Sexual behaviour and morphological variation in the millipede *Megaphyllum bosniense* (Verhoeff, 1897)

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Abstract

Sexual selection can be a major driving force that favours morphological evolution at the intraspecific level. According to the sexual selection theory, morphological variation may accompany non-random mating or fertilization. Here both variation of linear measurements and variation in the shape of certain structures can significantly influence mate choice in different organisms. In the present work, we quantified sexual behaviour of the millipede *Megaphyllum bosniense* (Verhoeff, 1897) as characterized by several sequences. These are: mating latency, duration of copulation, contact to copulation time, duration of contact without copulation, time from entrance (time-point when individuals were placed in boxes in which tests occurred) to contact with copulation, and time from entrance to contact without copulation. Further, we analysed the influence of morphological variation (both variation of linear measurements and variation in the shape of several structures) on mating success. Variation of body length, antennal length, length of the walking legs, trunk width, and trunk height was analysed by traditional morphometrics, while variation in size and shape of the antennae, walking legs, head, and gonopods (promeres, opisthomeres) was analysed using geometric morphometrics. More than half of all physical contacts detected among the millipedes resulted in copulation. Based on the value of sexual selection coefficients, preferences toward the previous partner were found to be prevalent in both female and male choice tests. Individuals with different mating status significantly differed in some morphological traits (body mass, head centroid size, head shape, and promere shape). Our study yielded new information about the sexual behaviour of millipedes and variation of morphological traits as a potential basis for mate preferences.

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Introduction

Sexual selection, *i.e.*, the mode of selection that emerges due to inequality in mating success (Arnold, 1994), can be seen as a major driving force that favours evolution of traits among members of the same species (Andersson, 1994; Levitan, 2004). Indeed, the role of sexual selection in shaping external morphological variation among conspecific males (*e.g.* Danielsson, 2001; Jimenez-Perez and Wang, 2004; Willemart *et al.*, 2009) and females (*e.g.* Stuart-Smith *et al.*, 2007; Bussiere *et al.*, 2008; Xu and Wang, 2010a) has been demonstrated in many studies. Also, a growing body of empirical data shows that, besides the two Darwinian mechanisms of sexual selection (mate choice and competition for mates) (Darwin, 1871), lock-and-key relations (Arnqvist, 1998) and sexual conflict (Eberhard, 1996; Arnqvist and Danielsson, 1999; Kahn *et al.*, 2010; Richmond, 2014) act as agents in shaping of genital traits. Size and shape of the genitalia can have significant influence on mating success, sperm transfer, sperm storage, and paternity in insects (reviewed in Simmons, 2014).

Both on the intraspecific and the interspecific levels, mate choice is one of the key components of the sexual selection theory (Andersson, 1994; McPeck *et al.*,

2008, 2009; Edward and Chapman, 2011; Dougherty and Shuker, 2014). Differences in mate choice presume the existence of variation of certain trait(s) in males and/or females and occurs when the effects of trait(s) expressed in one sex lead to non-random allocation of reproductive investment (*e.g.* mating or fertilization) with individuals of the opposite sex (Edward and Chapman, 2011; Edward, 2015). Among trait(s) that can be sexual selection subject(s) and which underlie preferences that lead to mate choice, morphological traits are probably the easiest to quantify. Apart from consideration of linear measurements, a geometric morphometric approach can be applied, and data obtained on size and shape of the trait(s) in focus can be separated and precisely analysed (Zelditch *et al.*, 2004; McPeck *et al.*, 2008, 2009; Gasparini *et al.*, 2011; Cooke and Terhune, 2015). In connection with this, a number of studies are available that link the shape of certain structures (both the “usual morphological” traits and secondary sexual characteristics) with success in mating in the most studied *Drosophila* species (Menezes *et al.*, 2013; Trajković *et al.*, 2013; LeVasseur-Viens and Moehring, 2014; Richmond, 2014), as well as in other taxa (*e.g.* Kelly, 2014).

Studies of the potential relationship between sexual behaviour and morphological variability in millipedes (Diplopoda) are mostly focused on representatives of the superorder Juliformia (*e.g.* Haacker and Fuchs, 1970; Mathews and Bultman, 1993; Barnett *et al.*, 1995; Tadler, 1996; Cooper and Telford, 2000; Jovanović *et al.*, 2017). Precopulatory behaviour typically involves the male’s ascending on the female dorsal side, moving toward the anterior part of her body, and positioning his ventral side on the ventral side of the female. During all these stages, the male actively taps the female with his antennae. Gonopods (male secondary sexual structures which represent modified legs of the seventh and/or eighth body ring) serve as sperm carriers (Enghoff, 1985, 1992a; Barnett *et al.*, 1993, 1995; Barnett and Telford, 1996). Since millipedes are characterized by multiple mating, gonopod size and shape could be under the influence of postcopulatory sexual selection.

