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Microhabitat preferences of triploid *Cobitis* fish and diploid progenitors in two streams in Slovakia (Danube River Basin)

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ABSTRACT

Loaches (genus *Cobitis*) in Slovakian tributaries (Danube River Basin) are represented by diploid-polyploid hybrid complexes of *C. elongatoides* and *C. tanaitica*. Uniquely, their coexistence is made possible by the presence of polyploid (mainly triploid) females that are sexually dependant on diploid male sperm (donors) for gynogenesis. Information on the spatial distribution of *Cobitis* with of differing ploidy remains relatively scarce. The main aim of this study, therefore, was to identify in ploidy in the species preferred microhabitat. Overall, 345 *Cobitis* were recorded in Rivers Okna and Ondava (Danube Basin) between 2011 and 2015, with 316 diploid and triploid individuals detected by flow cytometry examined were studied for the microhabitat preferences with the using the point sample method. The sex ratio differed significantly between localities, while diploid-polyploid ratio not. Moreover, each of ploidy-sex forms (i.e. diploid male, diploid female, triploid female) showed a preference for a specific substrate composition, velocity, depth and distance from the bank at both localities. Mixed effect linear models identified clear differences in fine substrate between seasons for individuals from the River Okna. Similarly, non-metric multidimensional scaling detected differences in environmental variables preference for both diploid and triploid forms. These finding indicate specific spatial and temporal microhabitat preference for diploid (sexual donors) and triploid (asexuals) *C. elongatoides* × *C. tanaitica* when coexisting as mixed a population.

1. Introduction

Spined loaches (genus *Cobitis*) are small bottom living fish species widespread in the Europe, Asia and North Africa (Kottelat and Freyhof, 2007, Kottelat, 2012). While many *Cobitis* sp. form "pure" diploid (sexual) populations (Boroń, 1992, Šlechtová et al., 2000), many European (e.g. Bohlen and Ráb, 2001, Janko et al., 2007) and Asian population (Ko et al., 2015) have now confirmed occurrence of mixed (hybrid) diploid-polyploid complexes (Vasil'ev and Vasil'eva, 1982).

Cobitis population in Slovakia (Danube River Basin) consist of diploid-polyploid hybrid complexes of *Cobitis elongatoides* (Băcescu and Mayer, 1969) and *Cobitis tanaitica* (Băcescu and Mayer, 1969) (Lusk et al., 2003; Lusková et al., 2004; Papoušek et al., 2008). The complex is made up of: 1) diploid sexually reproducing males and females of *C. elongatoides* (sexuals); 2) triploid and tetraploid (gynogenetically reproducing) females with two haploid genoms of *C. elongatoides* and one or two haploid genomes from *C. tanaitica* (Lusková et al., 2004). 3) rarely present are also triploid and tetraploid males which are probably sterile (Juchno et al., 2017). Presence of diploid hybrids with one haploid genome of *C. elongatoides* and one from *C. tanaitica* is not very likely. Formation of asexual (diploid) hybrids assume the presence of another maternal (sexual-diploid) *C. tanaitica* species, which was never been detected from the tributaries of Slovakia (Lusk et al., 2000; Lusková et al., 2004; Papoušek et al., 2008). Geographical distribution of *C. tanaitica* is more restricted for lower most part of Danube River (Janko et al., 2005; Kottelat and Freyhof, 2007). And globally, the presence of diploid hybrids in mixed populations is very rare (Boroń, 2003; Kotusz, 2008) and is conditioned by presence of minimum 2 maternal species.

Generally, triploid and tetraploid (asexual) females reproduce by

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gynogenesis and are depend on suitable parental "sperm donors" (Vasil'ev and Vasil'eva, 1982). The clones produced by such a union are capable of expanding beyond their original hybrid zones (Janko et al., 2005; Choleva et al., 2008). Overall, the asexuals (polyploids) are forced to coexistence with no suppression of their sexual (diploid) donors (Beukeboom and Vrijenhoek, 1998), what propose diversification of ecological niche and minimizing the overlapping of them. The ratio of diploid to polyploid *Cobitis* will be influenced by local environmental condition, which vary in different river zones (Lusk et al., 2000; Šlechtová et al., 2000; Lusková et al., 2004).

Individual *Cobitis* species are associated with different substrate types such as sand and mud (Delić et al., 2009), or clay and gravel flow (Bohlen et al., 2003), or with particular aquatic macrophytes assemblages (Robotham, 1978). Most *Cobitis* species are now protected (Koščo et al., 2014) due to the loss or deterioration of their specific habitat (Przybylski et al., 2002; Culling et al., 2003; Pekárik et al., 2008). Slovak populations have been protected under Slovak law since 2004 (Decree No. 24/2003, Law No. 543/2002C. of L.).

In this study, we identify differences in the ecological parameters characterising the niche of *Cobitis* individuals with different sex and ploidy forms (sexual males and females, asexual (triploid females) coexisting under natural conditions. In this sense, we define following objectives:

1.) determinate the key microhabitat factors limiting the distribution of the ploidy-sex forms (diploid males, diploid females, triploids (asexual) females)
2.) verified the seasonal impact (e.g. monthly, yearly) to microhabitat preferences of ploidy-sex forms.

