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Biological, Ecological and Taxonomic Characteristics of the Species *Pelasgus minutus* (Karaman, 1924) (Cypriniformes: Leuciscidae) from Belchishko Blato, Republic of North Macedonia

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Abstract: *Pelasgus minutus* is an endemic Macedonian ichthyofauna species inhabiting Lake Ohrid and its basin. It is an endemic species for the Balkan Peninsula and Europe. According to the European Red list, it is in the Data Deficient (DD) category. In the Habitats Directive, it is placed in Annex II. Kottelat and Freyhof (2007) consider that the population of *P.minutus* in Lake Ohrid is probably extinct. The Belchisko Blato is its last refugium. This study aims to investigate the species' main morphological, physiological, and ecological features due to a need for more information regarding its biological characteristics. A total of 71 individuals were analysed during the ichthyological research. Sixteen meristic counts are presented, and 41 morphometric measurements are performed with percentage relationships. The gender structure indicates a stable population. The percentage representation is 4.2% juvenile, 43.7% male and 52.1% female individuals, with age range from 0+ to 3+. The majority of the population belongs to individuals aged 2+ (47%), and the rest are as follows: 1+ (28.2%), 3+ (19.7%) and 0+ (5.1%). Sexual maturity is reached during the second year of life (1+). The most minor sexually mature male and female individuals are aged 1+. Spawning occurs during the late winter months (February- March). The average absolute fecundity is 1549 eggs, ranging from 1144 to 1549.

Key words: *Pelasgus minutus*, morphology and morphometry, age, maturation, fecundity, ecology status.

Introduction

To date, there are no studies about *P. minutus* biology and ecology. The species is considered among Europe's smallest cyprinid fish species (Karaman 1924). In the description of *P. minutus*, according to Karaman (1924), the small size and the low number of scales are the main characteristics that distinguish *P. minutus* from all other species of the genus. For the Macedonian ichthyofauna, *P. minutus* is a native species of Lake Ohrid (Talevski et al. 2009, Talevski et al. 2009a, Talevski et al. 2009b, Milosević & Talevski 2015). The authors determine that this species is native to Lake Ska-

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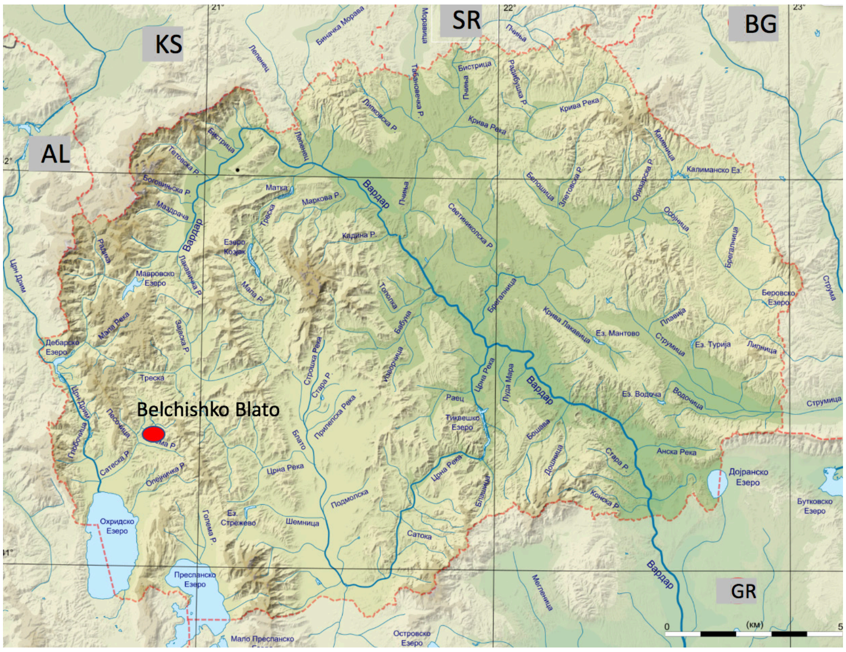