We assumed that morphological features which are in frequent contact between males and females before copulation occurs may be involved in some sort of mutual assessment and be subject to precopulatory sexual selection. The julid species *Megaphyllum bosniense* (Verhoeff, 1897) was used to test the impact of morphological variation (both variation of linear measurements, body mass and variation in the shape of

certain structures) on mate choice. Mating success of both sexes was tested not only in relation to morphology, but also in relation to previous mating experience. Knowledge of these relationships, together with the results of quantifying behavioural sequences, would contribute to a better understanding of intersexual selection and the mating behaviour of millipedes.

Material and methods

Analysed species

Studied species *M. bosniense* is one of the most common millipedes in Serbia (Makarov *et al.*, 2004) and is also distributed in other countries [Albania, Austria, Bosnia and Herzegovina, Bulgaria, Croatia, Greece, Italy, Hungary, Montenegro, Former Yugoslav Republic of Macedonia (FYROM), Romania, Slovenia] (Lazányi *et al.*, 2012). This species is characterized by sexual dimorphism (SD) in body length: females are approximately twice the size of males. For this study, we collected 114 animals (57 individuals of each sex) from Mt. Avala (near Belgrade, Serbia) (Čarapićev Brest, village of Beli Potok; N 44°41’32”; E 20°31’06”) during October of 2016. We confirm that no specific permissions were required for collecting millipedes on this site. Also, we confirm that no endangered and/or protected species was used in our study. Sex of the collected adults was immediately determined and males and females were placed in separate boxes filled with ground cover from the collecting site. Millipedes were kept for seven days in the laboratory (room temperature, relative humidity of ~60%, natural light regime) to acclimate them before the experiments.

Behavioural assays

Behavioural traits in *M. bosniense* were scored in three types of tests (*mating arena test*, *female choice test*, and *male choice test*). In the mating arena test, males and females were placed in plastic boxes (38 cm × 25 cm) filled with leaf-litter, pieces of bark, and soil from the sampling site. We put males and females together into plastic boxes for ~30 minutes. Each mating pair was scored for behavioural sequences (explained below, Table 1) and transferred to a separate plastic box. Mated and non-mated individuals were further kept individually in plastic boxes filled with leaf litter for 48 hours. Female and male choice tests were conducted after the mating arena test. In the “choice”

Table 1. Description and quantification of sexual behavior.

Sequences	Abbreviation	Description of sequences
Mating latency	ML	Time elapsed from placing millipedes in the mating experiments until the beginning of copulation.
Duration of copulation	DC	Time scored from the beginning to the end of mating.
Contact to copulation time	CC	Time scored from contact between female and male that finished with copulation.
Contact duration without copulation	CWC	Duration of contact between female and male which does not result in copulation.
Time from entrance to contact with copulation	ECC	Time elapsed from entry of individuals into plastic boxes until contact with copulation.
Time from entrance to contact without copulation	ECWC	Time elapsed from entry of individuals into plastic boxes until contact without copulation.

tests, an individual of one sex and two individuals of the opposite sex (the previous partner from the mating arena test and a new one) were placed in a plastic box (20 cm × 13 cm × 13 cm) filled with leaf-litter, pieces of bark, and soil from the sampling site. We marked the previous and new partners using UV dust, which is commonly employed to tag insects (Hagler and Jackson, 2001) without modifying their mating behaviour (Terzić *et al.*, 1994). A small amount of red-coloured dust was applied to the telson of the previous partner, while green-coloured dust was applied to the telson of the new partner. A fluorescent UV lamp was used in subsequent determination of the mating partner.

Sexual behaviour was quantified by determining several sequences (Jovanović *et al.*, 2017) (Table 1). Behavioural sequences were scored in the mating arena, female choice, and male choice tests. Additionally, mating success of previously mated and non-mated individuals in the mating arena test was scored in both types of subsequent “choice” tests. The time elapsed between the mating arena and female/male choice tests was 48 hours (Jovanović *et al.*, 2017). During this time, the individuals were kept individually in plastic boxes filled with leaf litter, regularly fed, and moistened, to relax them from the previous mating.

