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Original Article

Spatial morphometric plasticity of spirlin *Alburnoides bipunctatus* (Bloch, 1782) phenotype from the Nišava River, Serbia, Danube basin

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***Abstract*:**

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Variation of 22 morphometric and 4 meristic characters of the species *Alburnoides bipuuctatus* from Nišava River were analyzed. Specimens were collected from four sampling stations along the putative mesohabitat gradient of river influence and were grouped into the categories with similar total length. Morphometric characters varied immensely between different mesohabitats outlining morphometric plasticity. Among several morphometry features that were influenced by different mesohabitats the most observable differences were noted for: postocular distance, anal fin height, minimal body height and preanal length. Regarding the meristic features, number of soft rays in the anal fin did varied significantly among sampling stations while fourth hard ray in the anal fin for this species was reported for the first time ever. Pharyngeal teeth formula of spirlin also showed variability across different mesohabitats. In conclusion, it appears that spirlin express a big morphological plasticity in relation to spatial and mesohabitat distribution and there is a possibility that spirlin from Nišava River is a complex of neospecies in formation/morphotypes/ecotypes rather than a single population.

Key words: *Alburnoides bipunctatus*, Cyprinidae, mesohabitat, phenotype plasticity

Introduction

Spirlin *Alburnoides bipunctatus* (Bloch, 1782) – Cyprinidae is a freshwater species reaching a maximal length of 13-14 cm (L e l e k , 1987). It is a sub-montane species typically present in upper and middle rithronic communities. It is widely distributed in Europe and in North and West Asia including the Turkey and Iran (B o g u t s k a y a , 1997, L e l e k , 1987). Due to spirlin population abundance in majority of its areal, it is an important prey item of many predatory fish species, while its main food items are diatoms and green algae (T r e e r et al., 2006). Spirlin is listed in IUCN as a species of least concern, but locally threatened, and its status in Serbia is - LR (lc) (S i m o n o v i ć , 2001). Several subspecies have been described for European region: *A. bipunctatus rossicus* (Berg) from Volga basin; *A. bipunctatus strymonicus* (Chichkoff) from Marica drainage; *A. bipunctatus ohridanus* (Karaman) and *A. bipunctatus fasciatus* (Nordmann) from Ohrid Lake and Drim basin and *A.bipunctatus prespensis* (Karaman) from Lake Prespa (L e l e k , 1987). At least six subspecies are present in Asia (L e l e k , 1987). Nominate subspecies inhabit both Europe and Asia. Its areal is

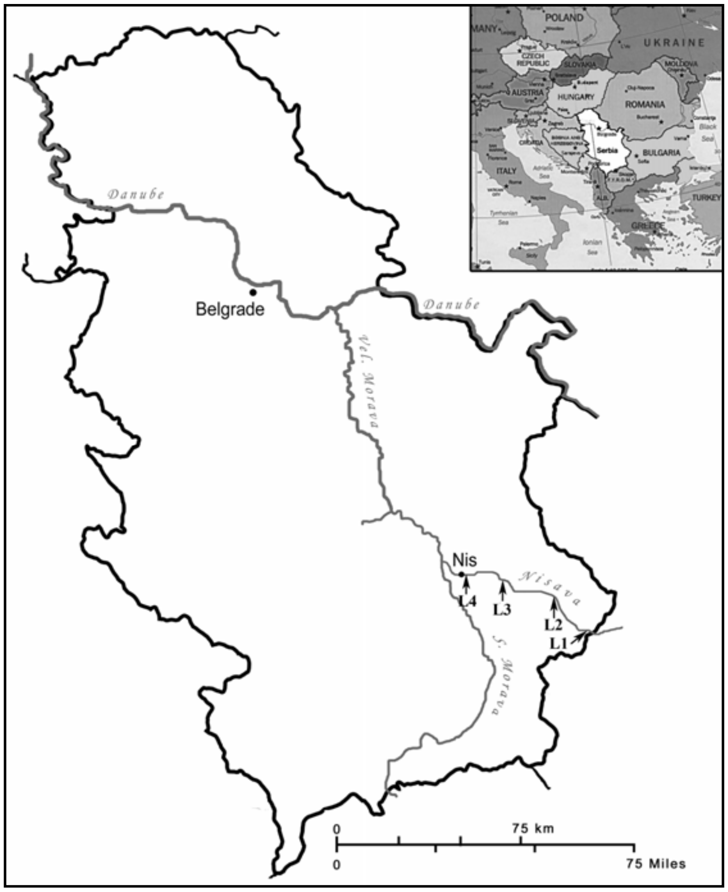

Figure 1. Geographic region of Nišava River. The four sampling stations (L1-L4) are indicated with dotted arrows

from Loire drainage in France eastward, in nearly all rivers draining to southern Baltic, North, Black and Azov Seas; Caspian basin, in upper Volga and from Kura drainage southward to Iranian tributaries of Caspian (Froese & Pauly, 2011). Nominate subspecies is also the one inhabiting Nišava River (Simonović, 2001).