Fig. 1. Map of the geographical location of Belchishko Blato.

dar, where the taxonomic status of the population remains unclear (Crivelli 2006). During research on the evolutionary history of the genus Pelasgus, *P. minutus* was recorded in the Ohrid-Drin-Skadar basin, located in the Lake Ohrid inflow (Rodríguez et al. 2021). *P. minutus* is an endemic species not only for the fish fauna of the Balkan Peninsula but Europe as well (Freyhof & Brooks 2011). According to the European Red List, it has been placed in the category of Data Deficient (DD) (Freyhof & Brooks 2011). Moreover, concerning the Habitats Directive, *P. minutus* is within Annex II (Freyhof & Brooks 2011). However, concerning the Bern Convention Annexes, *P. minutus* is not placed in Appendices II or III (Freyhof & Brooks 2011).

In an ichthyological survey of Lake Ohrid in 2002, Criveli noted that not a single individual of *P. minutus* was detected in Lake Ohrid (Kottelat M. Pers comm), emphasising that the last specimens were collected in 1973 (Crivelli 2006). Kottelat and Freyhof (2007) consider that the population of *P.minutus* is probably locally extinct in the waters of Lake Ohrid, and its distribution is limited to the river waters of the Adriatic basin. The population of *P. minutus* in Belčishko Blato is probably the only remaining population of this species in the country. The conducted research is highly significant because it provides data on the biology and ecology of this species for the first time, which will serve as the basis for the determination of the actual ecological status of this species and the implementation of measures for its protection in the future. The study aims to investigate the species' main morphological, physiological, and ecological features to provide more information about its biology and ecology.

Materials and Methods

Study Area: The present study was conducted in ten (10) localities of Belchishko Blato. It is one of the largest remaining wetlands in the Republic of North Macedonia, with an approximate area of 400 hectares. It is a relict remnant of the former Deseret Lake dated from the Pliocene and part of river Sateska, which currently flows into Lake Ohrid. During the field research of each locality of interest for the fish fauna, geographic coordinates of latitude and longitude were registered through the application Memento Database (Android edition, Version 4.10.3).

There are no relevant literary data about the ichthyofauna of Belchishko Blato. The ichthyofauna of Belchishko Blato is mentioned in a report named "Study on the valorisation of Belčishko Blato", dated 2022, but no sources or author of that data are given. In that report, the presence of seven species of fish is mentioned and shown in one table, namely: *Anguilla anguilla, Chondrostoma ohridanus, Cyprinus carpio, Pelasgus minutus, Phoxinus lumaireul, Scardinius knezevici* and *Squalius squalus*.

Fish sampling and processing: *P. minutus* samples were caught and collected from December 2021 to June 2023 using a combination of fishing techniques: standing nets, trap nets and electrofishing. Standing nets with different mesh sizes were placed in the afternoon or evening and pulled out the following day. "Umbrella" trap nets were put in accessible localities and left overnight like standing nets or were removed within the same day. Electrofishing was performed with electric shockers Samus 725G and Samus 725L. The total length (TL), fork length (FL) and standard length (SL) of each fish specimen was measured with a digital vernier calliper with a precision of 0.01 mm, and body weight (W) was determined to the nearest 0.01 g. After the measurements, the live fish were returned to the water of Belčishko Blato at the place where they were caught. Some of the fish were sacrificed for further research in the laboratory. After the dissection, the digestive tract contents and gonads were collected. By analysing the collected gonads, the stage of sexual maturity was determined. The mass of the collected gonads was also measured.

Scales from the left side under the dorsal fin were collected from each caught individual to determine the fish's age. The collected scales were analysed in the ichthyological laboratory of the UKIM Institute for Animal Sciences and Fishery in Skopje. The age analysis of the fish was performed by "reading" the scales using a digital microscope with a camera (Olympus MIC-D).