Descriptive statistical procedures were performed to describe the aforementioned sequences of sexual behaviour. Differences in values of **DC**, **ECC**, and **CC** in the mating arena test and in some of the “choice” tests were determined using the T-test. All statistical analyses were performed in the R program package (R Development Core Team, 2013; available at <http://www.r-project.org/>) and Statistica 7 (StatSoft Inc., Tulsa, OK, USA). To determine differences in success

of mating with the previous partner and mating with a new one, the sexual selection coefficient (**W**) was calculated for males and females separately using JMATING software (the highest mating success, **W** = 1) (Carvajal-Rodriguez and Rolan-Alvarez, 2006, available at <http://acraaj.webs.uvigo.es/JMsoft.htm>). The sexual selection coefficient (**W**) is widely used parameter for estimation of sexual selection effects and it represents the maximum likelihood fitness estimator of one class relative to another (Rolán-Alvarez and Caballero, 2000).

Morphological traits in *M. bosniense*

To explore the relationship between mating success and morphological variation of certain traits, traditional and geometric morphometric analyses were carried out.

In performing these analyses, we used the ImageJ program (Abràmoff *et al.*, 2004) to measure several morphological traits (Table 2; see Ilić *et al.* (2016) for descriptions of linear measurements). Body mass was weighted using a Scout Pro 123® scale (Ohaus Corporation, Pine Brook, NJ, USA).

Table 2. List of analyzed morphological traits.

Morphological trait	Abbreviation
Body mass	BM
Body length	BL
Antennal length	AL
Walking leg length (anterior pair of legs) taken from the 25th body ring	LL
Trunk width taken from the 24th body ring	TW
Trunk height taken from the 24th body ring	TH

Figure 1. Position of landmarks on antenna (A) and leg (B) of *M. bosniense*; and position of landmarks and semilandmarks on head (C), opisthomere (D1), and promere (D2). See supplementary data for descriptions of landmarks and semilandmarks.

T-test was used to test the differences in mean values of **BM**, **BL**, **AL**, **LL**, **TW**, and **TH** between sexes with different mating status. Eighty-four mated (42 individuals per sex) and 30 non-mated millipedes (15 individuals per sex) were used for that purpose.

To analyse shape and size of antennae, walking legs, head, and gonopods (promeres, opisthomeres), geometric morphometrics was applied as a method widely used in animal studies (Kaliontzopoulou *et al.*, 2007; McPeck *et al.*, 2008, 2009; Wojcieszek and Simmons, 2011; Brusatte *et al.*, 2012; Richmond, 2014; Changbunjong *et al.*, 2016; Sasakawa, 2016). For these analyses, we used: 1) the antennae in 56 males (41 mated and 15 non-mated, further presented as mated/non-mated) and 57 females (42/15); 2) the walking legs from the 25th body ring in 57 males (42/15) and 56 females (41/15); 3) the head in 57 males (42/15) and 54 females (40/14); and 4) the promeres of 57 males (42/15) and 5) opisthomeres of 56 males (42/14). All of the indicated structures were used to estimate morphological variation between individuals with different mating status.

The aforementioned structures (head, antennae, walking legs, gonopods - promeres, and opisthomeres) with a reference scale were photographed using a Nikon DS-Fi2 camera with a Nikon DS-L3 camera controller attached to a Nikon SMZ 1270 binocular stereomicroscope. To determine the position of semilandmarks, it was necessary to make fans at the head, promeres, and opisthomeres in the MakeFan program (available at <http://www3.canisius.edu/~sheets/IMP%208.htm>). TpsDig software (Rohlf, 2008, available at <http://life.bio.sunysb.edu/morph/soft-dataacq.html>) was used to position landmarks and semilandmarks. Different numbers of landmarks and semilandmarks were placed, depending on the type of morphological structure (Fig. 1).

Centroid size (**CS**) for the analysed morphological traits was calculated using the CoordGen6 program (Sheets, 2003, available at <http://www3.canisius.edu/~sheets/IMP%208.htm>). Variation in shape of the same traits was examined by Canonical Variate Analysis (**CVA**) in the MorphoJ program (Klingenberg, 2011, available at http://www.flywings.org.uk/morphoj_

Table 3. Descriptive statistics of behavioural sequences: *mating latency* – **ML**; *duration of copulation* – **DC**; *contact to copulation time* – **CC**; *contact duration without copulation* – **CWC**; *time from entrance to contact with copulation* – **ECC**; and *time from entrance to contact without copulation* – **ECWC**. The time is expressed in seconds. For explanation of behavioral sequences see Table 1.

