrast, sex change from male to female is marked by presence of oocytes in testes tissues resulting in observation of ovotestis. The ovotestis formation involves degradation of testes tissues and formation of ovarian cavities among seminiferous tubes. In later stage, by constant mitosis in some peripheral cells of ovarian cavities, pre-vittelogenic follicles are formed and among these some cells undergo meiosis and as a result oogonia and protoplasmic oocytes are developed. The vittelogenesis in follicles, frequency, maturity and type of oocytes depends on the species, individual age, type and quantity of chemicals in the water, duration of fish exposure to chemicals and extent of changes in testes tissues (7, 17, 25-29).

Most fishes are gonochoristic and therefore their sex determination and differentiation mechanisms are involved in determining the sex ratio of the population. Intersex males are formed through two mechanisms. First, and the most frequent ones, is intersex in the presence of endocrine disrupting chemicals (30) and the second one is a mosaic intersex, a natural phenomenon (4, 31-33).

The aim of the present study was to describe gonad development in *O. bergianus* histologically over the period of a year and monitor the presence of oocytes in testicular tissues.

Materials and Methods

To assess the existence of xenobiotic factors involved in sex change in fishes, water quality data of the Jajroud River was gathered from the Tehran Province Water and Wastewater organization. In addition, data were obtained from published papers on heavy metals, oil and pesticide-based pollutions. Water samples from the Jajroud River, a source of drinking water, are regularly analysed by Tehran Province Water and Wastewater organization (34) using national standard (35) by Institute of Standards and Industrial Research of Iran and International standards and protocols such as World Health Organization (36), Wisconsin Department of Health and Family Services (37) and United States Environmental Protection Agency (38).

About 50 individuals of *Oxynoemacheilus bergianus* were caught at monthly intervals from January to December 2003 (in total n=546) from Jajroud River (35˚ 34' to 35˚ 47' N, 51˚ 47' to 51˚ 40' E) northeast of Tehran. Fishes were normally hidden on the river substratum and therefore, by agitating the water, these were directed to a hand net (65 cm diameter and mesh size 1×1 mm). Then, caught fishes were transferred live to the laboratory of Zoology, University of Tehran and kept in tanks with aeration for a few days prior to data recording, dissection, and histological study. Also, for comparison, a single fish sampling was carried out from a River at Qom city about 150 km far from the main sampling locality, the Jajroud River.

Total length (L_T) was recorded for both juvenile and mature fishes. Their sex was verified after dissection by thorough stereoscopic examination of the gonads. In small fishes, a piece of gonad tissue was compressed on a slide and examined at higher magnification, since sex determination was difficult under low power microscopy or the naked eye. After sex determination, some female gonads at different stages of maturity and all male gonads were fixed in Bouins solution for 24 hr, washed in distilled water for 4-5 hr before their routine process for histology (39). Paraffin blocks were cut at 7 µm, and then sections were stained with Haematoxylin and Eosin before their permanent mount on slides by Canada Balsam (Merck).

Ovaries and testes maturity were determined by microscopic examination. The main criteria for maturity of the ovary were general morphology and the diameter of oocytes. This was also applicable to oocytes within testes tissues. The number of oocytes per testis lobe was estimated from the number of testicular oocytes per area of longitudinal and transverse sections of one testis lobe. In symmetrical fishes, there are normally two equal sizes of testes lobes (right and left) located laterally in the visceral cavity. Only one lobe of the testis was counted due to equal size and developmental stages of testis lobes. The other was used for longitudinal sectioning to record testis lobe length. The numbers of oocytes were estimated by calculating the maximum number of 7 µm transverse sections in the length of the testis lobe. To assess the rate of oocyte development in male fishes, developmental stages of ovaries and normal testes were considered. The oocytes in testes were measured, counted, and compared with ovarian oocytes.

In the intersex gonads, types and numbers of oocytes were recorded. A χ^2 test was applied to investigate differences between frequencies. The Kolmogorov-Smirnov test revealed normal distribution of data. Then, one-way ANOVA was applied for comparison of the oocytes means. All data were analysed with SPSS statistics software v. 9 (www.spss.com).

Results

Sex ratio

The monthly proportion of males and females in random fish samples showed fluctuations in sex ratio. Male to female ratios were 1:1 only in August and November with significant tendency toward male biased ratios in April and December of 2003 (Table 1).