Recently, a relationship between morphometry and microhabitat utilization by *A. bipunctatus* has been established (Copp *et al.*, 2010; Kovač *et al.*, 2006). The differences observed were mainly due to different preference for water velocity, depth and substratum, within the same sampling station, as the fish get older (Copp *et al.*, 2010). Such, segregation is likely connected with very specific abrupt isometric growth of *A. bipunctatus* - two periods of isometric growth with different proportional values interrupted with short interval of allometric growth (Kovač *et al.*, 2006). Such adaptation is increasing swimming performance of spirlin and provides better microhabitat utilization. While microhabitat analyses are based on identifying the segregation and preferences toward specific ecological factors within a habitat, mesohabitat modelling is based on a coarser scale covering longer sections of the river with mixture of ecological factors and adds the spatial component. The degree of spatial influence can be immense when analysing species with high morphometric plasticity potential. Spatial component as a function of mesohabitat was not evaluated in the past regarding variation in morphometry for this species. Therefore the aim of this study was to investigate whether the phenotypes of the spirilin will be significantly different along the same river.

Materials and methods

Stream and sampling site description

The Nišava River with its tributary Ginska is a 202 km long with a watershed area of 4068 km^2. It is situated in south-east Serbia and Bulgaria (upper one fourth of the river) and it is the largest tributary of the Južna Morava River, part of the southern Danube River watershed (Gavrilović & Dukić, 2002). The Nišava has two distinct parts: upstream of the town of Bela Palanka it is a narrow, rapid mountain stream ranging from 6 to 76 m in width and 0.6 to 2 m in depth with velocity from 1 to 2.5 m s^{-1} and discharge of 0.3-270 m^3 s^{-1} of water. After Bela Palanka and all the way to Južna Morava River, the Nišava becomes a meandering stream with increased width and depth, and flow that carries up to 700 m^3 s^{-1} of water (Branković, 1997).

Sampling of spirlin was performed on four sites (**Fig. 1**). The Nišava River at Ivko's watermill, 1 km upstream from the city of Dimitrovgrad was selected as site L1. This is the first section of the river as it enters Serbia and there are no larger settlements upstream to this sampling site. In this section the river has a width of 3-6 m, and a depth of no more than 1 m. The bottom is composed of gravel, and the banks are covered with thick vegetation. The section is composed of light riffles intersperse with glide sections. The majority of the benthofauna is composed of Ephemeroptera and Trichoptera taxons, while in the ichthyofauna the most dominant species are: spirlin, brook barbel *Barbus balcanicus* (Kotlik, Tsigenopoulos, Rab & Berrebi 2002) - Cyprinidae and chub *Leuciscus cephalus* (L. 1758) - Cyprinidae.