	N	Min.	Max.	Median	Variance	Std. Dev.	Quartiles 25%	Quartiles 75%
ML	42	205.00	8025.00	1318.50	3168232	1779.95	625.00	2510.00
DC	42	103.00	23935.00	17013.50	35581643	5965.04	14822.00	19463.00
CC	42	10.00	1127.00	162.00	52994	230.20	97.00	277.00
CWC	93	4.00	367.00	35.00	6299	79.36	19.00	78.00
ECC	42	104.00	7866.00	1075.50	3315427	1820.83	371.00	2252.00
ECWC	93	71.00	7325.00	1012.00	2165755	1471.65	571.00	2077.00

page.htm). Differences in linear measurements and CS of the aforementioned traits were tested using ANOVA in the R program package (R Development Core Team, 2013; available at <http://www.r-project.org/>).

Results

“Mating arena” test

In observation of the sexual behaviour of individuals in the mating arena test, it was noticed that males of *M. bosniense* (Fig. 2 A) were the more active when searching for mates, and attempted to establish numerous contacts with females. If the female is receptive, it will allow access to its gonopores (Fig. 2 B), which designate this sex as the “choosier” one in mate selection. Precopulatory behaviour in this species (Fig. 2 C-F) was quite similar to that recorded in the earlier studied species *Pachyiulus hungaricus* (Karsch, 1881) (see Jovanović *et al.*, 2017). Behavioural sequences (ML, DC, CC, ECC, ECWC, and CWC) explored by descriptive statistics are presented in Table 3. Out of all contacts, 51% resulted in copulation (Fig. 2 G-I).

Female and male choice tests

Out of all achieved copulation in the female choice test (64% out of 31 copulations), females mostly chose the previous partner for subsequent copulation (in 80% of cases). A similar pattern was detected in the male choice test: 47% of all contacts resulted in copulation (out of 21 copulation). Males also mostly chose the previous mating partner for copulation (in 85% of cases). When considering “contacts” (the male’s ascending on the female dorsal side and moving toward the anterior part of her body), 77% of all contacts

(with and without copulations) were observed with the previous males in the female choice test. In the male choice test, on the other hand, 68% of all contacts were observed with previous females. In most cases, one contact was sufficient for accomplishing copulation in both the female and the male choice tests (75 and 69%,

Figure 2. Sequences of sexual behaviour in *M. bosniense*. A – male; B – female; C, D – contact; E, F – extrusion of gonopods (arrows); G, I, H – copulation (photo: B. Ilić).

Table 4. Differences of ML, DC, CC, and ECC between two successive behavioral tests (mating arena and male/female choice tests). Significant differences are highlighted in bold. The time is expressed in seconds. For abbreviations see Table 1.

	Male	Female	Max.	Median	Variance	Std. Dev.	Quartiles 25%	Quartiles 75%
	N	I – mating arena	II “choice” test	P-value	N	I – mating arena	II “choice” test	P-value
ML	21	2122.29±427.07	1991.81±259.61	P= 0.7954	31	2010.39±323.20	942.36±131.81	P= 0.0033
DC	21	18081.00±739.99	16033.29±790.40	P= 0.0658	31	17520.13±609.18	19455.16±558.08	P= 0.0225
CC	21	230.62±34.64	279.10±52.39	P= 0.4447	31	241.81±42.83	164.94±22.81	P= 0.1184
ECC	21	1891.71±430.63	1712.71±275.43	P= 0.7280	31	1768.61±328.76	777.42±132.06	P= 0.0069

Table 5. Analyses of morphological traits (body mass and linear measurements), as source of variation, between males and females with different mating status. Significant differences are highlighted in bold.

	Mated/non-mated males		Mated/non-mated females	
Traits	T-values	P-values	T-values	P-values
Body mass (BM)	-0.3263	0.7454	2.8375	0.0063
Body length (BL)	-0.1033	0.9181	2.1283	0.0378
Antennal length (AL)	-0.1589	0.8744	1.8458	0.0703
Leg length (LL)	1.1517	0.2544	1.0982	0.2770
Trunk width (TW)	-0.2885	0.7740	0.6194	0.5382
Trunk height (TH)	-1.0988	0.2766	0.4681	0.6415

respectively). A smaller percentage of copulations was recorded after two and three contacts in both types of “choice” tests (24 and 30%, respectively).