Transient testicular tissue

The percentage of all examined males bearing ovarian tissues was 42.2%, with variation in numbers of previtellogenic oocytes throughout the year. In March and July 2003, no male fish was recorded with oocytes in testicular tissue (Table 1). Among males grouped according to L_T, a higher mean number of oocytes were observed in the size class of 6-7 cm (Fig. 1).

Minimums of one to a maximum of 705 previtellogenic oocytes per testicular lobe were recorded, with the frequency of oocytes per lobe of testis greater in June and October-December (Fig. 2). While in female fishes (Fig. 3A-C) oocytes were observed in different developmental stages, in the intersex males all oocytes were at the previtellogenic stage with a maximum diameter of 70 µm (Fig. 3E and F).

Table 1. Monthly samples of male and female *Oxynoemacheilus bergianus* in 2003, and significant departures from a 1:1 sex ratio.

Month	Male,* n	Female n	M/F	χ^2	*P*
January	16 (5)	34	1:2	6.480	0.011
February	10 (3)	32	1:3	11.524	0.001
March	17 (0)	36	1:2	6.811	0.009
April	21 (6)	9	2:1	4.800	0.028
May	16 (3)	10	3:2	1.385	N.S.
June	18 (2)	28	2:3	2.174	N.S.
July	12 (0)	34	1:3	10.522	0.001
August	27 (9)	23	1:1	0.320	N.S.
September	31(16)	19	3:2	2.880	N.S.
October	31 (24)	20	3:2	2.373	N.S.
November	28 (21)	24	1:1	0.308	N.S.
December	37 (23)	13	3:1	11.520	0.001

* numbers in bracket show number of intersex males
** N.S. = Non-significant

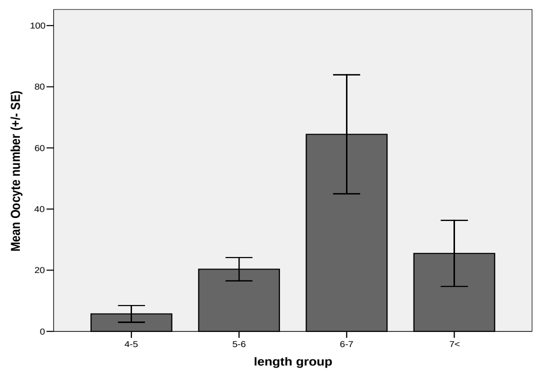

Figure 1. Mean number of oocytes per testis lobe in male *Oxynoemacheilus bergianus* for each L_T group.

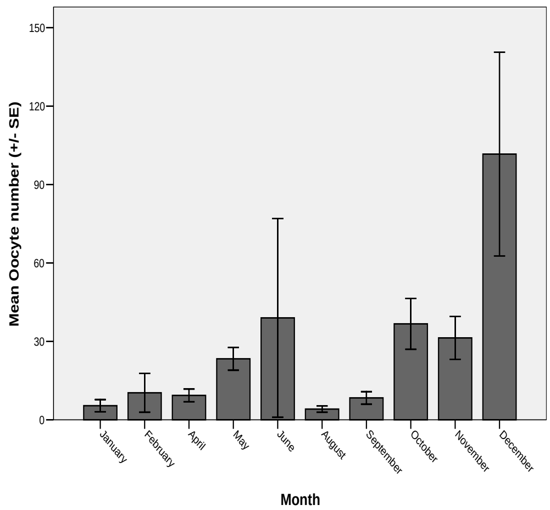

Figure 2. Monthly mean number of oocytes per testis lobe in male *Oxynoemacheilus bergianus* during 2003.

