



# What can we infer from the shell dimensions of the thick-shelled river mussel *Unio crassus*?

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**Abstract** We studied shell variation in the thick-shelled river mussel (*Unio crassus* Philipson, 1788) sampled from two sites of very different character: (1) a rocky channel (San river) and (2) a fine-sediment channel (Zborowianka river). The analyzed mussels differed significantly between the sampled channels in almost all analyzed phenotypic traits and shape indices. Intersexual variation was so low that it cannot be used effectively for sexing in this species. The growth rate was much higher in the fine-sediment and nutrient-rich channel than in the rocky one, but the asymptotic shell length for the rocky channel was larger than for the fine-sediment channel, suggesting higher survival in the mountainous, unmodified river. Shell size differed significantly within the rocky channel, depending on microhabitat: shell size and dorsal arching were much greater in mussels living in the strong current of the rocky midchannel than in those inhabiting still water at the nearest bank. The results demonstrate that microhabitat conditions significantly determine shell shape.

**Keywords** Morphology · Microhabitat · Phenotypic plasticity · Unionidae · Taphonomy · Energy allocation · Sexual dimorphism

## Introduction

Mollusca is the second-largest phylum on the Earth (Bank et al., 2014). The most characteristic feature of mollusks is their shell, the shape of which varies among taxa, partly reflecting their phylogenetic history and partly the habitat where they are living (Bogan, 2008; Perez & Minton, 2008). The shells and their characteristics are very useful in paleobiology and evolutionary biology because they form a rich fossil record (Sparks, 1961) furnishing invaluable information on the phylogenesis of this group, its past biodiversity (e.g., Kidwell, 2001, 2002), and extinction risk (Harnik, 2011). Because mollusks' relation to their paleohabitat can be extrapolated from the ecology of contemporary species, the ecological conditions of past geological epochs can be inferred from fossil shells (Leonard-Pingel et al., 2012, Yanes, 2012). The shells themselves have been used as environmental archives, and they also contain metabolic signals (Mutvei & Westermark, 2001; Geist et al., 2005a). Due to the dietary and cultural role of mollusks, shells can be used in archeology to assess the character of habitats exploited by ancient humans (Morey & Crothers, 1998).

Freshwater mussels form one of the most important groups of mollusks (Graf & Cummings, 2007); they

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can dominate the biomass in some freshwaters, and they perform key ecological functions (Gutierrez et al., 2003; Aldridge et al., 2007). Some species of the freshwater family Unionidae are widespread, but the whole family is exposed to large-scale threats to their existence (Lydeard et al., 2004). Despite their ubiquity and vulnerability, they are still among the under-studied groups of animals.

In the past, a confusingly huge number of Unionoida species were described, mainly on the basis of morphology (ca 4000 named species in the Nineteenth century, 1300 species at the beginning of the Twentieth century; Graf & Cummings, 2007). Recently the number of species has been greatly reduced—with only 16 for Europe (Lopes-Lima et al., 2016)—but the enormous morphological plasticity of the shells (e.g., Melnychenko et al., 2004; Gural & Gural-Sverlova, 2008) remains a problem in need of explanation, as it still leads to many cases of misidentification (Shea et al., 2011; Morais et al., 2014), hampering research on species distribution, conservation, and invasiveness (Sousa et al., 2007; Guarneri et al., 2014).

Variation of shell morphology can be explained on the basis of genetic factors and/or environment influences, and the interaction between them (Falconer & MacKay, 1996). Morphological differences may arise from genetic isolation of particular populations (e.g., by geographic distance); shell morphology may also be phenotypically plastic and shaped mainly by the environment (Zieritz et al., 2010). Many studies have reported a lack of match between intraspecific morphological and genetic patterns (Buhay et al., 2002; Machordom et al., 2003; Alvarez-Molina, 2004; Geist & Kuehn, 2005b; Zieritz et al., 2010; Guarneri et al., 2014). In considering environmental drivers, however, it needs to be remembered that the plasticity of the response of phenotype to the variability of environmental conditions also has some genetic basis (Via et al., 1995) and cannot be treated as a kind of environmental noise disturbing the relation between heredity and phenotype (Pigliucci, 2005). Research on environmental factors influencing shell morphology should focus on their influence on individual ontogeny, leading to different phenotypes in different environmental conditions.

It is widely accepted that the individual growth of mussels follows the pattern of a von Bertalanffy curve (Hochwald, 2001; Haag, 2009). This pattern is explained by the theory of allocation of energy to

growth and reproduction (Kozłowski, 1992; Haag & Rypel, 2011), according to which after reaching some critical size, it pays to allocate more and more energy to reproduction, at the expense of growth. The question of the factors governing allocation of energy is basic to many ecological and conservation biology studies of mussels (Aldridge, 1999; Haag & Staton, 2003): predictions of age, growth pattern, size at maturity, and the growth asymptote are crucial to understanding the ecology of mussels (e.g., Jokela & Mutikainen, 1995; Czarnoński et al., 2003; Ren & Ross, 2005) and population processes (e.g., Hastie et al., 2000). Freshwater mussels are good model organisms for this sort of study, as their age can be read from the yearly increments of shell growth (Negus, 1966).

The factors influencing mussel shell size and shape may be related to adaptations specific to this systematic group. Females of Unionidae store very large numbers of developing larvae, called glochidia, in the outer pair of gills, transformed into structures called marsupia. When filled with larvae, the marsupia are large, thick structures located inside the shell, which should affect its shape (e.g., *Anodonta anatina* (Linnaeus, 1758); Zieritz & Aldridge, 2011). The glochidia stored in marsupia should require increased oxygen consumption; this should be compensated by enlargement of the interior space, measurable as greater width of the female shell. However, the effect of sex is rarely studied in mussels (Aldridge, 1999, but see Haag & Rypel, 2011).

Lastly, we call attention to the influence of habitat on shell morphology, which is far from being explained for the huge variety of freshwater habitats and related adaptations in mussels. In the pearl mussel *Margaritifera margaritifera* (Linnaeus, 1758), shell growth rates differ between northern and southern parts of its range (Bauer, 1992; San Miguel et al., 2004; Helama & Valovirta, 2008; Varandas et al., 2013), and there is a marked influence of general characteristics of rivers: water temperature and productivity (Hastie et al., 2000). Hochwald (1997, 2001) found that body growth parameters of *U. crassus* were almost exclusively correlated to each other and to temperature, with no relation to other environmental factors. Haag & Rypel (2011) reported intraspecific differences in growth rates in many North American species and also demonstrated effects of hydrological conditions on growth. The relative height and

“obesity” of the shell are suggested to reflect the anchorage ability of mussels, influenced by river discharge (Hornbach et al., 2010). In lakes, Amyot & Downing (1991) found water-depth-related differences in shell morphology. Green (1972) and Green et al. (1989) found that exposure to water energy and turbulence caused variation of shell morphology in *Lampsilis radiata* (Gmelin, 1791) in lakes; such variation has also been linked to sediment type (Hinch et al., 1986).