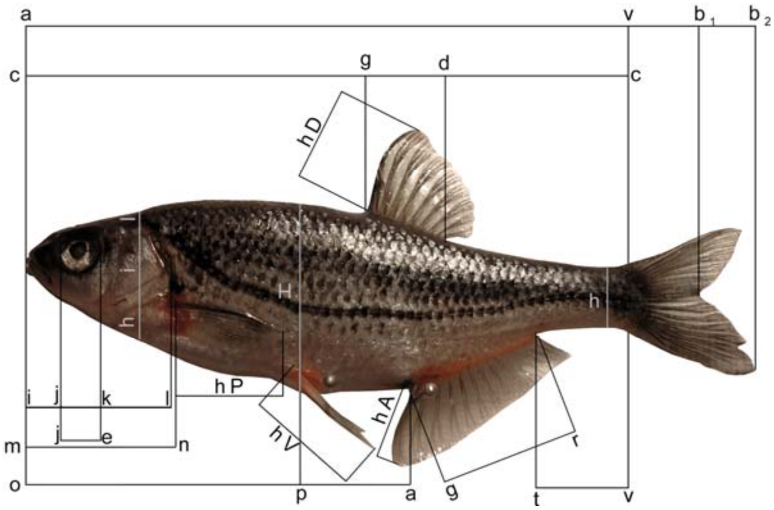

Figure 2. Morphometry of *A. bipunctatus*
a-b$_2$: Total length (*Longitudo totalis*)
a-b$_1$: Fork length (*Longitudo caudalis*)
a-v: Standard length *(Longitudo corporis)*
c-g: Proposal length *(Longitudo praedorsalis)*
g-d: Base length of dorsal fin *(Longitudo dorsalis pinnae)*
hD: Dorsal fin height *(Altitudo dorsalis pinnae)*
dc: Postdorsal length (*Longitudo corporis postdorsalis pinnae)*
i-l: Head length *(Longitudo capitis)*
i-j: Preocular distance *(Spatium preorbitale)*
j-e: Horisontal diameter of eye *(Diameter oculi horizonialis)*
k-l: Postocular distance *(Spatium postorbitale)*
h-i-l: Maximal head height *(Altitudo capitis)*
m-n: Prepectoral length *(Longitudo praepectoralis)*
o-p: Preventral length *(Longitudo praeventralis)*
o-a: Preanal length *(Longitudo praeanalis)*
g-r: Base length of anal fin *(Longitudo analis pinnae)*
hP: Pectoral fin height *(Altitudo pectoralis pinnae)*
hV: Ventral fin height *(Altitudo ventralis pinnae)*
hA: Anal fin height *(Altitudo analis pinnae)*
t-v: Caudal trunk length (*Longitudo pedunculi caudalis*)
H: Maximal body height *(Altitudo corporis maxima)*
h: Minimal body height *(Altitudo corporis minima)*

Site L2 – Tributary of Temska stream to Nišava River. Temska stream is the typical salmonid stream and the biggest right tributary of Nišava River. The tributary section is enveloped in thick jungle-like vegetation, and river bottom is rocky with cobble size stones. The average water discharge is 10.5 m^3 s^{-1} (Živić *et al*., 2005). Mouth of the tributary is about 4 m wide with depth of 0.5 m and the whole section is with light riffles. Benthofauna is almost exclusively composed of over dominant order Trichoptera with occasional representatives of Nematomorpha, Hirudinea, Odonata and Megaloptera (Živić *et al*., 2005). Ichthyofauna is similar to site L1 with addition of bleak *Alburnus alburnus* (L. 1758) - Cyprinidae, barbel *Barbus barbus* (L. 1758) – Cyprinidae and nase *Chondrostoma nasus* (L. 1758) - Cyprinidae.

Site L3 - Nišava River after town of Bela Palanka and before Sićevo Gorge. This section of river has biggest width, and it is essentially one long glide with mixture of sandy and muddy bottom with dept often over 1.5 m.

As site L4, the Nišava River before its entry of the major city of Niš was selected (suburbia Donja Vrežina). The selected site is located on the last section of river, about 12-15 km before its mouth. In this section the Nišava River is between 0.2 and 1.2 m deep and is a mixture of small riffle areas and stretches of slow moving water. The bottom is composed of gravel with copious *Cladophora sp.* algae clinging on the bottom. Benthofauna is composed predominantly of classes Gastropoda, Hirudinea, Clitellata and Malacostraca (Gammaridae). Icthyofauna is rich, with the predominant species such as: spirlin, barbel, brook barbel, chub, bleak, nase, perch *Perca fluviatilis* (L. 1758) – Percidae, bitterling *Rhodeus sericeus* (Pallas, 1776) - Cyprinidae, *Carassius gibelio* (Bloch, 1783) - Cyprinidae, carp *Cyprinus carpio* (L. 1758) - Cyprinidae, roach *Rutilus rutilus* (L. 1758) - Cyprinidae, gudgeon *Gobio spp.*, as well as other typical lowland cyprinid species.

Morphometry

Several hundreds of spirlin specimens were sampled at each site ignoring the differences in microhabitats and sampling along the whole profile section. After the sampling, 40 specimens > 60 mm were chosen at random from each of the sampling sites and 22 morphometric and 4 meristic characters were measured or counted. List of measured morphometric characteristics is provided in **Fig. 2**. All measurements were taken with 0.1 mm precision using vernier. List of meristic features counted is outlined in **Fig. 3**. In adition mass of specimens were recorded using digital scale (KERN 440’33, readability d = 0.01 g).