Morphology and morphometry: The most commonly used methods in the taxonomic keys were used to perform a general analysis of the morphological properties of the fish body's external appearance. The external morphology was analysed, and morphometric and meristic characters were presented. In general, morphometric lengths and meristic counts were performed according to the methodology of Kottelat & Freyhof (2007) using a digital calliper with a precision of 0.01mm.

Sampling of gonad and Gonado Somatic Index (GSI) calculation: The ventral side of the samples was cut and opened from the anus toward the lower jaw by using scissors, and the belly was opened. Then the muscle of the abdomen was cut from the anus toward the ventral column vertically. Muscle and fat tissues, digestive organs and blood vessels were removed. Then, the ovaries were taken out by using forceps. The weight of the ovaries was measured with electronic balance in grams, and the length was recorded in mm. A small portion of the gonad was weighed and presented as a sub-sample to estimate fecundity. The gonadal somatic index (GSI) was calculated using the following formula: GSI=100×GW/BW, where GW is gonad weight, and BW is body weight (Howaida et al. 1998).

Fecundity estimation: In the present study, the gravimetric method was applied (Simpson, 1959). The ovaries from each specimen were removed by dissecting the samples. Only matured ovaries were selected for fecundity analysis. The matured ovaries were weighed with an electronic balance (g). These ovaries were spilt out longitudinally and kept in a petri dish. Three samples, one from the anterior, middle and posterior region of each ovary, were taken, and the weight of each was measured with an electronic balance (g). All ovaries were preserved in a 4% formalin solution for a more effortless separation of the eggs from the ovary wall. After a few hours, the eggs enlarge, and a small amount of water is added to separate them from each other with the help of laboratory needles. The eggs varied in size. They were photographed by a binocular digital camera. The number of eggs in each sub-sample was counted from the image, and the mean number of eggs was calculated. The average number in the sub-sample was multiplied by the weight of the ovary. The absolute fecundity of the species was then calculated according to the following formula: AF=N×GW/SW, where AF is the absolute fecundity, N is the number of eggs in the sub-sample, GW is the gonad weight, and SW is the total weight of the sample (Bagenal 1978). Relative fecundity (RF) is expressed as the number of eggs per 100 grams of body weight of fish according to the formula: RF = 100 x AF/W, where RF is relative fecundity, AF is absolute fecundity, and W is the mass of the fish (g) (Bagenal 1978).

Fulton's Condition Factor (K): Fulton's condition factor (K) was calculated using the following formula: K = (W/TL3) × 100, where W is total body weight (g), and TL is the total length (cm) (Le Cren 1951).

Results

Meristic and morphological measurements.

A total of 71 specimens of *P. minutus* were collected during the project period. Among the specimens, 31 were males, 37 were females, and 3 were juveniles. Over the study period, the lowest standard length for males was recorded at 27.2 mm, while the highest was 59.1cm. The average standard length was 38 mm (Table 1). For the female individuals, the lowest standard length was recorded at 18.2 mm, the highest was 73.6 mm, and the average was 39.7 mm (Table 2). Regarding the body weight of male individuals, the lowest value was 0.44 g, and the highest was 2.71 g. The female individual's lowest body weight was 0.14 g, and the highest was 9.01 g.

Table 1. Fish sizes of male *Pelasgus minutus* Karaman, 1924.

Mark	Fish sizes	min	max	average
W (g)	Weight	0.44	2.7	1.16
TL (mm)	Total body length	34.9	62.7	45.1
FL (mm)	Length of body to smallest ray of caudal fin	29.8	60.2	42.1
SL (mm)	Standard body length	27.2	59.1	38.0

Table 2. Fish sizes of female *Pelasgus minutus* Karaman, 1924.

Mark	Fish sizes	min	max	average
W (g)	Weight	0.14	9.01	1.67
TL (mm)	Total body length	22.7	83.5	47.1
FL (mm)	Length of body to smallest ray of caudal fin	19.7	79.6	44.1
SL (mm)	Standard body length	18.2	73.6	39.7

Table 3. Number of rays in the fins of *P. minutus* Karaman, 1924.