As so many aspects of mussel biology are reflected in shell morphology, it would be worthwhile to identify which traits reflect influences of age, sex, and environmental conditions, and to determine whether the growth rate is influenced by these factors, as Hochwald (2001) posited. To test that suggestion, we studied the shell morphology of one of the most threatened European mussels, *U. crassus* (Lopes-Lima et al., 2016). We examined the effects of age, sex, and environment (two contrasting microhabitats) on its shell growth and dimensions.

## Study area and methods

The study was conducted at two sites (Fig. 1): (1) the Zborowianka river (tributary of the Biała Tarnowska river) in the Carpathian foothills, near Góra Wieś village and (2) the San river in the Bieszczady Mts., near Żurawin and Procisne villages. The sites are 140 km apart, but both are in the northern part of the Carpathians (SE Poland) within one geological structure (Carpathian flysch), with most of the rivers still inhabited by numerous populations of *U. crassus* (Zajac, 2004).

The Zborowianka river flows through a narrow valley (ca 110 m wide, 262 m a.s.l. at study site) with a flat bottom. Its channel (5–7 m wide) is a typical pool-riffle structure, slightly meandering, eroded in fine sediment deposited on the flat valley bottom, with sparse riffles built of fine gravel (<10 cm) or fragments of bare rock exposed by lateral erosion of the channel. Slow-flowing water occurs in long deep pools (flow velocity 0.04 m/s, <1 m depth at low water level) between shallow riffles (<15 cm depth). It harbors a population of ca 10,000 *U. crassus* (unpubl. data).

The San river is a typical natural mountain river (ca 542–554 m a.s.l. at study site) with a plane-bed

channel according to Montgomery & Buffington’s (1998) classification, ca 30 m wide, constrained by the geological structure of the hills. The bed substrate is partly bare rock and partly rock debris and gravel, with low sandy banks covered with *Carex fusca* Reichard and *C. silvatica* Hudson. The river bed is shallow (<30 cm depth at usual water level), with fast flow (0.3–0.5 m/s in midchannel) and turbulence (diversified bottom relief). The studied reach harbors a population of ca 30,000 *U. crassus* (unpubl. data).

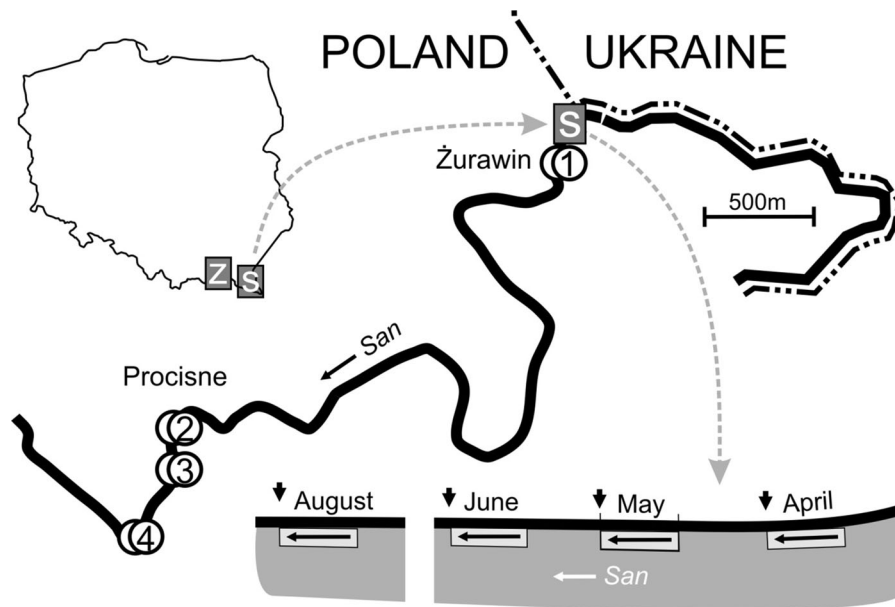
## Sampling

### *Zborowianka river*

*Unio crassus* individuals (no other Unionidae occur in this watercourse) were sampled at one reach (49°44′49.12″N, 20°58′59.18″E, 262 m a.s.l.) where mussels occur at high density in river pool banks (30–50 individuals per 1 m of one side bank). Twelve samples containing 245 mussels in total (age over 4 years) were collected after ice melting (March–April) approximately every week in 2013 until the end of the breeding season, excluding periods of high water level. On each occasion, a sample was collected from the bank in the pool section of the river; the bank was inspected carefully by hand, and any mussel felt with the fingers in sediment or among roots was taken until 30–40 specimens were collected. They were individually marked with an oil marker, and relocated to selected places for the purpose of another study. The next sample was taken from a new place (downstream; procedure similar to that for the San river, see Fig. 1), and new individuals were collected, measured, marked, and relocated. According to this procedure, we could not take the same mussel twice, because each specimen that was measured was also clearly marked and relocated in another place. One sample of 43 individuals from March 2014 was used for photographic analysis of shell arching (see below).

### *San river*

*Unio crassus* was sampled at two sites: Żurawin and Procisne (Fig. 1). At the Żurawin site (49°13′08″N, 22°43′09″E, 555 m a.s.l.), the mussels (age over 4 years) were sampled every 2–4 weeks depending on water level (6 samples, 164 individuals in total) in



**Fig. 1** Location of sampling sites and sampling scheme: z—Zborowianka river, s San river; 1–4 paired samples collected for shell arching analysis. At bottom, sampling scheme for general sample at Żurawin shown; samples were taken sequentially. Each sampled section of the channel (gray rectangles) was searched along the bank (long black arrows). After collecting a

mussel sample, the end of the sampling site was marked with a stick (short arrow). On the next occasion, another sample was taken downstream in a new bank area. A similar sampling scheme was used for the Zborowianka, but the collected mussels were marked and relocated after measurement

2013 from a reach ca 100 m long (gray square in Fig. 1) above the ford. *U. crassus* occurs here at very high density (30–100 per 1 m of one side bank). The mussels were not marked here; instead, the sampling point was marked on the bank with a wooden pole (short arrow on sampling scheme in Fig. 1), and on the next occasion, the sampling point was moved downstream (or the other side of the river was used in the same manner) to avoid sampling at the previous place. We collected up to 40 individuals, inspecting the bank carefully by hand, but if mussels occurred in the gravel near the bank, they were also collected. From another study, it is known that the dispersal distance of *U. crassus* adults in this river is very short (<2 m; Zajac, 2017).

To disclose the ecological factors responsible for shell arching, we collected 4 pairs of samples from the San river, (doubled circles in Fig. 1): (1) ca 60 m downstream from the Żurawin ford (49°13'03"N, 22°43'04"E), (2) ca 0.7 km upstream from the mouth of the Wołosaty river near Procisne (49°12'12"N, 22°41'07"E), (3) ca 0.4 km upstream from the mouth of the Wołosaty (49°12'03"N, 22°41'05"E), (4) at

Procisne near the mouth of the Wołosaty (49°11'52"N, 22°40'57"E). Sample 1 was collected on Jun. 10, 2014, sample 2 on July 18, 2014, and samples 3 and 4 on Sept. 20, 2014.