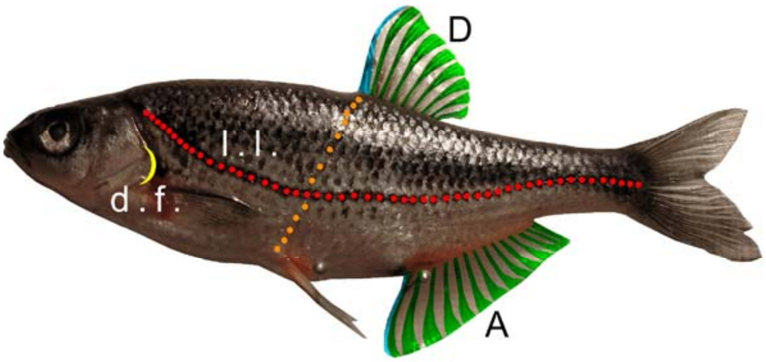

Figure 3. Meristic characters of *A. bipunctatus*.
D: Number of rays in dorsal fin - separately for hard and soft *(Numerus radiorum pinnae dorsalis)*
A: Number of rays in anal fin - separately for hard and soft *(Numerus radiorum pinnae analis)*
ll: Number of sacles in the lateral line *(Squamae linea lateralis)* shown in red; number of scales above and below the lateral line *(Squamae linea trasversalis superior et Squamae linea trasversalis inferior)* shown in ocher.
Df: Number of pharyngeal teeth (*Dentes pharyngeales)*

Statistics

For morphometry data sample from each sampling site was divided into two size categories based on the total length of the individuals: category A 60 - 80 mm; category B 80 - 100 mm. Similar approach was previously used with success to assess difference in morphometry characteristics in mixed age sample for spirlin (K o v a č *et al.*, 2006; C o p p *et al.*, 2010). There were only five specimens in total longer than 100 mm (the longest was 122 mm) and these specimens were discarded from any further analysis. Analysis of variance (ANOVA) was used to depict any differences in characters between different sampling sites for each category separately followed by Tukey – HSD post hoc comparison of means test. Only values with $P < 0.05$ were considered statistically significant.

Results

Morphometrics

Morphometric results for each category are presented in **Tab. 1**. Total length of specimens in category A did not varied significantly between sampling sites (ANOVA; df total = 63; F = 2.50; $P > 0.05$); however there was a significant difference in morphometry of 9 out of 22 characters (**Tab. 2**). Standard length (a-v) of specimens from site L1 was significantly smaller than the ones from L2 and L4 indicating a longer tail fin. Specimens from L1 had shorter base of the dorsal fin (g-d) comparing to L4 specimens; L1 specimens also had shorter postdorsal length (dc) comparing to the L2 and L4 specimens. There was a small difference in preventral length (o-p) and preanal length (o-a) with o-p being shorter in L1 compared to L2 specimens and o-a being shorter in L1 as opposed to L4. Caudal trunk length (t-v) was also shorter in L1 vs. L2 specimens. The most observable differences were for postocular distance (k-l); anal fin height (hA); and minimal body height (h) (**Fig. 4**). Postocular distance from L4 is significantly smaller comparing to specimens from any other sampling site. Anal fin height was considerably bigger in L2 specimens comparing to any other sampling location while minimal body height of L1 was significantly smaller comparing to other samples. Mass of the specimens varied slightly between localities for category A (ANOVA; df total = 63; F = 4.59; $P < 0.01$). Average mass with standard deviation of the specimens from L1-L4 were: 3.31 ± 0.74; 4.64 ± 0.53; 4.24 ± 1.26; and 3.95 ± 1.02 g respectively.

Category B significantly varied in total length between sampling locations (ANOVA; F = 13.04; df total = 82; $P << 0.001$) (**Fig. 5**). Specimens from L1 and L4 sites had the same mean total length and were significantly different from L2 and L3 specimens. Sample L2 and L3 had the approximately the same mean total length. Since there was obvious difference in total length the rest of 21 morphometric features were compared only between the samples with the same mean. L1 vs. L4 and L2 vs. L3. Results are presented in **Fig. 6**. Mass of the specimens varied significantly between localities for category B (ANOVA; df total = 82; F = 16.55; $P < 0.001$). Average mass with standard deviation of the specimens from L1-L4 were 6.34 ± 1.23; 8.19 ± 1.65; 9.32 ± 1.46; and 6.42 ± 1.20 g respectively.

Meristics

Number of scales in lateral line did not varied significantly between sampling sites (ANOVA; df total = 159; F=0.41; $P >> 0.05$) and the average scale number formula for the whole river profile is 43-50 $^{8-10}/_{3-5}$. There was no difference in average number of hard or soft rays in the dorsal fin between sampling sites (ANOVA; df total = 159;