The number of copulations was significantly higher with the previous partner than with a new partner in both types of “choice” tests [female choice test: W1 (new mating partner) = 0.24, SD = 0.1149, $P < 0.0001$, W2 (previous mating partner) = 1; male choice test: W1 (new mating partner) = 0.17, SD = 0.1129, $P < 0.0001$, W2 (previous mating partner) = 1].

Furthermore, no significant differences of DC, ECC, CC, and ML were recorded in males that mated twice in the mating arena and male choice tests. On the other hand, significant differences of DC, ECC, and ML were obtained in females that mated twice in the mating arena and female choice tests (Table 4).

Analyses of morphological traits in M. bosniense

We detected the existence of variability in the morphology of certain structures between males and females with different mating status. Mated females were larger with higher values of body mass and head CS than non-mated females (Figs. 3 and 4, Tables 5 and 6). Except in the case of body mass and body length in females, no differences of linear measurements were

observed between mated and non-mated individuals (Table 5). CVA showed clustering of the groups by CV2 axis, illustrating that the proximal part of the head was sharper in non-mated individuals of both sexes (Fig. 5). Promere shape differed significantly between males with different mating status (Table 6). Namely, the bases of promeres were wider in non-mated males (Fig. 8). In the case of other parts of the body (antennae, walking legs, opisthomeres), significant differences in size and shape were not observed (Table 6; Figs. 6, 7, and 9).

Discussion

In laboratory conditions, *M. bosniense* established numerous body contacts, more than half of which resulted in copulation. Sexual selection coefficient (W) calculated in this study indicated that preferences toward the previous partner were expressed in both “choice” tests. We presumed that females use a combination of different types of stimuli in recognition of suitable mating partner: morphological traits under the action of sexual selection, chemical/olfactory cues (such as volatile pheromones, cuticular components,

mandibular secretion, secretion from the coxal glands) or auditory stimuli (Haacker, 1969; Carey and Bull, 1986; Tadler, 1996, Wesener *et al.*, 2011). In the context of sexual selection, exchange of visual information is common in diurnal animals living in open habitats. In millipedes, because of their way of life in natural conditions, the visual stimuli themselves could not play an important role in determining mating partner. However, the quality of morphological traits could be detected indirectly, through other sensory domains (*e.g.* through the quality and/or intensity of tactile/mechanical stimulation provided by certain morphological structures).

A tendency toward mating with previously chosen partners was reported in millipedes in laboratory conditions (Telford and Dangerfield, 1993a; Jovanović *et al.*, 2017). Mating with the same partner could be the

result of assessment of his/her good health condition or his/her high genetic quality. Moreover, mating with the same partner could be explained as a form of mate guarding. The opposite strategy was observed, for example, in some insect and pseudoscorpion species [Tregenza and Hosken (2005) and references therein]. Selection of a different mating partner may ensure successful fertilization if the first male was sterile or of poor quality. Mating with a different partner could provide genetic diversity of the progeny as well.

Significant differences were observed in some morphological traits that could be involved in sexual selection (body mass, head size, and head shape) between mated and non-mated females, as well as in head and gonopod promere shape between mated and non-mated males. These results are consistent with data obtained in many arthropods indicating that

Figure 3. Morphological variation of body mass (A), body length (B), antennal length (C), leg length (D), trunk width (E), and trunk height (F) between males and females with different mating status. The median with the first and the third quartiles is shown (in boxes), together with the range of variation and outliers.

Figure 4. Morphological variation in centroid size (CS) of head (A), antennae (B), walking legs (C), promeres (D), and opisthomeres (E) between individuals with different mating status. The median with the first and the third quartiles is shown (in boxes), together with the range of variation and outliers.

Table 6. Analysis of centroid size (CS) and shape of certain morphological traits, as source of variation, between males and females with different mating status. Significant differences are highlighted in bold.