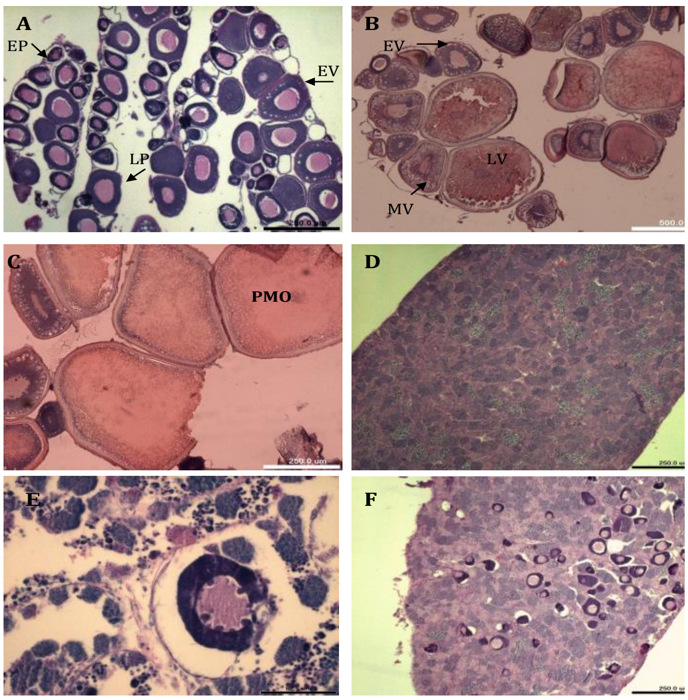

Figure 3. *Oxynoemacheilus bergianus* ovary (A-C) and testis (D-F). Transverse section of immature (A) and mature (B, C) ovaries. EP: Early pre-nucleus oocyte; LP: Late pre-nucleus oocyte; EV: Early vitellogenic oocyte; MV: Mid-vitellogenic oocyte; LV: Late vitellogenic oocyte; PMO: Prematuration oocyte. Transverse sections of normal testis with only testicular tissue (D) and testis with oocytes in intersex male (F). Ovarian tissue in male gonad consisted of only previtellogenic oocytes. (E).

Frequency of testicular oocytes

Fishes with L_T >6 cm showed higher numbers of oocytes in the testis (Fig. 1). Our histological data based on the maximum estimated number of oocytes show that the testicular tissues were in the process of transforming to a type of intersex gonad with 1/3 of testis tissue occupied by previtellogenic oocytes (Fig. 3F). The mean number of oocytes (Fig. 2) in December 2003 was significantly higher than other months (n=4308, F=2.844, $p < 0.0005$).

Histology of gonads

To assess the developmental stages of oocytes within testes tissues, oocyte development was evaluated using ovarian histology. Our histological data show two stages of development. First, most of the oocytes were previtellogenic, including early (~80 µm) and late (~150 µm) pre-nucleus oocytes (Fig. 3A). A few early vitellogenic oocytes (~200 µm) were present in some ovaries. Second, in addition to previtellogenic oocytes, many vitellogenic oocytes including early, mid, and late vitellogenic stages were also present (Fig. 3B). Some oocytes (~650 µm) with partially coalesced yolk granules (an indication of maturation) were also observed in the second type (Fig. 3C).

Finally, our histological analysis revealed two types of testes. Some were filled with seminiferous tubules within several stages of spermatogenic cells organized in cysts, which show synchronous development (Fig. 3D). Both testicular and ovarian tissues were observed in the second type (Fig. 3F). The testicular tissue exhibited typical organization and development, whereas ovarian tissues were less prominent and observed in the interspaces of seminiferous tubules (Fig. 3E). The oocytes in the ovarian tissue were previtellogenic including oogonia and early and late pre-nucleus stages (Fig. 3F).

Discussion

Sex differentiation involves hormonal regulation and environmental factors such as temperature, pH, photo-period, salinity, and population density (6, 9, 40-42).

Stelkens and Wedekind (11) and Baroiller et al. (23) reported the genetic and environmental sex determination factors. They also presented the evidence of fish sex reversal explaining that sex differentiation could be influenced by both factors. It was also demonstrated that the sex ratio of natural populations might be skewed by human influences on temperature related sex determination and temperature induced sex reversal.

Oxynoemacheilus bergianus is a gonochoristic fish with a breeding season confined to March-May and main breeding activity in March to April (2-3). Onset of maturity is at 3.7-4.3 cm L_T.

Sex ratio

Our present data show no significant differences between the frequency of males and females in May and June and in August to November. The sex ratio 1:1 was only observed in August and November (Table 1). The fluctuations in sex ratio show dominance of males in the population in four months (Table 1). The tendency for sex change in males seemed to be associated with imbalance of sexes in the population. Previous observations revealed significant differences in sex ratios in May and July in favour of males (3). Hence, there is not a fixed sex ratio in this fish species and fluctuation in the sex ratio occurs throughout the year but this is more pronounced in May-July due to breeding season.