Number	Merist hallmark	Scope
1	Number of main caudal rays – total	16-18
2	Number of rays in the dorsal fin	9-10
3	Number of rays in the anal fin	9-10
4	Number of rays in pectoral fins	13-14
5	Number of rays in ventral fins	7

Table 3 illustrates the morphometric lengths of the collected individuals. The total body length (TL) of *P. minutus* was between 46.11 and 53.59 mm. The standard length (SL), up to the hypural set, was 84.43 % TL, while the length of the body, together with the scaly tail cover, was 88.09 % TL. The length of the body to the smallest ray of the caudal fin had a mean value of 43.49 to 49.85 mm.

Table 4 presents the meristic counts of *P. minutus* and their range. The collected individuals are characterised by 9-10 rays in the dorsal fin and the anal fin. Seven rays, distinguished abdominal fins and 13-14 rays were counted in the pectoral fins. A total of 16-18 main caudal rays were counted in the caudal fin (Table 3).

The remaining morphometric lengths were measured and presented as percentages of TL, SL, and lateral head length (HL) (Table 4). HL was 30.85 % of SL. Distances within the cephalic portion of the body were measured as a percentage of the HL. The interorbital distance percentage was 38.35% of the HL, the eye diameter was 30.28% of the HL, and the postorbital distance amounted to 50.55% of the HL (Table 4).

Age and gender structure of the population

Most of the caught individuals (47%) were in their third year of life (2+). The number decreased for the categories of the first and fourth years. The most minor sexually mature male individual is aged 1+, with a length of 34.94 mm and a mass of 0.44 g. The most minor sexually mature female is aged 1+, with a length of 29.88 mm and a mass of 0.38 g. During the first year of life, all individuals from the population of *P. minutus* are juveniles. The first sexually mature individuals appear in the second year (1+) in both males and females. The entire population of *P. minutus* during the third year of life (2+) is already sexually mature and ready for spawning. The sex ratio for *P. minutus* was calculated as 1.19:1.00, which did not deviate from the expected ratio of 1:1

Spawning Season and GSI

To obtain initial data on the spawning season of *P. minutus*, although the material was not collected with monthly dynamics, the GSI was determined in the females. The GSI presented the highest values in February, 22.5 on average in 2022 and 15.63 on average in February 2023 (Table 5). The results indicated that *P. minutus* from Belchishko Blato was already prepared for spawning in February, which occurred at the end of February and the beginning of March. All examined individuals had already spawned in May.

Table 4. Morphometry of (10) *P. minutus* individuals Karaman, 1924 with a total body length of 46.11 – 53.59 mm.

Mark	Morphological measurement	min	max	Percentage (%)
TL	Total body length	46.11	53.59	
				% of TL
FL	Length of body to smallest ray of caudal fin	43.49	49.85	98.67
SL	Standard body length	36.23	42.63	84.43
				% of SL
pD	Post dorsal length	13.3	14.36	34.36
dh	Dorso-hypural distance	17.46	19.85	50.38
pl	Tail stem length	9.05	9.26	24.01
C?l	Length of upper lobe of caudal fin	9.58	10.51	25.95
C?l	length of lower lobe of caudal fin	9.58	10.51	26.79
aD	Predorsal distance	9.69	23.62	58.08
HL	Lateral head length	10.63	12.5	30.85
dHL	Dorsal length of head	8.11	10.02	23.98
pV	Abdominal length	19.42	22.07	56.35
pA	Pre-anal length	25.69	29.72	71.90
PV	Distance between pectoral and ventral fins	9.44	10.88	29.15
VA	Distance between pectoral and anal fin	6.08	8.14	16.85
Dl	Dorsal fin length	3.98	5.9	12.67
Dh	Dorsal fin height	8.83	9.28	24.25
Al	Anal fin length	3.71	4.46	10.72
Ah	Anal fin height	7.25	7.87	20.51
Pl	Pectoral fin length	7.13	7.73	17.54
Vl	Ventral fin length	5.77	6.5	14.19
w	Tail width (minimum body width)	2.18	2.67	5.36
W	Maximum body width	5.23	6	12.86
H	Body height	10.28	10.76	28.85
h	Tailstock height (minimum body height)	4.26	5.21	11.63
				% of H
ch	Head height at the level of the occiput	6.83	7.97	75.69
hw	Head width at gill cap level	6.28	6.58	55.59
io	Interorbital distance	3.82	4.83	38.35
s	Before eye distance	3.15	3.44	29.60
e	Eye diameter	3.55	3.62	30.28
phl	Behind the eye distance	5.01	5.85	50.55
lMx	Upper jaw length	3.33	3.44	30.19
lMd	Lower jaw length	3.06	3.09	29.27