Morphological traits	Mated/non-mated males		Mated/non-mated females	
	CS	Shape	CS	Shape
Head	0.9984	0.0026	0.0012	0.0004
Antennae	0.9824	0.8728	0.9999	0.1103
Walking legs	0.9163	0.6397	0.5176	0.2073
Promeres	0.3435	0.0064	/	/
Opisthomeres	0.6456	0.1548	/	/

Figure 5. Canonical Variate Analysis (CVA) of differences in head shape between males and females with different mating status (rectangles: black – non-mated males, white – mated males; circle: black – non-mated females, white – mated females).

morphological variability of sexual and non-sexual traits influenced their mating success (Polihronakis, 2006; Zhou *et al.*, 2012; Menezes *et al.*, 2013; Kamimura, 2014; Outomuro *et al.*, 2016; Bertram *et al.*, 2017).

Behavioural traits were scored and quantified in mating arena and “choice” tests. We obtained differences in some behavioural characteristics (ML, DC, and ECC), as well as in certain morphological traits (linear measures, centroid size, and shape values) between individuals with different mating status. It seems that mate choice in this species is quite complex, since evaluation of mating partners was based on a number of traits tested in this study (previous mating experience, several important behavioural steps, and size and shape of certain sexual and non-sexual structures).

The sequences of precopulatory behaviour in this species are quite similar to those previously described in other juliform millipedes (Haacker and Fuchs, 1970; Tadler, 1996; Jovanović *et al.*, 2017 and references therein). The male uses his anterior pairs of legs to get a grip on the female's head, after which the extrusion of gonopods follows. An intermediate step between the grip and actual copulation is the charge of gonopods with spermatophores (Barnett and Telford, 1996; Minelli and Michalik, 2015). The copulating pair can assume different positions, from parallel to those where the male is coiled around the female in varying degrees with the anterior or posterior part of his body. Components of mating behaviour, mating latency (ML), and copulation duration (DC) in some

Figure 6. Canonical Variate Analysis (CVA) of differences in antennal shape between males and females with different mating status (rectangles: black – non-mated males, white – mated males; circle: black – non-mated females, white – mated females).

Drosophila species were indicators of mate choice based on previous mating experience [Pavković-Lučić and Kekić (2009) and references therein]. Our results showed that ML and ECC lasted for a significantly shorter time in the female choice test than in the mating arena test. However, the pairs formed in the female choice test copulated significantly longer than in the mating arena. In other studies, it was found that the duration of copulation can be prolonged as a result of a male-biased operational sex ratio (OSR), considering that there were two males and only one female available (Telford and Dangerfield, 1990; Cooper, 2016).

Long copulation duration, a possible adaptation in connection with mate guarding and sperm competition, was previously observed in spiders (Schneider *et al.*, 2006; Herberstein *et al.*, 2011), diplopods (Telford and Dangerfield, 1990, 1993a,b; Barnett and Telford, 1996; Rowe, 2010; Cooper, 2016), chrysomelids (Dickinson, 1997), and many insects (*e.g.* Sillén-Tullberg, 1981; Schöfl and Taborsky, 2002; Afaq and Omkar, 2017). In *M. bosniense*, the long copulation (~5 hours) could be explained, at least partially, in terms of mate guarding. During this long-lasting copulation, the male protects his investment, a behavioural trait that could result from the need for manifestation of

Figure 7. Canonical Variate Analysis (CVA) of differences in leg shape between males and females with different mating status (rectangles: black – non-mated males, white – mated males; circle: black – non-mated females, white – mated females).

sperm precedence (Harari *et al.*, 2003; Cooper, 2016). A positive correlation between the amount of sperm in the female receptacula, and the duration of copulation with the last male was detected in some millipedes (Barnett and Telford, 1994) and insects (Simmons, 2001). It is possible that a male millipede can manipulate his own ejaculate, distributing it closer to the part of the oviduct where fertilization occurs (Barnett and Telford, 1994), *i.e.*, performing sperm repositioning, which is common in insects (Simmons, 2001; Córdoba-Aguilar *et al.*, 2003; Xu and Wang, 2010). In some millipedes, the volume of the second male ejaculate significantly increased within 24 hours after copulation (Cooper, 2015), or the volume of the first male ejaculate significantly decreased during the same period (Barnett *et al.*, 1995). Sperm competition may act as an agent in the shaping of genital structure(s) involved in sperm removal (Kamimura, 2000) and sperm mixing (Barnett *et al.*, 1995; Simmons, 2001). In millipedes, the flagellum can have such a role (Tadler, 1996; Minelli and Michalik, 2015). Wojcieszek and Simmons (2011) revealed a relationship between paternity success and the presence of a spiral, spike-like shape of the tip of the gonopods, which could be responsible for sperm manipulation and/or female stimulation. We assume that the opisthomere processes have a similar

function in *M. bosniense*. In some millipedes, one of the effective mechanisms for preventing subsequent copulation is the use of copulation plugs preventing future copulations with another male (Barnett and Telford, 1994; Cooper, 2016). However, we did not observe the presence of copulation plug in our sample. Before the female utilizes ejaculates, the male must maximize the likelihood of reproduction, which requires time spent not just in guarding the female, but also in maintaining genital contact. In that manner, the male could establish himself as one of the potential “choices”, if not the most successful one.