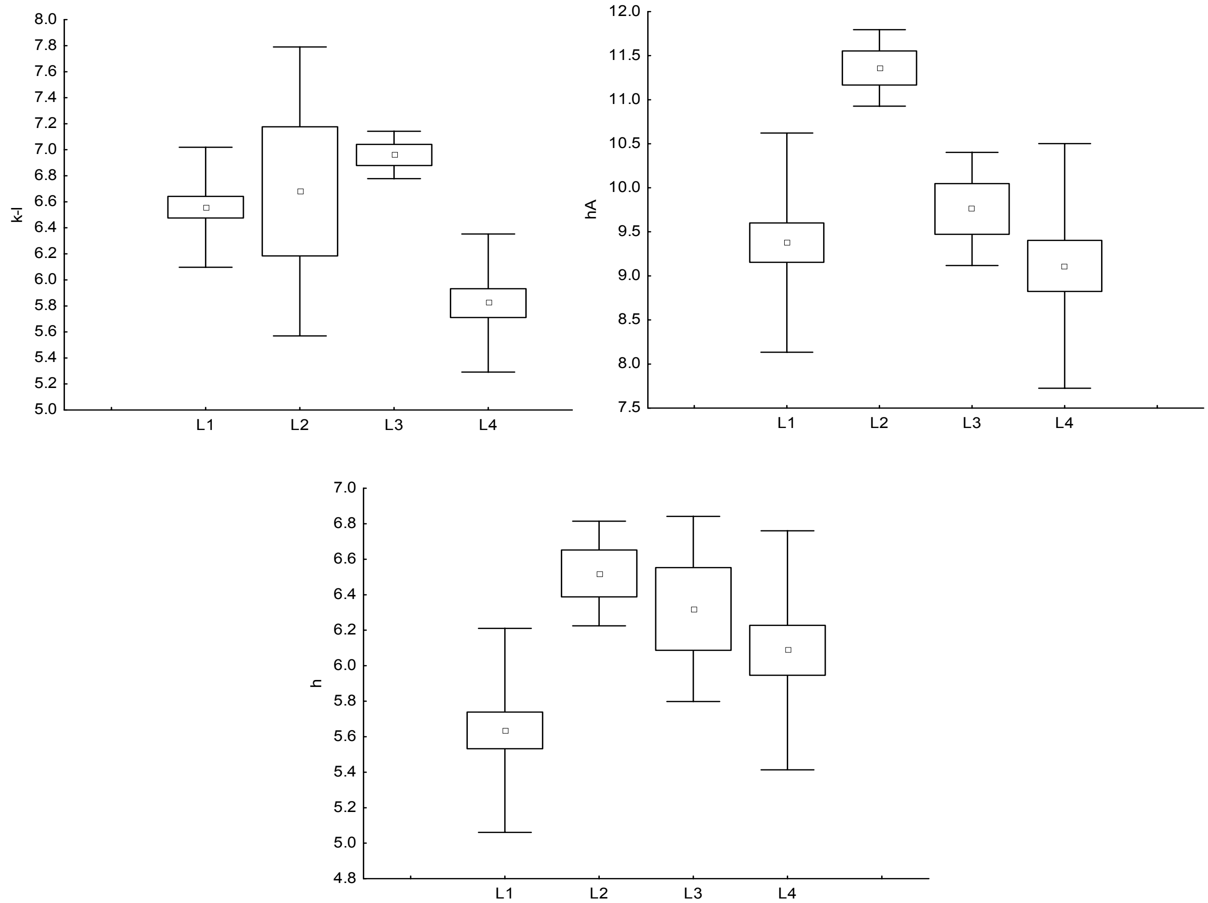

Figure 4. Difference in *A. bipunctatus* category A morphometry characteristics between four sampling locations. Y axis is in mm corresponding to the proper measurement (Figure 1). L1 – L4 are different sampling locations. Boxes refer to standard error and whiskers to standard deviation

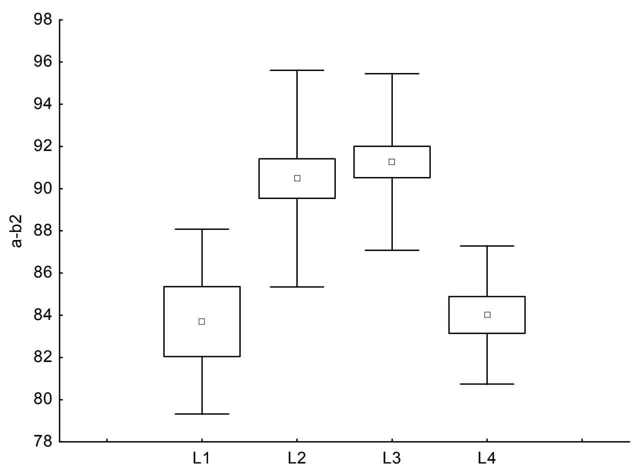

Figure 5. Distribution of *A. bipunctatus* category A total length morphometry across four sampling locations. Y axis is in mm of total length. L1 – L4 are different sampling location. Boxes refer to standard error and whiskers to standard deviation

Table 1. Morpfometric analysis of *A. bipunctatus*. All data except column % are in mm. % refers to the percent of given character compared to total length of the specimen. STD refers to standard deviation.