Fecundity

Absolute fecundity (AF) and relative fecundity (RF) were determined on 13 specimens of *P. minutus* caught in February, which had mature eggs. The AF average was 1549 eggs and varied from 295 to 3143. The RF average was 1.078.052 eggs and ranged from 164.167 to 1.540.000. It can be noted that AF was lowest in age group 1+ and highest in age group 3+ (Table 6).

In contrast to AF, RF presented the highest values in age group 1+ and the lowest in age group 3+, thus suggesting that it decreases with age. It can also be noted that AF is positively correlated with the fish's mass and length, while relative fecundity decreased with an increase in fish length and body mass (Table 6).

Table 5. Body weight (W), Gonad weight (Wg), and Gonadosomatic index (GSI).

Date	n	W	Wg	GSI min.	GSI max.	GSI avrg
15.02.2022	2	5.16	0,96	17.31	27.69	22.5
25.05.2022	7	0.61	0.01	0.72	7.14	2.73
16.09.2022	16	1.65	0.1	0.5	12.94	6.48
15.02.2023	12	1.57	0.25	8.82	23.3	15.63

Table 6. Body weight (W), Total length (TL), Absolute Fecundity (AF) and Relative Fecundity (RF).

Age	W avrg	L avrg.	No of spec.	AF Min.	AF Max.	AF Aver.	RF Min.	RF Max.	RF Aver.
1+	0.66	3.89	2	295	154	225	1475000	1540000	1507500
2+	1.22	4.29	5	1263	2688	2122	1120000	1263333	1165600
3+	3.4	5.49	6	1875	3143	2301	164167	766667	561056
Aver.	1.76	4.56	13	1144	1995	1549	919722	1190000	1078052

Table 7. The Fulton's condition factor (K) values of *P. minutus* by sexes.

Sex	n	Average	Min.	Max.	S.D.
Juv	3	0.96	0.52	1.2	0.380
Male	31	1.23	0.87	1.60	0.189
Female	37	1.35	0.85	1.94	0.276
All	71	1.18	0.52	1.6	0.096

Fulton's Condition Factor (K).

The K values of *P. minutus* ranged from 0.85 to 1.94 in females and from 0.87 to 1.60 in males (Table 7). The results indicate that this species was in good growth condition and that the habitat is adequate for fish growth. For a species or population, the condition factors are not constant throughout its life cycle and may be influenced by both biotic and abiotic factors.

Discussion

The current study reported the sex ratio, fecundity, spawning season, GSI, and K values for *P. minutus* for the first time. No reference has previously been reported with which the results of this study can be compared.