One of the basic prerequisites for “choosiness” is morphological variation of the potential partners (Bonduriansky, 2001). Different morphological traits (*i.e.*, linear and shape measurements, centroid size values) were analysed as the potential basis for mate preference and mating success in a number of previous studies (Zhou *et al.*, 2012; Head *et al.*, 2013; Menezes *et al.*, 2013; Ali *et al.*, 2014; Outomuro *et al.*, 2016; Bertram *et al.*, 2017). In the present study, body mass and body length differed significantly between mated and non-mated females, *i.e.*, mated females had a larger values of both traits. We investigated mating success as one of the components of reproductive success, others being fecundity, paternity success and parental care. However, explanation of our results may

rest upon literature evidence of positive correlations between fecundity and body mass in millipedes (Heath *et al.*, 1974), Orthoptera (Sturm, 2016) and in oviparous species of several insect orders (Honěk, 1993). Larger females with thicker abdomens (Yang and Wang, 2004), greater body length (Lu *et al.*, 2013), and body width (Cooper, 2017) were more attractive to males in different species. This could result not only from possessing a greater number of eggs, but also from being capable of receiving a larger volume of ejaculate (Lu *et al.*, 2013; Cooper, 2017). In the family Julidae, paired ovaries extend from 15th to the last leg-bearing segment, and eggs can take up more than half the segment's volume (Hopkin and Read, 1992). Further, *M. bosniense* is an euanamorphic species (individuals moult throughout entire life), body size is linked with age (Enghoff, 1992b). It implies that the age of an individual could also contribute to mating preference/success.

Morphological variation of antennae and legs between sexes with different mating status

In millipedes, the male's tapping the head of the female with his antennae can be described as male courtship and/or mate assessment (Carey and Bull, 1986). This type of behaviour is also common in insects (Møller and Zamora-Muñoz, 1997; Bonduriansky, 2001). In

the process of courtship and/or mate assessment, the antennae have an important role in millipedes, as shown by Carey and Bull (1986) in an experiment with the julid *Ommatoiulus moreleti* (Lucas, 1860). Males whose whole antennae or just the antennal tips were removed mated less frequently than did males which were not subjected to this procedure, while females with different degrees of antennal removal mated as frequently as did ones whose antennae remained intact. On the other hand, the length of undamaged and intact antennae was not significantly different between mated and non-mated longhorn beetle *Stenurella melanura* (Linnaeus, 1758) males (Møller and Zamora-Muñoz, 1997). This was corroborated in our study; namely, no significant effect of antennal size and shape (females: $P = 0.9999$, males: $P = 0.9824$; females: $P = 0.1103$, males: $P = 0.8728$; respectively) on mating decisions was observed.

It is known that possession of longer legs used in grasping (Garga *et al.*, 1997; Sugano *et al.*, 2008) or courtship behaviour (Amin *et al.*, 2012) is positively correlated with male mating success in insects and spiders. Since males “walk” across the whole length of the female's body, we supposed that the length and/or shape of the legs could be important factors providing suitable tactile stimulation in *M. bosniense*. A similar type of behaviour is observed in the hymenopteran *Cephalonomia tarsalis* (Ashmead, 1893) (Cheng *et al.*, 2004). However, our results gave no support to the

Figure 8. Canonical Variate Analysis (CVA) of differences in promere shape between males with different mating status (bars: white – mated males; grey – non-mated males).

Figure 9. Canonical Variate Analysis (CVA) of differences in opisthomere shape between males with different mating status (bars: white – mated males; grey – non-mated males).

presumption that this type of stimulation is important for successful mating (CS: females, $P = 0.5176$, males, $P = 0.9163$; shape: females, $P = 0.2073$, males, $P = 0.6397$). The significance of walking legs for the male mate search effort was previously noted in the paradoxosomatid *Cladethosoma clarum* Chamberlin, 1920 (Rowe, 2010). In this species, males possess longer legs than females. Nevertheless, since in *C. clarum* copulation lasts only several minutes, the number of receptive females could be the main driving force influencing male mating success.