	Category A					Category B				
	Mean	%	STD	Min	Max	Mean	%	STD	Min	Max
a-b_2	70.9	100.0	5.3	60.7	79.0	89.1	100.0	5.3	80.0	100.0
a-b_1	64.5	91.0	4.9	54.5	72.4	81.4	91.4	5.1	72.1	96.0
a-v	58.0	81.8	4.5	50.0	65.7	73.3	82.3	4.6	64.2	83.0
c-g	30.6	43.2	2.7	25.5	37.3	38.6	43.3	2.8	33.0	44.5
g-d	7.29	10.3	0.9	5.2	9.7	9.4	10.5	0.8	7.7	11.5
hD	11.7	16.5	1.2	8.2	14.2	15.2	17.1	1.4	12.0	19.0
d-c	19.7	27.8	1.8	16.0	23.6	24.6	27.6	1.9	20.0	29.0
i-l	14.5	20.5	1.2	12.0	17.0	18.0	20.2	1.2	14.0	20.6
i-j	3.9	5.5	0.6	2.6	5.2	4.9	5.5	0.4	4.0	6.0
j-e	4.2	5.9	0.5	3.3	6.5	5.0	5.6	0.3	4.0	5.7
k-l	6.3	8.9	0.7	4.9	8.0	8.0	9.0	0.9	4.9	10.1
h-i-l	11.1	15.7	0.9	9.5	13.0	13.9	15.6	1.0	12.2	16.7
m-n	14.5	20.5	1.3	11.8	17.4	18.1	20.3	1.4	14.0	21.3
o-p	27.1	38.2	2.8	20.0	32.6	34.3	38.5	2.3	28.6	40.0
o-a	37.2	52.5	3.0	30.5	43.0	47.5	53.3	3.6	40.0	57.2
g-r	9.6	13.5	1.2	7.0	12.0	12.6	14.1	1.3	10.0	17.0
hP	11.2	15.8	1.1	8.6	13.2	14.2	15.9	1.2	12.0	17.4
hV	8.8	12.4	1.0	7.0	11.4	11.0	12.3	1.1	9.0	13.0
hA	9.5	13.4	1.3	6.0	12.0	11.8	13.2	1.3	9.0	15.2
t-v	11.9	16.8	1.3	9.7	15.2	15.1	16.9	1.3	12.7	18.0
H	15.7	22.1	1.6	12.6	20.0	21.0	23.6	2.1	16.4	25.0
h	5.9	8.3	0.67	4.4	7.0	7.5	8.4	0.7	5.8	9.0

Table 2. Analysis of variance and post hoc comparison of means of *A. bipunctatus* category A morphometry. L1 – L4 refers to the sampling site. +; ++; +++ means that the effect is statistically significant with *P* value being < 0.05; < 0.01; and < 0.001 respectively. NS means that the effect is not statistically significant.

		a-v	g-d	dc	k-l	o-p	o-a	hA	t-v	h
p - value ANOVA		+	+	+	+++	+	+	++	+	++
p – value Tukey HSD	L1 vs. L2	+	NS	+	NS	+	NS	++	+	+
	L1 vs. L3	NS	NS	NS	NS	NS	NS	NS	NS	+
	L1 vs. L4	+	+	+	+++	NS	+	NS	NS	+
	L2 vs. L3	NS	NS	NS	NS	NS	NS	+	NS	NS
	L2 vs. L4	NS	NS	NS	+	NS	NS	++	NS	NS
	L3 vs. L4	NS	NS	NS	+++	NS	NS	NS	NS	NS

Table 3. Distribution of hard and soft rays in the anal fin of *A. bipunctatus* from Nišava River. L1 – L4 refers to sampling locations. Roman numerals refer to hard rays while Arabic refers to soft rays. Data are presented as % of sample.

	II	III	IV	10	11	12	13	14	15	16
L1	12.5	87.5	0	0	0	5	45	37.5	12.5	0
L2	12.5	82.5	5	0	0	2.5	12.5	42.5	37.5	5
L3	5	92.5	2.5	0	0	10	30	50	10	0
L4	5	95	0	2.5	0	15	17.5	50	12.5	2.5
Average	8.75	89.38	1.875	0.625	0	8.13	26.3	45	18.125	1.875

Table 4. Distribution of pharyngeal teeth of *A. bipunctatus* from Nišava River. L1 – L4 refers to sampling locations. Data are presented as % of the sample.