One sample of each pair consisted of all the *U. crassus* we found in the middle of the river channel ( $\geq 10$  m from the bank) in shallow riffles (10–20 cm depth, turbulent flow, mean water velocity 0.4 m/s) among cobbles in an area of ca 0.25 ha overgrown with *Potamogeton* sp. stems, indicating stable substrate. The other sample of the pair was taken from an area of still water at the bank nearest to the site of the first sample, built of fine sediment (dust and silt, smooth to the touch) overgrown by *Carex* sp. at the top of the bank, the leaves of which were hanging into the water, slowing the current. The bank was inspected by hand until all specimens from a section covering ca 1 m were collected.

The San and Zborowianka were sampled every month at the same locations (Góra Wieś for the Zborowianka, Żurawin for the San) in 2013 for water chemistry analyses ( $\text{NO}_3$ ,  $\text{PO}_4$ , K, Ca) during the period of mussel growth (Apr.–Sept.); the water

samples were collected in plastic bottles (0.33 l), refrigerated immediately, and analyzed the next day using the laboratory chromatography oven in the Institute of Nature Conservation, Polish Academy of Sciences.

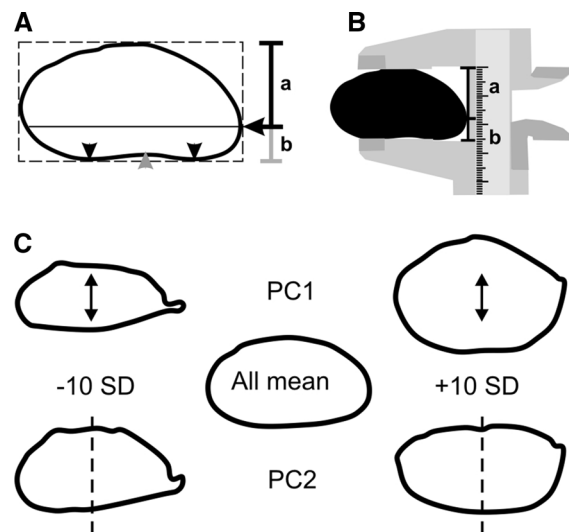
### Specimen measurements

Mussels collected during sampling of the Zborowianka and San rivers (gray rectangles in Fig. 1) were punctured with a syringe, and a tissue sample was taken from the gonads and inspected with a field microscope to determine sex by the presence of eggs or sperm (the single-puncturing procedure seems not to harm the mussels). Then, the mussels were measured precisely using calipers. Three standard measurements (shell length, height, width) were taken. Yearly increments, as their radii in the posterior part of the shell (Zajac, 2010), were also measured. On the top of the shell, one arm of the caliper was fitted firmly in the tiny hollow at the beginning of the growth rings and the other arm was fitted to the outermost end of the consecutive growth rings and finally at the siphons. This method obviates difficulties in finding the beginning of the growth ring in the forepart of the shell, where the growth rings tend to merge. In the longer, siphon part of the shell, the rings are more separated and thus less prone to misidentification. After that procedure, the mussels were returned to the same site in the river.

The sample from the Zborowianka (March 2014) and the sample from the San at Żurawin (paired sample No 1: midchannel/bank) collected for shell arching analysis were photographed together with a 1 cm<sup>2</sup> square for size standardization. To secure the horizontal position of the shells, they were placed in a row in plastic substrate, which allowed us to correct for valve arching when positioning the mussels. Then photographs from the same position were taken and imported into CorelDraw. A rectangle was fitted along the lower edge of the mussel silhouette on the photo (see also Alvarez-Molina 2004): one longer side at the lower edge of the shell outline and the other longer side at the uppermost point of the top of the shell (Fig. 2A); the shorter sides of the rectangle were fitted, respectively, to the top of the forepart and the top of the posterior/siphon part of the shell. Then, two measurements were taken: (1) vertical distance

between the outermost end of the shell in the siphon part and the upper horizontal of the fitted rectangle ( $a$  in Fig. 2A), and (2) distance from the outermost end of the shell in the siphon part and the lower edge of the rectangle ( $b$  in Fig. 2A). The measurements were imported to Excel and transformed to real dimensions corresponding to the standard (1 cm<sup>2</sup> square). It can happen that the lower edge of the shell is rounded and cannot be fitted exactly to the lower side of the rectangle (which was not the case with our studied samples). In that case, the same measurements can be made with reference to the longest axis of the shell.

Because this method of measuring shell shape is quite complicated, a field modification employing calipers (Fig. 2B) was applied. One of the large caliper arms was fitted to the bottom of the shell and the other to the top. Usually the siphon margin of the shell is quite soft, and so it had to be slightly flattened to reach the hard part of the shell. The distance between the



**Fig. 2** Methods of measuring shell shape indices: **A**—photo method (short arrows indicate maximum fit of lower edge of shell to adjusted square; long arrow indicates point of contact between fitted rectangle and outermost posterior edge of shell);  $a$ ,  $b$ —distance between longer sides of fitted rectangle and the topmost point of posterior part of shell; **B**—caliper method (field): the  $a/b$  ratio can also be measured with large calipers in the field, but the measurements will not be compatible with the photo method, due to the need to flatten the siphon part in order to position the caliper; **C**—mean shell shape reconstructed using principal components obtained from the elliptical Fourier descriptors in order to visualize the shape aspect described by the given PC; its mean score was modified by  $\pm 10$  SD. PC1 represents shell height, PC2 represents bilateral symmetry of shell outline

right and left caliper arms to the point of contact with the shell indicated the relation between the upper ( $a$  in Fig. 2B) and lower parts ( $b$  in Fig. 2B) of the shell. The ratio of these two distances indicated the level of downward dorsal arching. This method was used to measure shell arching in samples 2–4. Fitting the rectangle or the lower arm of the caliper allowed us to determine unequivocally whether the ventral part of the shell was concave (gray upwards arrow in Fig. 2A).

### Statistical analysis

We separately analyzed the measurements of mussels from the general samples collected in the Zborowianka river and the San river at Żurawin (“z” and “s” in Fig. 1,  $n = 409$  total), and the data from paired samples collected for arching measurements (samples 1–4 in San in Fig. 1), because samples 1–4 were taken purposely from microhabitats selected a priori.