Although the annual mean of sex ratio in other fishes were 1:1 (similar to the present study), temporal variations in sex ratios due to the genetic structure of the population (43), migration (44), mortality in general (45) and predation in particular (46-47) have been reported. Currently, as far as we know, data on life history of *O. bergianus* to reveal the sex ratio fluctuations is limited to a previous work (3). Therefore, we assumed that the change in the testes tissues of *O. bergianus* is associated with the lower frequency of females in the population. The role of population density and sex ratio in sex determination and differentiation has been shown in other fishes (see 8, 48). Based on our histological sections of testes, most observed oocytes were previtellogenic. The estimated number of oocytes was 1 to 705 per testis lobe with no evidence of dominance of oocytes within testis tissue; therefore, the tissues were considered as transient testicular tissue with no sign of ovotestis formation, and the fish as gonochoristic with non-functional gonad (intersex). Our data on the number of oocytes in male fishes show that these are most frequently observed fishes in October to December with no intersex fish in March and July (Fig. 2). This was associated with the peak of breeding in March and the absence of males greater than 5.3 cm L_T in July (2-3). Dominant size class of fishes with greater L_T than 5.3 cm, were recorded in September to December and bear more oocytes per testis lobe due to larger gonad size.

Our results show a male-biased sex ratio in September through December. In those months, frequency of females larger than 5.3 cm L_T was roughly around 1/3 that of males in a similar size class. According to

Skryabin (49), predominance of males is possibly common in short-lived fishes, which is applicable to *O. bergianus* as a fish with short lifespan.

Onset of maturity and predominance of males

Individuals of *Oxynoemacheilus bergianus* reach sexual maturity at 3.7-4.3 cm L_T. Group of sample fishes within this size range comprised 45% of females and 55% of males. After maturity, the population mostly include fish of L_T 5 cm and larger. This group size range shows male:female sex ratio of 3:2. Before maturity, the gonads of *O. bergianus* in both sexes undergo normal development towards well-defined ovaries or testes, morphologically and histologically. It has been reported in other fish species, in which intersexuality is mostly observed during developing stages of gonads before maturity (see 4). But, our current observation of intersex gonad seems to be related to an unnatural event such as effect of endocrine disruptors.

Gonad development

A typical structure of teleost fishes with spermatogenic cells organized into cysts was observed in testes. All stages of spermatogenic cells were observed histologically, indicating normal spermatogenesis taking place in seminiferous tubules. Process and pattern of oogenesis in teleost fishes were observed in ovaries. In most teleost fishes, the process of oocyte development is divided into four to eight stages based on yolk formation (50-54). In the current study, our data present seven stages of oocyte development, which was consistent with the classification of ovarian stages reported by Stahl and Kruse (53).

Intersexuality

The intersexuality is specified by presence of both ovary and testis in a gonochoristic fish individual (55). Although there are many field and laboratory studies on effects of endocrine disrupting chemicals in some fishes, there was always a debate about role of natural background on intersexuality. Previous studies from fish species living in urban chemically polluted water, industrial and agricultural sources and also experimentally polluted waters in the laboratory show intersex fishes developments while in non-polluted waters, fishes showed normal sex differentiation (56). Chemically polluted waters induce sex change but not in all species. Previous studies also show only one or few species affected by pollution and present intersexuality (57-59). In natural habitats, assuming a chemical free environment, assessment of pollution effects on intersexuality is difficult due to fish mobility and water current. Fish age, degree of adulthood, population density and sampling season should be also taken into account (8, 60-61).

In the present study, we find out that there was a sex ratio imbalance associated with biased male:female ratios (Table 1) in majority of the year (April-May and August-December). In August through December most male fishes have larger body size (6-7 cm L_T) showing higher numbers of oocytes per testis than those with smaller size, possibly due to larger gonad size. Similar with our finding, Bernet et al. (4) also reported gonad deformity in whitefish *Coregonus lavaretus* in all age classes greater than two years with higher frequency in older fish.