	Left branchial arch					Right branchial arch				
	3	4	5	6	7	3	4	5	6	7
L1	12.5	22.5	30	27.5	7.5	5	7.5	20	17.5	50
L2	2.5	17.5	35	35	10	5	12.5	20	25	37.5
L3	5	10	35	45	5	0	2.5	5	12.5	80
L4	2.5	17.5	37.5	37.5	5	5	5	35	37.5	17.5
Average	5.625	16.875	34.375	36.25	6.875	3.75	6.875	20	23.125	46.25

F = 0.06; P >> 0.05). The overall number of rays in dorsal fin was D, II-III + (7-8) 9 (10). Number of hard rays in anal fin did not varied statistically among the samples (ANOVA; df total = 159; F=0.84; *P* >> 0.05), however for the first time in science a fourth hard ray in anal fin was detected for this species which has never been documented elsewhere. The fourth hard ray was present only in the specimens from sampling station L2 and L3. On the other hand, number of soft rays in the anal fin did varied significantly among sampling stations (ANOVA; df total = 159; F = 5.61; *P* < 0.01). There was significantly higher number of soft rays in the specimens from L2. Distribution of hard and soft rays from anal fin across sampling stations is presented in **Tab. 3**. Formula of anal fin rays is: A, (II) III (IV) + (10) (12) 13-15 (16).

Pharyngeal teeth of spirlin are in two rows. First row have two teeth, while the number of teeth in second raw is variable. Specimens from L3 had more pharyngeal teeth on the right branchial arch than specimens from any other sample (ANOVA; df total = 159; F = 9.08; *P* < 0.001). Commonly these specimens had 7 teeth on the right branchial arch while specimens from other samples had 5 or 6 (**Tab. 4**).

Discussion

It is important to note that for the overall sample (category A + B) individuals from L2 and L3 on average have the biggest total length and mass. Also, the biggest total length of any individual was 120 mm for L2 and 104.6 for L3 while the same parameter was only 90 mm for L1 and 91 mm for L4. One of the possible reasons for this might be better feeding conditions on sites L2 and L3. The studies performed in the fair vicinities of these localities demonstrated abundance of diatoms (Trajkovic *et al.*, 2008) which are spirlin's primary food item (Treer et al., 2006) as well as caddis flies (Živić *et al.*, 2005) which are secondary food item (Treer et al., 2006). Better feeding can lead to faster growth rate which for this species is particularly characterized with two periods of isometric growth with different proportional values interrupted with short interval of allometric growth (Kovač *et al.*, 2006). Thus, a difference in a rate of allometric growth can result in drastic changes of morphometry. In general, L2 and L3 spirlin had biggest anal fin height, as well as the biggest maximal body height and maximal observed total length, indicating better growth than spirlin