In the analyses of the main shell dimensions (Tables 1, 2), age was used as a continuous variable because it well approximates a normal distribution due to the large number of classes and the large size of the general sample. We also used two indices of shell proportions, following Hornbach et al. (2010)—shell obesity (ratio of shell width to shell length) and relative shell height (shell height/length ratio)—as well as two other indices reflecting shell size: (1) square root of length multiplied by height, reflecting shell size as the square root of the approximated shell projection area (sqrt ASPA), and (2) shell width, standardized by sqrt ASPA. All the main shell measurements and shape indices were analyzed as response variables in general linear mixed models. Each of them was analyzed in a separate model versus the same set of predictors: river and sex as nominal predictors, age as continuous predictor, with sample ID as random factor (Table 1). The analysis was repeated with the same set of predictors for paired samples in the San river (samples 2–4), but excluding sex, because those samples were collected without sexing (Table 2).

To determine the significance of shell differences between males and females that might be used for sex determination, we applied discriminant function analysis using length ( $l$ ), width ( $w$ ), and height ( $h$ ) data from the samples of mussels from both rivers (gray

squares in Fig. 1,  $n = 409$  total), as well as the ratios of shell width to shell length (obesity) and of shell width to sqrt ASPA ( $w/\sqrt{l \cdot h}$ ). In the case of two-group/sex, a linear equation was fitted (Eq. 1):

$$\text{Group} = a + b_1x_1 + b_2x_2 + \dots + b_mx_m \quad (1)$$

where  $a$  is the constant, and  $b_1$  through  $b_m$  are regression coefficients. The model was built using a backward stepwise method.

Growth curves were calculated for the same general samples collected from the San at Żurawin and from the Zborowianka (gray squares in Fig. 1,  $n = 409$  total). The yearly increments in the siphon part of the shell were fitted to von Bertalanffy’s model (Eq. 2):

$$l_t = L_\infty \left( 1 - e^{-K(t-t_0)} \right) \quad (2)$$

where  $l_t$  is the shell length,  $L_\infty$  is the asymptotic shell length assuming growth rate equal to zero,  $K$  is the growth rate,  $t$  is the age,  $t_0$  is the age of organism of length equal to zero. The values of  $K$  and  $L$  were determined by nonlinear estimation, with the assumed function  $v2 = L \cdot (1 - \exp(-K \cdot (v1)))$ .

### Shell arching analysis

Elliptical Fourier descriptor (EFD) analyses were preformed with SHAPE software (Iwata & Ukai, 2002). We used the sample from the Zborowianka (March 2014) and paired sample no. 1 from the San (Fig. 1). The photographs for the  $a/b$  ratio analysis were used to obtain closed contours of mussel shells. After noise reduction, the closed contours of mussel shells were chain-coded (Freeman, 1974). The EFD coefficients were calculated by discrete Fourier transformation of the chain-coded contours in accordance with the procedures given by Kuhl & Giardina (1982). EFDs were normalized with a procedure based on the first harmonic ellipse that corresponds to the first - Fourier approximation to the contour information. Mussel shell shape was approximated by the first twenty harmonics. Such an approximation leads to a large number of normalized EFD coefficients; so, in order to summarize the information contained in them, we performed principal component analyses (PCA) based on variance-covariance matrices. The homogeneity of variance of each principal component score between groups was tested with Levene’s test. The effect that principal components describe for mussel

**Table 1** Differences in *U. crassus* shell dimensions between mountain and foothill watercourses; analyzed with GLMM models,  $n = 409$ 

Response	Predictor	Nominal factors	Mean $\pm$ SE	Relative difference (%)	Estimate	SE	<i>t</i>	<i>P</i>
Length (mm)	River	Zborowianka	55.65 $\pm$ 0.31	11	5.96	1.60	10.97	<0.001
		San	49.69 $\pm$ 0.39					
	Sex	F	52.97 $\pm$ 0.31	1	0.61	0.44	1.40	0.161
Width (mm)	Age		–		2.47	0.17	14.31	<0.001
		River	Zborowianka	19.39 $\pm$ 0.17	9	1.70	0.27	6.35
		San	17.69 $\pm$ 0.22					
	Sex	F	18.84 $\pm$ 0.17	3	0.60	0.20	3.07	0.002
		M	18.24 $\pm$ 0.18					
Height (mm)	Age		–		1.02	0.08	12.90	<0.001
		River	Zborowianka	29.42 $\pm$ 0.19	7	2.10	0.31	6.82
		San	27.32 $\pm$ 0.25					
	Sex	F	28.57 $\pm$ 0.20	1	0.41	0.23	1.77	0.078
		M	28.16 $\pm$ 0.20					
Obesity (width/length)	Age		–		1.18	0.09	12.71	<0.001
		River	Zborowianka	0.348 $\pm$ 0.002		0.007	0.003	2.51
		San	0.355 $\pm$ 0.003					
	Sex	F	0.355 $\pm$ 0.002		0.006	0.002	3.13	0.002
		M	0.348 $\pm$ 0.002					
Relative height (height/length)	Age		–		0.003	0.001	3.21	0.001
		River	Zborowianka	0.529 $\pm$ 0.002	4	0.021	0.003	6.72
		San	0.550 $\pm$ 0.003					
	Sex	F	0.540 $\pm$ 0.002	0	0.001	0.002	0.40	0.689
		M	0.539 $\pm$ 0.002					
Sqrt(length*height)	Age		–		0.003	0.001	3.16	0.002
		River	Zborowianka	40.45 $\pm$ 0.23	9	3.66	0.39	9.40
		San	36.79 $\pm$ 0.29					
	Sex	F	38.86 $\pm$ 0.23	1	0.49	0.31	1.59	0.113
		M	38.38 $\pm$ 0.23					
Width/Sqrt (length*height)	Age		–		1.70	0.12	13.94	<0.001
		River	Zborowianka	0.478 $\pm$ 0.003	0	0.001	0.004	0.21
		San	0.479 $\pm$ 0.003					
	Sex	F	0.483 $\pm$ 0.003	2	0.008	0.003	3.0	0.003
		M	0.475 $\pm$ 0.003					
	Age		–		0.005	0.001	4.24	<0.001

shell shape was visualized in relation to the mean effect only for significant principal components by inverse recalculation of EFDs using an eigenvector matrix, letting the score of the significant PCs be equal to mean  $\pm$  10 SD to show an exaggerated effect (Fig. 2C), enabling full visualization of the shape aspect described by the given PC.