P. minutus was initially described by Karaman in 1924 as a new distinct species (*Paraphoxinus minutus*). Today, the population is known by the scientific name *Pelasgus minutus* (Karaman, 1924). According to Kottelat & Freyhoff (2007), species of the genus Pelasgus are small fish living in springs, small and shallow streams, backwaters and ditches on the Balkan Peninsula. They can survive summer droughts in small eddies on riverbeds, springs or wells (Leris et al. 2022). Their taxonomic diversity is considered to be underestimated due to their small size, which could lead to misidentification, and was the reason for their long-term neglect in ichthyological research (Kottelat & Barbieri 2004). *P. minutus* is defined as a species distributed in the stagnant waters at the bottom of Lake Ohrid and the shallow coastal parts of the lake itself (Karaman 1924). During the ichthyological research in Belchishko Blato, *P. minutus* was not detected in the spring of the Matica River. Its presence has been noted only in large eddies of stagnant water. Karaman's findings (1924) show that this fish species avoids rapid water flows and prefers stagnant or slow waters.

The sex ratio for *P. minutus* was calculated as 1.19:1.00, which did not deviate from the expected ratio of 1:1. The data on the sex distribution in fish populations is most important for the reproduction of the population. The sex ratio in fishes may generally vary from species to species, between different populations of the same species and from year to year in the same population as well (Nikolsky 1963). The sex ratio of the population of *P. minutus* in Belcishko Blato indicates a stable population of fish with all the prerequisites and environmental conditions for successful spawning and survival.

Fecundity is the general term used to describe the number of mature eggs produced by an individual female per breeding cycle. Knowing a fish's fecundity is essential for evaluating its stock, life cycle, culture, and fishery management potential (Lagler 1966, Doha & Hye 1970, Das 1977). The present study shows that the high fecundity values of *P. minutus* indicate that the population is stable, with a high potential for survival.

The research showed that the population of *P. minutus* from Belchishko Blato was prepared for spawning in February, and the spawning was performed at the end of February and the beginning of March. All analysed individuals had already spawned in May. The spawning period of *P. marathonicus* is from May to September, while for *P. stymphalicus*, it is from December to April (Kottelat & Freyhof 2007). The data provided by Kottelat and Freyhof (2007) about the spawning period of *P. minutus* in Lake Ohrid contradict our research. According to them, it spawns in the period from June to July. Considering specific environmental conditions in Belchishko Blato, it is a matter of adaptation of the species to the conditions of the environment, which is probably why spawning timing has shifted and takes place relatively early during the late winter months and the beginning of spring.

The condition factor is an index reflecting the interaction between biotic and abiotic components in the physiological conditions of fish. This factor may vary among fish species in different localities (Blackwell et al. 2000), and the fluctuation in this index is also based on the seasonal variations of the gonads and feeding intensity (Biswas 1993). The condition factor increases in both sexes during the spawning season due to the increased weight of the gonads (Kostov et al. 2021). Higher values of K indicate the suitability of a specific water body for growth, a good feeding regime, and appropriate environmental conditions in favour of fish. In contrast, a lower K value indicates the reverse conditions (Le Cren 1951, Ujjania et al. 2012). Our results suggest that this species is in good growth condition, and the habitat conditions favour growth. The differences in mean K values between the sexes in the present study may be due to the variations in weight and age/size of individuals sampled, number of specimens, feeding ratios and gonadal development.

The present study aims to contribute to the limited knowledge of some biological, ecological and taxonomic aspects of *P. minutus* from Belchishko Blato. This study is the first one on this species. It will offer significant contributions to the current literature, providing important information regarding the monitoring and sustainability of the population in its distribution area. The International Union for Conservation of Nature (IUCN) has not yet assessed the status of this species, so it is listed as data deficient (DD) (Crivelli 2006). Evaluation of some bio-ecological characteristics of the species in this study will also make an important contribution to assessing *P. minutus* according to IUCN criteria. The results of the present study will be helpful for researchers and fisheries management authorities to understand better the life history patterns and ecology of this species. For now, there is no conservation action directly related to *P. minutus*. There is a proposal to place this species on the endangered species list in the Vulnerable Category (VU) (Talevski et al. 2010). Considering the results of our research and the distribution of the species in a relatively small area, we support the proposal for *P. minutus* to be redefined from DD status to Vulnerable or Critically Endangered.

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