The term “abnormal hermaphroditism” was used for the presence of oocytes in testes or spermatocytes in ovaries, but the frequency of hermaphroditism was low in all cited cases (62). Hence, the phenomenon was considered abnormal. A greater number of male *O. bergianus* (42%) show intersexuality throughout the year with the exception of March and July. It was also reported that abnormal hermaphroditism is caused by environmental factors such as temperature and xenobiotics (62). The study by Nakamura et al. (63) revealed that higher temperature led to a biased sex ratio in favour of males in the Japanese loach *Misgurnus anguillicaudatus* (Cantor, 1842). A higher frequency of male progeny after elevation of temperature in most examined fish species has been already reported (64). According to Fishelson (65) and Robertson (66), sex change may arise as a result of social interaction of conspecifics. Among the factors involved in sex change (67-69), behavioural interactions between sexes and the sex ratio of groups seem to be more relevant to *O. bergianus*. However, behavioural interactions between sexes occur only in the absence of strong sex determination systems (62). Previous studies on *O. bergianus* show the plastic nature of sex determination of this fish species (A. Farazmand, pers. comm. at the Department of Genetics, University of Tehran). But, as stated earlier no testis was observed to show full differentiation towards ovotestis or an ovary.

Fish sex is determined by different factors. These are categorized into genetic systems, intrinsic changes, and changes in environmental factors (62). This leads to equal or biased sex ratios, depending on rate of selection action on fish sex determination factors.

Monthly analysis of Jajroud River waters by Tehran Province Water and Wastewater Organization using national and International standards (see Materials and Methods) and also Bagheban and Naseri (70) and Kashefiolasl and Zaeemdar (71) show this water source is potable. In addition, there are published data for water quality from several surveys on the Jajroud River to establish possible impacts of xenobiotic on sex change. These studies on heavy metals (72), polycyclic aromatic hydrocarbons (70), organochlorine pesticides (73) show no contaminations at the sampling locality and adjacent areas close to Latian Dam (located upstream of the Jajroud River). Only in the areas with agricultural activities about 50 km downstream of the Dam, some residues of pesticides were recorded (73). The water in the dam that comes from snowmelt and mountain streams has been used, after standard treatments, as drinking water for parts of the capital city of Tehran. In addition, in the sample population of *O. bergianus* from the drainage basin in Qom (about 150 km from the River Jajroud), the presence of oocytes was also observed in some male individuals. The fishes are continuously moving between locations at up- and downstream below the Latian dam. Alongside of the river, there are many gardens and croplands. Therefore, based on this evidence, the gonad change in male fishes suggests to be an abnormal phenomenon during the life cycle of *O. bergianus* in both localities possibly due to pollution related to agricultural activities. Histology sections and monthly sex ratio data analysis revealed that, functionally *O. bergianus* is a gonochoristic fish regardless of gonad morphology (according to definitions by 74-75), or gonochoristic with differentiated gonadal development.

Although there are few studies on sex determination in loaches belonging to families Cobitidae and Nemachilidae, our morphological finding in changes in gonad differentiation is in agreement with other studies on bony fishes (24, 76-80). In some loaches like *Misgurnus anguillicaudatus* from family Cobitidae, decrease in germ cells show no effect in gonadal changes and sex determination consistent with development of each fish toward separate sex (24). A study on mud loach, *M. mizolepis* by Nam et al. (81) shows a treatment by 17 α-methyl testosterone resulted in nearly unisex all-male population.

In addition to above criteria, intersexuality is also a natural evolutionary phenomenon in some fishes to compensate for sex ratio (61, 82-86). But, this is happening mostly in response to environmental changes and endocrine disrupting chemicals. Accordingly, in male and female fishes under effects of these chemicals, some hormonal, physiological and anatomical changes occur and gonad tissue shift to opposite sex (7, 87-90). This intersexuality, which is induced by environmental stress, could be used as an early warning bio-indicator of water pollution due to chemical accumulation in fish tissues and its consequences as gonadal change (87, 91).

The present study, as our knowledge, present for the first time, histological evidence on the gonad development in *O. bergianus* in normal development and differentiation in female and some male fishes. However, some males show intersexuality. Since no real ovotestes have been observed, histological changes were considered to be a transient stage in the testicular tissue with no evidence of sex change in fish. As Mitcheson et al. (75) reported, this could be categorized as non-functional sex change. As this phenomenon was also observed in a population of *O. bergianus* in Qom province at polluted locality, this suggests being due to the effect of endocrine disruptors. Also, further studies of social behaviour, genetics of sex determination, and factors involving in sex ratio imbalance may shed more light in natural and abnormal sex changes in fish in general and in *O. bergianus* in particular.

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Editorial Note

Volume 7, issue 2 of Progress in Biological Sciences was initialy scheduled to be published in December 31, 2017. However, some administrative changes leaded to a major delay in processing of the manuscripts. This issue is actually published in May 1, 2020. Editor-in-chief apologizes deeply for any inconvenience caused to the authors.