The paired samples collected in the San river near Procisne (nos. 2–4 in Fig. 1) were tested for the effect of the two different microhabitats on their *a/b* arching ratio as response variable in the GLM model, where site (nos. 2–4, Fig. 1), microhabitat (midchannel with fast-flowing water, coarse sediment with *Potamogeton* sp. (*mp*), and bank with fine sediment and still water

**Table 2** Differences in *U. crassus* shell dimensions between microhabitats within the San river channel, analyzed with GLMM models for sample nos. 2–4, with age influencecontrolled,  $n = 143$ ; bf—bank with fine sediment, mp—midchannel with *Potamogeton* L. spp. vegetation

Response	Predictors	Nominal factors	Mean $\pm$ SE	%	Estimate	SE	<i>t</i>	<i>P</i>
Length (mm)	Microhabitat	bf	45.99 $\pm$ 1.32	5	2.32	0.71	3.28	0.001
		mp	48.31 $\pm$ 1.33					
	Age		–		2.50	0.22	11.39	<0.001
Width (mm)	Microhabitat	bf	15.74 $\pm$ 0.29	5	0.82	0.26	3.0	0.003
		mp	16.57 $\pm$ 0.29					
	Age		–		1.0	0.09	11.81	<0.001
Height (mm)	Microhabitat	bf	25.21 $\pm$ 0.57	6	1.53	0.35	4.34	<0.001
		mp	26.74 $\pm$ 0.057					
	Age		–		1.20	0.11	11.03	<0.001

(*bf*), and mussel age (and interactions between them) were used as predictors. Because not all age classes were represented in the site/microhabitat array, only age classes 6–9 years were included in this analysis.

## Results

### Environmental differences between study locations

The Zborowianka river was much more nutrient-rich than the San, although the two rivers followed similar trends. The Zborowianka showed a large supply of  $\text{NO}_3$  in early spring (7.73 mg/l), depletion in May (3.54 mg/l), a large increase in June (5.96 mg/l), and then a steady decrease towards September (respectively: 3.85, 1.55, 0.68 mg/l). In the San, the concentrations of that anion were almost four times lower than in the Zborowianka during almost the whole growing season (respectively: 2.36, 0.18, 0.81, 0.13, 0, 0 mg/l). In the Zborowianka, during the spring and early summer (May–July), there was a very large and long-lasting peak of  $\text{PO}_4$  (0.11, 0.15, 0.07 mg/l, respectively; 0.02 mg/l in August, 0.00 mg/l in September), several times larger than in the San (0.03 mg/l in April, 0.01 mg/l in May and Jun, 0.02 mg/l in July, 0.003 mg/l in August, 0.03 mg/l in September). The calcium concentrations during the spring and summer were about three times higher in the Zborowianka (from 103 mg/l in May to 97.7 mg/l

in July) than in the San (from 39.8 mg/l in May to 42.0 mg/l in July).

### Main shell dimensions

All main shell measurements differed between the mussels inhabiting the two analyzed rivers (Table 1). On average, those inhabiting the Zborowianka river had longer shells (difference of ca 5.96 mm or ca 11% of mean shell length). The difference in mean shell width (1.7 mm) was also statistically significant, and the relative difference was similar (9%); they also had significantly higher shells (difference of 2.1 mm or 7% of shell height).

The differences in the combined indices of shell primary measurements were less evident (Table 1). For the indices based on shell width, the relative differences between their means were small (ca 2% difference in obesity) or even negligible (0.2% difference in w/sqrt ASPA). Relative shell height differed significantly between the rivers, and the mean difference was moderately high (ca 4% for relative height). We found a 9% difference in sqrt ASPA.

Males did not differ significantly from females in shell length and shell height (Table 1), regardless of their age or the river inhabited, but there were significant differences in shell width: the females were larger than males by 0.6 mm (ca 3% of mean female shell width). Discriminant function analysis of sexual size dimorphism suggested that none of the shell dimension parameters or the indices based on



them have any discriminant power (Wilks' lambda parameters close to 1). The stepwise backward removal procedure revealed a significant difference between sexes only for the ratio of shell width to sqrt ASPA ( $w/\sqrt{l \cdot h}$ , Wilks' lambda: 0.97,  $F(2,406) = 5.7$ ,  $P < 0.0037$ ). However, in the classification matrix, only 56% of the cases were correctly classified. For all analyzed measurements, age very significantly affected shell size (Table 1).

The mussels inhabiting the San river also differed in all main shell measurements between the microhabitats of that river (Table 2). On average, those inhabiting the midchannel habitat had longer shells (ca 2.3 mm difference, 4.8% of mean shell length). The difference in mean shell width (0.83 mm) was also significant, and the relative difference (5.0%) being very similar to that of shell length. The mussels inhabiting the midchannel also had significantly higher shells (1.5 mm; relative difference 5.7% of mean shell height). The combined indices of shell shape (analyzed in the same model as for the main measurements) showed no significant differences between microhabitats (obesity  $t = 0.02$ ,  $P = 0.99$ ; height/length  $t = 1.47$ ,  $P = 0.14$ ; width/sqrt(ASPA)  $t = 0.51$ ,  $p = 0.609$ ) except for sqrt ASPA, which showed a 5% difference between the mean values for the two microhabitats; that difference was significant ( $t = 3.9$ ,  $P < 0.001$ ).

#### Growth curves

We tested whether the elongation of the posterior part of the shell (siphon part) in successive years of the mussels' life follows von Bertalanffy curves. For the Zborowianka, we estimated  $L$  at 61.7 (SE = 1.03,  $t = 59.7$ ,  $df = 1571$ ,  $P \ll 0.001$ ) and  $K$  at 0.197 (SE = 0.005,  $t = 36.3$ ,  $df = 1571$ ,  $P \ll 0.001$ ). The model is very well fitted: for the Zborowianka data, the proportion of variance accounted for is very high: 0.89 ( $R = 0.95$ ; Fig. 3A). For the San river,  $L$  was estimated at 64.0 (SE = 1.29,  $t = 49.5$ ,  $df = 1311$ ,  $P \ll 0.001$ ) and  $K$  at 0.139 (SE = 0.004,  $t = 31.4$ ,  $df = 1311$ ,  $P \ll 0.001$ ). The model for the San specimens is also very well fitted; the proportion of variance accounted for is very high: 0.90 ( $R = 0.95$ ; Fig. 3B). A comparison of standardized  $K$  values indicates a much higher growth rate in the Zborowianka than in the San (only 71% of the value for the Zborowianka). On the other hand, the asymptotic shell

length ( $L$ ) for the San is larger than for the Zborowianka (96% of the value for the San).

