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COMPARATIVE ANALYSIS OF LIGULAS OF SLUGS *ARION LUSITANICUS* FROM LITHUANIA AND DENMARK

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Comparative analysis of ligulas of slugs *Arion lusitanicus* from Lithuania and Denmark. —Mantas Adomaitis, Grita Skujienė.—Observations on the proximal portion of the oviduct and ligula morphology and morphometric analysis of the distal genital apparatus were made in separate and mixed populations of *Arion lusitanicus* and *Arion rufus* from Lithuania and Denmark. In both countries, significant bigger variation of ligulas structure was observed in pure populations of *A. lusitanicus*. Our results show that *A. lusitanicus* ligula morphology is insufficient feature for distinguishing hybrids. Our results suggest that variations of ligula form and inside number of folds in *A. lusitanicus* seems to be determined both by size and reproduction stage (i.e. is it before or after copulation) factors.

Key words: *Arion*, morphology, ligula, invasive species, hybridisation.

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Порівняльний аналіз лігул слизнів *Arion lusitanicus* з Литви і Данії. — Мантас Адомайтіс, Гріта Скуєненю.—Проведено порівняльний аналіз проксимальної частини овідукта і морфології лігули та морфометричний аналіз дистальних відділів статеві системи у чистих та змішених популяціях *Arion lusitanicus* та *Arion rufus* із Литви та Данії. Значно вища варіабельність структури лігули спостерігалась у чистих популяціях *A. lusitanicus* обох країн. Наші результати доводять, що морфологія лігули *A. lusitanicus* є недостатньою ознакою для виділення гібридів. Вони свідчать також про те, що варіабельність форми лігули та кількості внутрішніх складок у *A. lusitanicus*, ймовірно, визначаються розміром тварини та репродукційною стадією (наприклад, до чи після копуляції).

Ключові слова: *Arion*, морфологія, язичок, інвазивні види, гібридизація.

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Introduction

The slug *Arion lusitanicus* (Mabille 1868) is highly invasive gastropod and is listed as one of the hundred most invasive alien species in Europe [23; 3; 15; 22]. It is known that this species has negative effect to native slug species [29; 24; 9].

In Lithuania, first records of *A. lusitanicus* date to the year 2008 and it emerged in about ten years after the occurrence of *A. rufus*. Both species are not native for Lithuania, although one large *Arion* – *A. ater* – lives here and is included into the list of rare species of the Lithuanian Red Book. Now *A. lusitanicus* is successfully invading in several locations [1] and raises some questions: what morphological type of slugs is spreading in Lithuania and is it dangerous for *A. ater*; are spreading slugs polymorphic or not; that are similarities with *A. lusitanicus* in surrounding or further countries.

The part of reproduction system of slugs, localized in the distal-proximal portion of the oviduct and described as a ligula, has identification value for large *Arion* spp. [9; 25; 4; 21]. First it was mentioned by Hesse [18] for *Arion empiricorum* Furussac, 1819. Object of this work is morphology of ligulas of slug

Arion lusitanicus Mabille, 1868, also regarded as *Arion vulgaris* Moquin-Tandon, 1855, as this species is spreading over Europa and possibly is crossbreeding with others large Arionids [10; 2]. This crossbreeding is often mentioned as a successful spreading factor for *A. lusitanicus* and parallels is often drawn that at first *A. rufus* displaces *A. ater* and later *A. lusitanicus* – *A. rufus* [11; 8; 21; 19; 14; 16; 26]. Although some authors earlier stated that *A. lusitanicus* couldn't have interspecific matings with other species as there are natural reproductive barriers due to differences in the morphology of ligulas, spermatophores transfer and copulation behaviour [6; 9]. There are some indications about hybrids between *A. lusitanicus* / *A. vulgaris* and *A. rufus*, but experimental data shows very low effectivity – in captivity only 16% of all interspecific copulations were successful and only some individuals were able to lay eggs [12; 26; 10]. Ability of mating between *A. lusitanicus* / *A. vulgaris* and *A. rufus* were analysed by comparing genital morphology and copulation behaviour [30; 2; 17]. Nobody will not reared slugs from eggs after interspecific copulation till the second or third generation, so it remains unclear whether hybrids

are fertile and which genital features reliably indicate hybrids.

As variation of the ligula morphology usually is regarded as hybrid feature [13; 21; 17], we studied the ligula morphology and made morphometric analysis of the distal genitalia in separate and mixed populations of *A. lusitanicus* with *A. rufus* from Lithuania and Denmark.

Material and methods

Material was collected from Lithuania and Denmark. Lithuania is located in the centre of Europe and belongs to the Baltic-Scandinavian region like Denmark [5] and has a continental climate with high precipitation (550–850 mm annually) throughout the year, cold (up to $-20 - 30^{\circ}\text{C}$ and -5°C in average) and long (105–135 days) winters and warm summers ($+17^{\circ}\text{C}$ in average) [7]. Majority of slugs in such conditions are active until frosts [1]. Denmark has an oceanic climate with similar precipitation but milder winters (the most humid time of the year, 0°C in average) and cooler summers [28] so active period of slugs continues protractedly.