2. Materials and methods

2.1. Study area and sampling

On the basis of previously gathered distribution data (Koščo et al., 2008), we based our study on two contrasting rivers in the east of Slovakia (Tisa River Basin), the 4 m wide River Okna and 48 m wide River Ondava (geographical and hydrological characteristics provided in Table 1). All fish were sampled using electrofishing gear ((200–300 V, 0.2–0.5 A) between 2011 and 2015 (River Ondava 2011–2013, River Okna 2014–2015). At each positive sample point (i.e. *Cobitis* present) microhabitat characteristic (Table 2.) were recorded using the point sample method of Copp and Peňáz (1988) modified according to Pekárik et al. (2012). Maximal velocity at the positive sample point (presence of fish = 1 measurement of velocity) were measured 5 cm above the bottom with the using of flow probe. All sampling points were chosen to include all available microhabitat types. During sampling, fish were stored separately in labelled containers corresponding to each sampling point. Immediately after sampling, the fish were anaesthetised with solution of clove oil, measured to the nearest 0.1 mm (standard length, SL) and a fin clip taken and fixed in 70% alcohol for laboratory analysis of ploidy. Sex was determined morphologically through visual presence of Canestrini's scales (*lamina circularis*) on the pectoral fin, and by the more pointed shape of the male's pectoral fins (Kotusz, 2008; Buj et al., 2015). All fish were then released back to the stream.

In all cases, fish sampling was authorised through a permit from the Ministry of Environment and special yearly permits for electrofishing.

2.2. Ploidy determination

Level of ploidy was determined as the DNA content of fin clip cells via flow cytometry (Partec CCA I; Partec GmbH, Munster, Germany) using 49.6-diamidino-2-phenylindol (DAPI). As a reference standard, we used fresh blood from coloured form of diploid goldfish (*Carassius auratus*). Fish evaluated for microhabitat preferences was categorised as either: 2 m − diploid male, 2f − diploid female or 3f − triploid female. Conversely, polyploid males (3 m − triploid, 4 m − tetraploid), tetraploid females (4f) and individuals disposing with both triploid and tetraploid cells (3 + 4) were excluded from further analysis due to their low frequency.

2.3. Data analysis

The diploid-polyploid (D:P) ratio was assessed using the chi-square test. Differences between ploidy-sex forms (2m, 2f, 3f) was analysed both on the overall data matrix of environmental variables (EV) and for each EV separately. Autocorrelations were tested for according to Fox (2016), variables with correlation coefficients > 0.7 being excluded from the analysis as highly autocorrelated. The effect of each ploidy-sex form on the overall EV set was assessed using permutational multivariate analysis (PERMANOVA) using Euclidean distance and 9999 permutations. Permutations were restricted for each year separately to account for yearly replications in the samples. Non-metric multidimensional scaling (NMDS) was used to reduce the *n*-dimensional set of EV into two dimensions. The envfit function (vegan package, R software ver. 3.1.2; Oksanen et al., 2017) of vegan package and squared correlation coefficient (R^2) were used for measuring of environmental vector importance for each ploidy-sex form. Differences in season microhabitat preferences for each ploidy-sex form was analysed using the Generalised Linear Mixed Models (GLMM), with presence or absence of each ploidy-sex form modelled using binomial error distribution. Replications within years or months were controlled for by adding year to the random part of the model. Multi-model inference, using the Akaikéinformation criterion corrected for small sample sizes (AICc), was used to select the best combination of explanatory EV for individual linear models. The effect of sampling month on microhabitat selection from the Okna site was assessed by adding month as a two-way interaction term to each EV. Multi-model selection was undertaken using the glmulti package (Calcagno, 2013) and the lme4 package (Bates et al., 2015) was used for GLMM. The results of the selected models were visualised using visreg (Breheny and Burchett, 2013). All analyses were performed using R software (ver. 3.1.2) and packages therein, with $\alpha = 0.05$ as the level of significance for all tests.

3. Results

Altogether, 345 loaches were sampled between 2011 and 2015 (Table 3). Of these 316 fish (81 from the Ondava and 235 from the Okna) were analysed for differences at the microhabitat preferences

Six ploidy-sex forms were recorded from the River Okna between 2014 and 2015, and from the River Ondava between 2011 and 2013 also. Both populations had similar M:F sex ratios (Okna 1:2.3, Ondava 1:3), with both differing significantly from 1:1 (Okna, $\chi2 = 38.1$, df = 1, $P < 0.001$; Ondava, $\chi2 = 23$, df = 1, $P < 0.001$). The D:P ratio (Table 4) also differed significantly from 1:1 in the Okna (2.3:1; $\chi2 = 36.5$, df = 1, $P < 0.001$), but not in the Ondava (1.2:1;

Table 1
Geographical and hydrological characteristics of the sampling sites.

River	No. samples	Coord. N, E	m a. s. l.	Width (m)	Period	x̄ Cond. μS.cm^{-1}	x̄ Temp.°C	x̄ O_2 mg l^{-1}	x̄ pH
Okna	8	48.716, 22.119	103	4	2014–2015	200	17.7	10.1	7.8
Ondava	3	48.702, 21.776	104	48	2011–2013	324	18.9	8.5	8.3