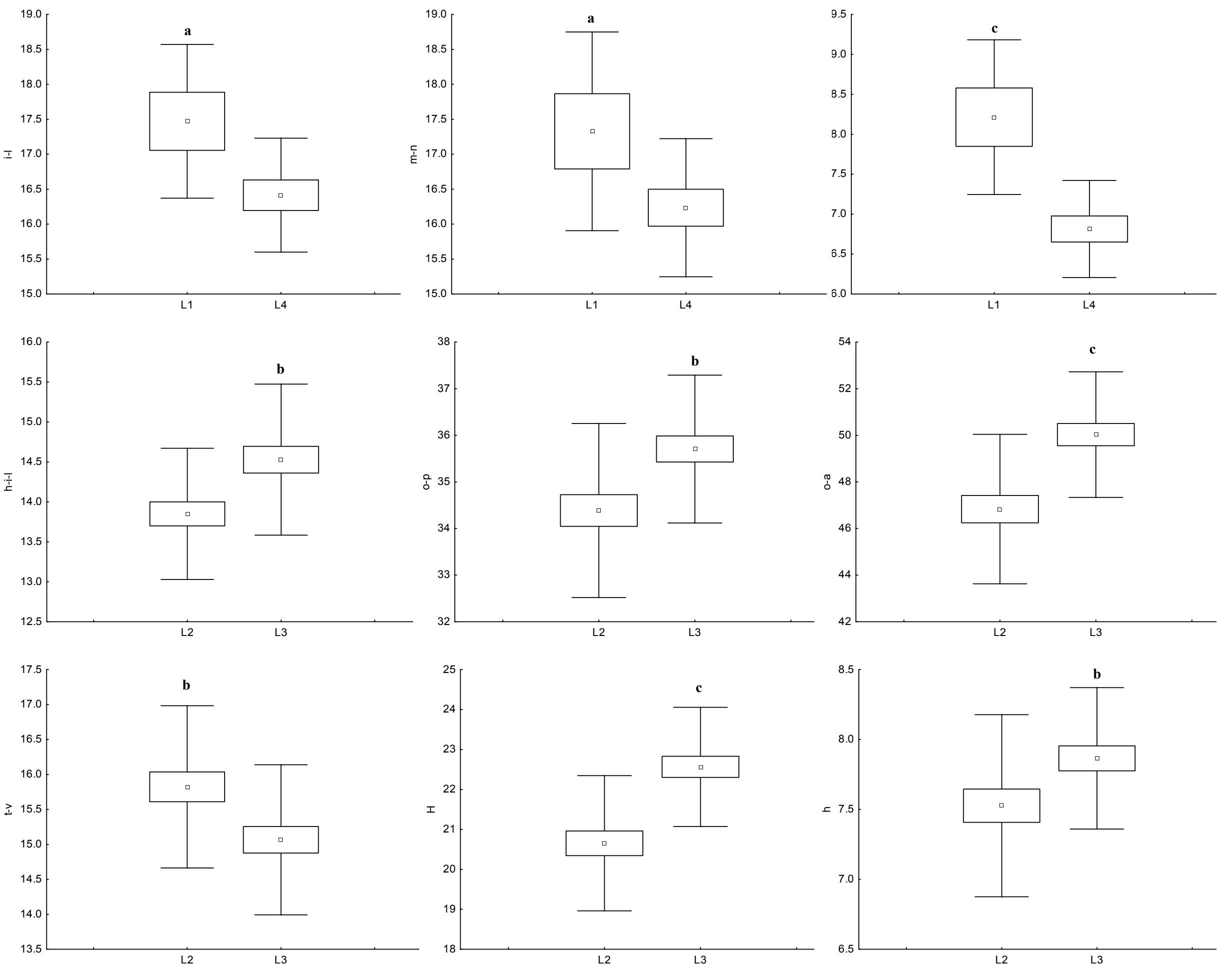

Figure 6. Difference in *A. bipunctatus* category B morphometry characteristics. Y axis is in mm corresponding to the proper measurement (Figure 1). L1 – L4 are different sampling locations. Boxes refer to standard error and whiskers to standard deviation

from other localities. Spirlin of both categories from the L1 locality compared to spirlin from other samples had shorter base of the dorsal fin, shorter postdorsal, preventral, preanal, caudal trunk and minimal body length while having longer head, post ocular and prepoctaral length and theoretically longer tail fin. Essentially this gives them a streamlined, torpedo-like shape with powerful head to pectoral region and slender, narrow streamline body for propelling themselves through the faster water current. Fish with more streamline body have less drag (by minimizing viscous force) in a faster current, spending less energy by utilizing continuous, rather than burst-and-coast swimming mode (Müller *et al.*, 2000; Petrell & Jones, 2000).

Results of this investigation suggest that morphometry of spirlin can vary significantly for some characters, while others are not affected. Comparison of this study with previous investigation of nominate subspecies features inhabiting different mesohabitat type (River Gradac) identified head length (i-l); maximal head height (h-i-l); base length of dorsal fin (g-d); caudal trunk length (t-v); maximal body height; and minimal body height (h) as characters with biggest variability (Stanisavljević *et al.,* 1999).

In conclusion, it appears that spirlin express a big morphological plasticity in relation to spatial and mesohabitat distribution. Whether the plasticity was based on the influence of environmental factors, or it resulted from the expression of an organism's genes as well as the interactions between the two so far is not known. However, it is known that the karyotype of spirlin varies significantly on a spatial scale (Kilic-Demirok & Ünlü, 2004).

Coupled with the number of known subspecies (Lelek, 1987); the number of newly discovered sister species which were until recently classified as nominate subspecies (Bogutskaya *et al.*, 2010; Coad & Bogutskaya, 2009); and the new meristic characteristics presented in this manuscript it is reasonable to conclude that taxon *A. bipunctatus bipunctatus* is most likely complex of cryptic species/subspecies/neospecies in formation/morphotypes/ecotypes rather than a single nominate subspecies. It is possible (especially for species in formation) that species complex has the same genetic material (genome) which can be accounted for successful reproduction among different members, but completely different gene expression (transcriptome). It is hard to carry out such experiments for the species for which genome is not yet sequenced and most of the time pieces of information can be obtained through Expressed Sequence Tag (EST) sequencing coupled with genomics which is a laborious process based on pure luck of proper EST extraction (Jovanović *et al.*, in press). However, the recent advances in "Next Generation" sequencing platforms and technologies, for quantitative comparative transcriptomics allowed for successful differentiation in the hepatic transcriptomes between two lake trout ecotypes, and demonstrated that this approach could be used to simultaneously discover and quantify differentially expressed genes in a nonmodel organism lacking a sequenced genome (Goetz *et al.,* 2010). This would be the right approach to discern whether spirlin from Nišava River is a complex of different transcriptomic populations/ecotypes or not, and determine the level of crypticism within a complex if any.

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