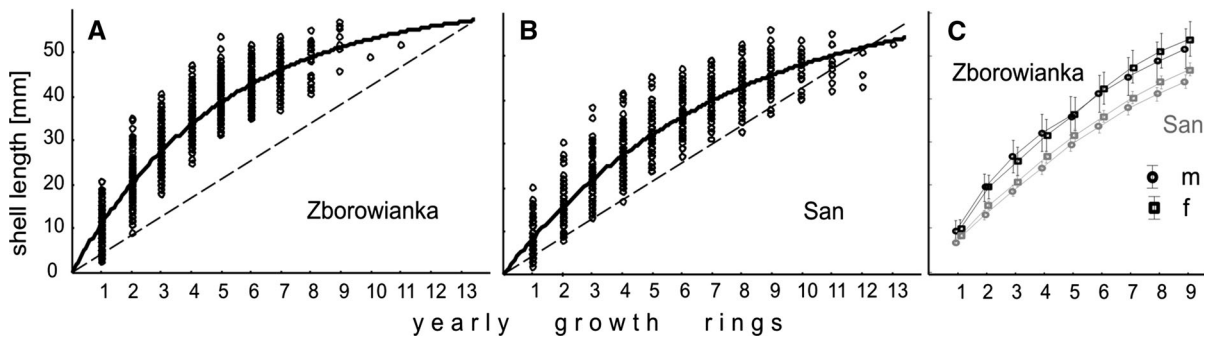
Growth measurements in successive years of a mussel's life are not independent of each other and should be analyzed using a general linear model (GLM) for repeated measurements of the same individuals. We analyzed such a model for the differences in shell length between rivers in relation to age classes, controlling for the effect of sex. We analyzed individuals with nine annual rings ( $n = 62$ ), because after this age, the number of mussels decreases rapidly. This analysis showed that in each age class, the mussels in the Zborowianka had longer shells than those in the San (Fig. 3C). For each year class, the difference was statistically significant, with a  $\beta$  value of 0.29 for age 1 ( $P < 0.05$ ); for age classes 2 to 9, the  $\beta$  values were 0.51, 0.52, 0.48, 0.43, 0.51, 0.51, 0.49, and 0.49, respectively ( $P < 0.001$ ). Also, in the San, the males were consistently smaller than the females (Fig. 3C) but not significantly. In the Zborowianka, the growth patterns of the sexes differed between age classes but again not significantly. There was no significant interaction between river and the sex of individuals.

#### Shell shape

Among the samples collected in 2014 for the shell arching analysis, 16 of the 50 mussels collected from the San river at the Żurawin location had a concave ventral edge of the shell (gray upwards arrow at lower edge of shell in Fig. 2A); only one such mussel was found in the sample from the Zborowianka ( $n = 43$ ; Fisher exact test,  $P = 0.002$ ).

A comparison of shell height above ( $a$ ) and below ( $b$ ) on the horizontal axis of the shell ( $a/b$  ratio in photo method; Fig. 2A) showed that mussels in the San ( $n = 50$ ) were much more variable with respect to shell asymmetry (i.e., dorsal arching) than the Zborowianka mussels were ( $n = 43$ ; Bartlett test:  $F = 13.0$ ,  $df = 49, 42$ ,  $P = 0.0003$ ; Fig. 4A), and the  $a/b$  ratio was significantly higher in San mussels (Welch ANOVA for unequal variances:  $F = 15.5$ ,  $P = 0.0002$ , Fig. 4A).

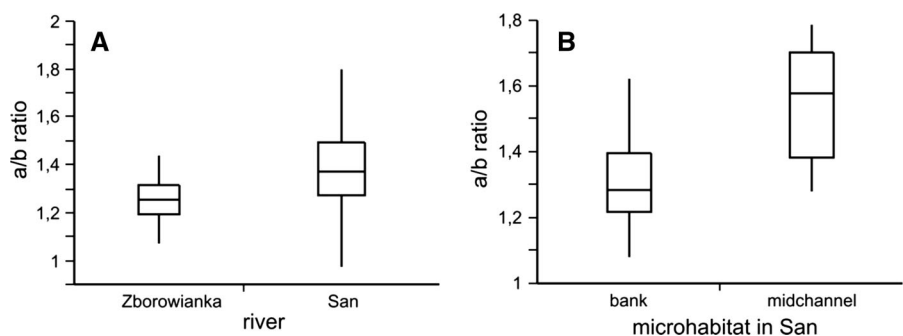
In terms of microhabitat, the mussels collected in the midchannel of the San river differed significantly in  $a/b$  ratio from the mussels collected at the bank ( $a/b$  ratios estimated using photo method from paired



**Fig. 3** Growth curves of mussels: **A**—in the Zborowianka, **B**—in the San river: fitted von Bertalanffy curves (dashed line is straight line of proportional growth, enabling visual comparison

of curve shapes); **C**—difference in growth curves between sexes in relation to differences between rivers the Zborowianka (black) and San (gray)

**Fig. 4** Comparison of shell arching ( $a/b$  ratio measured from photographs—see Methods): **A**—between the San and Zborowianka rivers, **B**—between microhabitats of the San; means, box—SE, whiskers—SD



sample no. 1 at Żurawin, Fig. 4B;  $F = 42.0$ ,  $n = 60$ ,  $P \ll 0.0001$ ). The same sample was analyzed with EFD. Their first four principal components accounted for 81.8% of total variance (Table 3). The differences between the analyzed microhabitats were statistically significant for only two of seven principal components. The effect of those principal components on mussel shell shape was visualized for the first two significant principal components (Fig. 2C), indicating that PC1 can be interpreted as shell height (arrows in Fig. 2C), whereas PC2 should rather be interpreted as reflecting symmetry of contour on the vertical axis (dashed lines in Fig. 2C).

The arching ratio ( $a/b$ ), measured with calipers (Fig. 2B), gave results similar to the photo method. GLM analysis with the  $a/b$  ratio as response variable against site, microhabitat, and age indicated that the  $a/b$  ratio did not differ between sampling sites ( $F = 1.07$ ,  $df = 2,100$ ,  $P = 0.187$ ) or age classes ( $F = 1.23$ ,  $df = 3,100$ ,  $P = 0.302$ ); nor was the interaction between predictors significant ( $F = 0.718$ ,  $df = 6,100$ ,  $P = 0.636$ ), but the  $a/b$  ratio

did differ significantly between microhabitats ( $F = 20.3$ ,  $df = 1,100$ ,  $P < 0.0001$ , Fig. 5).

## Discussion

### Basic shell measurements and combined indices

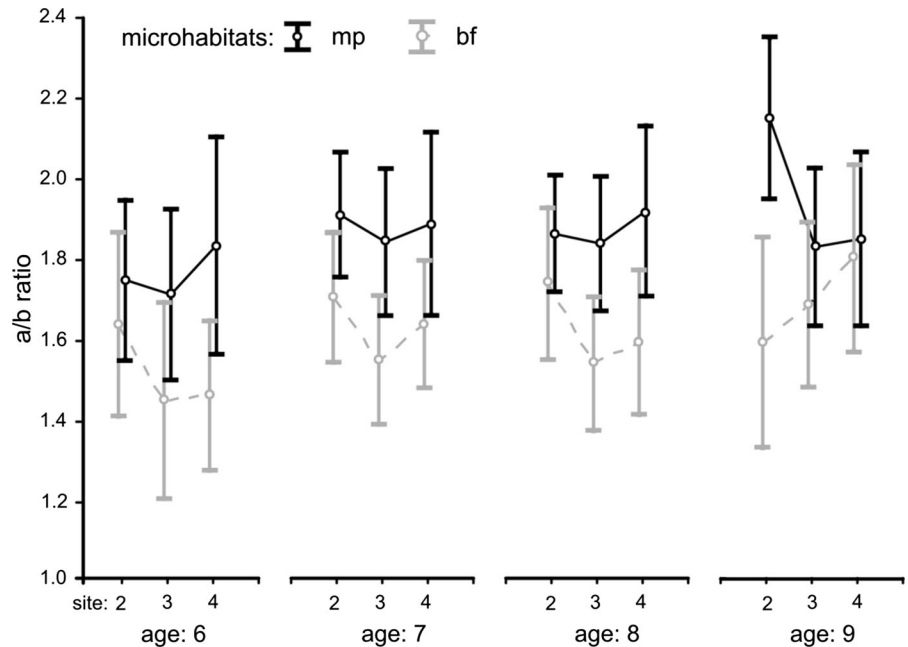
In an analysis of a large sample, even very small but consistent differences in shell dimensions resulting from given factors will be revealed as statistically significant, but it does not necessarily mean that the differences are important. As was predicted, there was a small but consistent difference in shell width which should be related to the growth strategy of females, which creates an additional space for the enlarged anatomical structure of marsupia (Zieritz & Aldridge, 2011), and/or to compensate for the larger oxygen consumption of brooded glochidia. Although sex-related differences in shell size are statistically significant in a large sample, the small absolute