Apart from tactile stimuli, courtship behaviour in millipedes can include exchange of stimuli from other sensory domains. Other cues were not investigated here, although it is presumed that volatile pheromones or cuticular components may play an important role in the mating behaviour of some millipedes (Carey and Bull, 1986). Mandibular secretion is observed in the julid *Julus terrestris* Latzel, 1884 (Haacker, 1969) and in the pachybolid *Centrobolus* Cook, 1897 after copulation has already started (Cooper and Telford, 2000). In *Cylindroulus boleti* (C. L. Koch, 1847) and *Brachiyulus lusitanus* (Verhoeff, 1898), males released a secretion from the coxal glands of the second pair of legs in the mouth of females during the first minute of copulation (Tadler, 1996). Males of the family Sphaerotheriidae or the genus *Loboglomeris* (both belonging to order Glomerida) use specific sound to induce vibrational patterns that would appear to counter the coiling behaviour of females (Haacker, 1968; Wesener *et al.*, 2011).

Morphological variation of the head between sexes with different mating status

Precopulatory sexual selection can affect morphological variation of the head in insects (Rodríguez *et al.*, 2002; Bonduriansky and Rowe, 2003). Females of some insects distinguished their potential mates on the basis of head morphology, eye length, face width (Rodríguez *et al.*, 2002) or on the basis of head elongation (Bonduriansky and Rowe, 2003).

As already mentioned, the head of the female in *M. bosniense* is an area where the male spends some time tapping with his antennae, as it is “the attachment region” after formation of a mating pair. In the present study, we found that mated and non-mated individuals of both sexes differed significantly in head shape (females: $P = 0.0004$; males: $P = 0.0026$). As the male positions himself above the female grasping her head, shape of the head could

be important for achieving the position needed for a successful copulation. The larger heads in mated females provide larger areas for males to perform courtship and/or mate assessment.

Morphological variation of gonopods (promeres and opisthomeres) between males with different mating status

Morphological variation of somatic and genital traits may have significant impact on mating success in many animal groups (Langerhans *et al.*, 2005; Pavković-Lučić *et al.*, 2009; Ponlawat and Harrington, 2009; Cator and Zanti, 2016). Somatic traits are under the influence of precopulatory sexual selection (Jones and Ratterman, 2009), while sexual traits can be subjected to both pre- and postcopulatory selection (Polihronakis, 2006; Kamimura, 2014; Simmons, 2014). When considering genital traits, for example, in water striders, non-intromittent genital traits are under precopulatory, while intromittent genital traits are under postcopulatory sexual selection (Rowe and Arnqvist, 2012).

Millipede gonopods are morphologically complex integrated units with different roles during copulation (Wojcieszek *et al.*, 2012). In several julid species, the function of the promeres are probably only to open the vulvae. On the other hand, the opisthomeres - onto which a sperm droplet is secreted - are necessary for insemination (Tadler, 1996). This author considered that the Julid gonopods evolved toward being opisthospermous (sperm transfer by posterior gonopods). Promeres are regarded as structures without a direct sperm transfer function. When reaching vulva, promeres are pressed against the operculum, which “covers the gonopore from anterior” (Koch, 2015). Therefore, it is important for members of the family Julidae to possess well developed promeres. Gonopods of *M. bosniense* are characterized by a well-developed promeres, as well as opisthomeres with two smaller processes and a larger posterior one (Lazányi and Vagalinski, 2013). Our results show that only promere shape differs between mated and non-mated males (promere shape: $P = 0.0064$; opisthomere shape: $P = 0.1548$), suggesting that shape of “genital” parts has more important role in mating in comparison with the size of the same structures. These findings corroborate results of the study on water striders where the shape of genital structures has greater importance in insect genital evolution (Rowe and Arnqvist, 2012).

Conclusions

In *M. bosniense*, it seems that both sexes can exert precopulatory as well as cryptic choice. Here we examined morphometric traits that could determine a suitable mating partner and measured some sequences of mating behaviour that imply cryptic choice on the background of extremely long copulation during which mate guarding and possible ejaculate manipulation occur. Using a combination of different types of stimuli, males of *M. bosniense* recognize females with greater body mass and head shape corresponding to their own to achieve a firm grasp and head-above-head copulation position. Furthermore, differences in promere shape contribute to male mating success. Previous mating experience also influence mate choice in this species.

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