**Table 3** Differences between effective Principal Component scores of bank and midchannel mussel shell shape in the San river; only effective components presented (explaining over

80% of variation; PC5 to 7 not shown due to negligible explanatory power) and tested with Student’s *t* tests, *df* = 58. SD—standard deviation of mean

PCs	Eigenvalue	% of variance	Cumulat. (%)	Stream		Bank		<i>t</i>	<i>P</i>
				Mean	SD	Mean	SD		
PC1	0.00055	53.7	53.7	0.0154	0.019	−0.0154	0.017	6.7	<0.0001
PC2	0.00017	16.4	70.0	0.0055	0.014	−0.0055	0.010	3.6	0.0007
PC3	0.00008	7.4	77.4	0.0019	0.009	−0.0019	0.008	1.7	0.0898
PC4	0.00005	4.5	81.8	0.0006	0.007	−0.0006	0.006	0.7	0.4887

**Fig. 5** Comparison of shell asymmetry between two San river microhabitats: mp (midchannel-*Potamogeton*) versus bf (bank fine sediment) located in distant river sections (sites nos. 2–4) for the selected age classes (6–9 years); circles indicate means, whiskers—0.95 CI, (*a/b* ratio calculated from caliper measurements, see Fig. 2B and Methods for details)



differences (Table 1) make sexing of *U. crassus* on the basis of shell dimensions an unreliable method.

The relative differences in the main shell measurements were much larger between rivers than between sexes, but they were still quite small and were similar between measurements (7% height, 9% width, 11% length). A similar level of relative difference between rivers was shown by the square root of approximated shell projected area (ASPA; 9%), an obvious result since this measure is based on simple multiplication of shell length and height. The differences in the remaining combined indices of basic shell measurements were much smaller (<5%); this indicates that the shells maintain more or less similar proportions in the two rivers. Despite the large differences in water

velocity between the rivers, the shell obesity measure, suggested to be a trait reflecting mussels’ anchoring ability (e.g., Hornbach et al. 2010), was only slightly higher (by ca 2%) in the four times faster San river. The lack of a difference in obesity may be attributable to the high share of mussels sampled from the bank (microhabitat with still water in both rivers), but such a bias should also have been reflected in the basic shell measurements (Table 2), which differed much more than obesity did in the same sample.

The basic measurements also differed between microhabitats within the San River, showing surprisingly stable relative differences: ca 5% in length and width, and 6% for height, which again indicates stable shell proportions. It is not surprising, then, that

all the combined indices except one (sqrt ASPA: sqrt of multiplied height and length) showed no significant differences between microhabitats. Generally, this means that mussels living in the strong midchannel current grew larger but grew proportionally, enlarging all the main shell dimensions by apparently the same coefficient. Thus any ratios of the main shell measurements remain the same for individuals inhabiting still water in channel margins and strong current in midchannel. The single significant one, sqrt ASPA, is only the product of multiplication of two basic dimensions; hence its significance is derivative of the basic measurements.

### Shell growth

Under optimal allocation of resources between growth and reproduction, higher survival in a given population should allow longer growth and lead to larger absolute body size (Kozłowski, 1992). Our analysis of the Bertalanffy curves indicated a higher asymptotic shell length for the San than for the Zborowianka. Such an analysis should be treated with caution because it is based on only the two rivers; however, Fig. 3 does show that after the eighth year of life, the number of mussels in the Zborowianka decreased, whereas in the San, such a decrease was not seen until after the eleventh year of life. The morphology of the San channel is not modified by man and seems very stable; during the three years of observations, there were virtually no structural changes in the studied channel section, despite the river's large size. Its catchment is a protected area (Bieszczady National Park, Eastern Carpathians Biosphere Reserve, Natura 2000 site), with extensive forest areas, which stabilizes water discharge (Gallart & Llorens, 2004). The Zborowianka catchment drains agricultural areas located on hills, the forest cover is sparse, and some river sections are under strong anthropoppression (numerous bridges, roads, settlements). During the same three years of observations, we noted significant changes in channel morphology at our Zborowianka sampling site, due to lateral erosion and large-scale movement of sediment during spates. Such phenomena might cause high mussel mortality (Hastie et al., 2001).

Another important conclusion comes from a comparison of standardized  $K$  values between the rivers. It shows that the growth rate is much higher in the

Zborowianka than in the San (only 71% of the value for the Zborowianka). This large relative difference confirms Hochwald's (1997, 2001) approach, which assumes that life history traits are the best features reflecting habitat effects.

### Dorsal arching

In the San river microhabitats, *U. crassus* dorsal arching in the posterior part of the shell showed enormous phenotypic variation over a spatial scale of only a few meters. One reason for larger arching of the shell may be exposure of the posterior part of the shell to water current, which may physically cause twisting the shell towards the bottom due to distortion of the shell-secreting mantle margin (Zieritz & Aldridge, 2009). Regardless of the factors causing it and regardless of its adaptive significance, it seems to be a good indicator of mussels' microhabitats. For example, in *Unio pictorum* (Linnaeus, 1758) inhabiting a large lowland river, the Thames, Zieritz et al. (2009, 2010) found similar differences in shell morphology between sampled microhabitats (marina vs. river channel).

However, shell arching is not strikingly bimodal when sampled throughout the channel of the San. The ranges of arching of individuals sampled from the different microhabitats even overlapped to some extent (Figs. 4, 5). This overlap might be due to movement of individual *U. crassus* within the channel (Zajac & Zajac, 2011, but see Zajac, 2017), exposing the mussels to different microhabitats at various periods of life. These movements and also passive translocation can make them traverse longer distances, especially during episodes of bank erosion and flood (unpubl. data).

Although shape analysis tools like elliptical Fourier analysis (EFA) doubtless offer some advantages, morphological differences between microhabitats can be detected without such sophisticated tools. It is well known that multivariate coefficients are frequently difficult to interpret. Caution is always needed in analyzing composed statistical indices, which are prone to misinterpretation because some PCs can be redundant to simple measurements or even meaningless. In our case (Fig. 2C), PC1 seems to be redundant to the shell height/length ratio, whereas PC2 offers an original and interpretable shape descriptor (lack of symmetry in side contour in reference to vertical

axis—dashed line in Fig. 2C). Such an analysis should be supported by a clear ecological mechanism or should be concordant with other independent ecological features, as suggested by Douđa et al. (2014). Applying EFA to mussel shells requires extensive testing against many different ecological backgrounds in a large number of rivers and populations, in order, for example, to test the number of harmonics applied (e.g., 8 suggested in the methodological paper by Crampton, 1995 versus 20 used by Preston et al., 2010) and to standardize meaningful shape patterns useful for identifying habitat characteristics or for taxonomy. Additionally, unlike direct measurements (e.g.,  $a/b$  ratio), EFA descriptors cannot be directly compared between different studies: any comparison between different samples needs a completely new, common shape analysis for a new pooled dataset, which is not always possible.

#### Ecological factors influencing shells

The role of hydrology in shaping mussel shells has long interested ecologists (Ortmann, 1920), who have suggested that downstream sites usually harbor larger mussels. Special attention has been paid to shell cubature (also called obesity), the ratio of shell width to length, which tends to increase downstream (Roper & Hickey, 1994; Hornbach et al., 2010). Many researchers note that shell size, shape, and sculpture influence burrowing and anchoring (Hornbach et al., 2010), but this is an area needing further work (Levine, 2014). However, shell morphology analyses are usually based on the three basic dimensions: length, height, and width. It should be noted that these dimensions (which are correlated anyway) and the indices derived from them are very general and thus difficult to interpret: larger shell size found in lower river courses may result from hydrology (anchoring) but also from increased trophic or higher temperature of slower river parts. We agree that the *U. crassus* shell does not offer many traits to measure—it is a very simple shape. The three main dimensions can yield data for answering some questions (e.g., size and water trophic) but not all of them; for example, the height/length ratio performs worse than the  $a/b$  ratio in microhabitat analysis. More informative might be purpose-oriented measurements such as growth curves (which could elucidate not only trophic but also survival) or specific measurements of siphon position,

lower shell margin curvature, and shell thickness. Mussel shell shape usually does not offer a platform for design of new measurements, but in our opinion the standard measurements cannot be applied mechanically to any ecological question. The measurement method should be adapted or even designed for the problem to be solved.

Shell size/shape distributions are not molded by growth conditions alone. It is well known that such distributions in adult freshwater mussels can be shaped by predation, which is frequently size-dependent (Neves & Odom, 1989; Tyrrel & Hornbach, 1998; Owen et al., 2011; Zajac, 2014). In the two studied rivers, we never observed the characteristic signs of predation (see Zajac, 2014) on *U. crassus*. Mussel mortality caused by hydrological events may also be size-dependent, as when the smallest mussels or those less arched are crushed among cobbles or buried in fine sediment.

Recent publications indicate that *U. crassus* occurs within an interval of nutrient content: it avoids both too oligotrophic and too eutrophic waters (Hus et al., 2006; Douđa, 2010). The influence of physicochemical parameters on shell growth is generally difficult to detect because their effect on mussel growth is usually indirect; for example, nutrient loads from tributary rivers may influence the growth of lake mussels through food availability (Riccardi et al., 2016). In our case, a comparison of river nutrients ( $PO_4$ ,  $NO_3$ ) suggests that the higher growth rate of *U. crassus* in the Zborowianka may be a simple result of the higher trophic of this habitat (3–4 times higher nutrient content), with a higher supply of calcium as well. However, the nutrient content of these rivers differs by ca 300%, whereas the growth rate estimated from Bertalanffy curves differs by 29% and the basic shell dimensions differ by ca 10% between the rivers. The difference in nutrient concentration has low explanatory power, because both rivers can supply enough nutrients for *U. crassus* to grow at the maximal rate. Moreover, nutrient content showed no effect on the asymptotic shell length: ultimate shell length was higher in the nutrient-poorer San, though actual shell length in a given age class was higher in samples from the Zborowianka. This suggests that factors other than habitat trophic are at play, for example the influence of survival on life history traits.

Our data suggest an essential guideline for proper sampling. Failure to relate morphology with habitat in

ecomorphological studies (also in paleontology, archeology, etc.) may result from improper sampling that mixes microhabitats in one global sample: the differences between microhabitats within one river (e.g., two microhabitats in the San) may be much stronger and much clearer than the differences between rivers of different sizes and types.

These data have relevance to taxonomical studies. The changed approach to mussel taxonomy which reduced the number of taxa has relied on the implicit assumption that mussels have a large range of phenotypic variability of morphological traits, but the repetition of some shapes across species has complicated the task of determination. No single morphological trait can be used for species determination (see critique of the comparative method by Graf, 2007) because, as demonstrated here, species morphology can be greatly altered in response to differences in environmental factors within one habitat. *U. crassus* was one example of proliferation of subtaxa: in his review, Graf (2007) demonstrated six “versions” of *U. c. crassus*. Starobogatov (1977) proposed the separate genus *Batavusiana* (syn. *Crasiana*) for *U. crassus*, and Stadnychenko (1984) proposed five subtaxa within that genus. Here, we demonstrated that even such an obvious difference in shell morphology as kidney shape should be considered in depth, as to whether it has a genetic basis in line with the biological species concept (Graf, 2007) or is only environmentally induced phenotypic variation leading to species proliferation in approaches like the comparative method (Shikov & Zatravkin, 1991).

If there are no taxonomically significant genetic differences between populations, all the differences in shell morphology between habitats should rather be attributed to phenotypic plasticity, which might be adaptive *per se* anyway. Evidence for this has been given by Hinch et al. (1986) as well as Zieritz et al. (2010), whereas Kesler et al. (2007) and Zieritz & Aldridge (2009) gave some indirect evidence for plasticity or rather for the influence of habitat on phenotype. Zieritz et al. (2010), who studied both genetic and morphological diversity, failed to identify even a single locus that could be associated with shell shape differences between ecomorphotypes. According to a recent study by Sell et al. (2013), the Carpathian populations of *U. crassus* form a genetically distinguishable but quite uniform group; thus, it is unlikely that its phenotype diversified for

phylogeographical reasons. Moreover, it is doubtful that individuals of the same species inhabiting two microhabitats only a few meters apart are genetically isolated, especially considering the mode of fertilization in unionids.

We conclude that the *U. crassus* shell shows moderate variation of the main shell measurements in relation to environment, unlike the growth rate and shell dorsal arching. This means that morphological measurements should be purpose-oriented, based on specifically designed measurements. The latter is confirmed in our microhabitat analysis: mussels living in fast-flowing water have a more arched shell. The detected occurrence of different morphological forms in one river indicates significant diversification of the microhabitats there.

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