Slugs were randomly collected from July to September, 2014–2015, at eleven sites (Fig.1; Table 1), nine of which were in Lithuania (Fig.1: 1–9) and two in Denmark (Fig. 1: 10–11). Sites with mixed populations are mentioned as sites with presence of *A. rufus* (Table 1: 1, 2, 5, 11). Coloration of *A. lusitanicus* and *A. rufus* in both countries was opposite and making it impossible to assign dark brown or orange specimens to a species solely on coloration. Slugs were identified according the form of genitalia using Rowson *et al.* [27] identification guide and ligulas characters described by Noble [21].

All specimens were killed by cooling at -20°C and then were stored in 70 proc ethanol prior to dissections. All slugs were weighted (on a scale graded in 0,1 g), measured and dissected. Morphometric analysis was carried out using 11 characters: length, width and weight of animal, length of thick part of oviduct, epiphallus, vas deferens, bursa copulatrix with duct, ligula, number of folds of ligula, connection between ligula and atrium. As slugs from Denmark were dissected earlier and for this study we had only genitals, so we couldn't check body size, length and width.

Organs were measured under gentle tension. Number of folds of ligula was chosen as the clearest criterion for assessment of ligula morphological forms: 1) two symmetrical large without any inside wrinkles folds as described by Noble [21] for *A. lusitanicus* taxa (including Mabilie, 1868); 2) two large outside, irregular rough folds (assigned for „non-typical” ligula of *A. vulgaris* or more precisely „hybrids” by Hatte land *et al.* [17]) and one or more inside folds we assigned for ligula of potential hybrids of *A. lusitanicus* / *A. rufus*.

Statistical analysis was performed using STATISTICA 8. Results were analysed using non-parametric Spearman Rank Order Correlation for measuring the strength of populations between two selected ranked characters and Mann-Whitney U test to

compare two unpaired groups of data. This test computes p values that test the null hypothesis that the two groups have the same distribution.

Results and discussion

Overall 219 samples from Lithuania and 44 from Denmark were analysed (Table 2). Mann-Whitney test showed that only length of vas deferens and bursa copulatrix significant differ and is bigger of slugs from Denmark. This can correspond to later collection data (Table 1) and possibly post-copulative stage as bursa copulatrix is bigger. Length of vas deferens of Lithuanian samples much more varies, but in average is less than this is of Danish slugs.

Spearman Rank Order Correlations showed that weight of Lithuanian slugs have strong positive correlation (the correlation coefficient was between 0.7 and 0.9, $p < 0.05$) with all other quantitative variables. A scatter plot (Fig. 2) show distribution of length of oviducts against body mass and as the length of oviducts of Danish slugs distribute between is 0.6 and 1.2 cm we can made presumption that collected slugs from Lithuania and Denmark are similar in size as majority of slugs are from the same size group but some slugs from Lithuania are bigger.

Mann-Whitney U test showed that neither the body weight nor the ligulas' morphological forms ($p = 0.49$) differed between slugs caught in July or August in Lithuania, but ligulas' morphological forms differed significant ($p < 0.05$) depending on weight (Fig. 3). As we know that big Arions are protandric and during the first month of life each individual is male, from two months onwards it is a true hermaphrodite and finally it becomes female at the age between five to twelve months when it produce eggs and weight of the body increases [20], we can presume that differences of ligula's morphological forms depends on using of the ligula – if it is not used (before copulation) it has less folds and has more regular form and this corresponds to the lower body weight; later, after copulation slugs increase their weight as are producing eggs and after copulation form of ligula became more irregular and we can see that ligula becomes more polymorphic in shape and inside structures like folds.

Contrary to our expectations that older collection time of slugs in Denmark will generate higher ligulas polymorphism, Lithuanian slugs were much more polymorphic and had more folds inside the ligulas (Fig. 4, left). Possible this polymorphism belong to size of slug and it reproductive stage as mentioned above (we presume that the more number of folds appear after copulation). Moreover Mann-Whitney U test showed that specimens representing pure *A. lusitanicus* and mixed populations of *A. lusitanicus* and *A. rufus* (sites with mixed populations are from both countries and are mentioned as sites with presence of *A. rufus* in Table 1) significantly differ in number of folds (Fig. 4, right).

Considering to precondition that number of folds were related with other characters of hybrid ligula' form described by Hatte land *et al.* [17] our results are contrary to literature data.



Fig. 1. Study Area: **Lithuania:** Kaunas – Ķhuolynas (1), Mickeviius valley (2), Amaliai (3), Botanical garden (4), Ķhaliakalnis (5); Vilnius – Balsiai (6), VU Botanical garden (7); Panevėžys – Skaistakalnis park (8); Marijampolė – Puskelniai (9); **Denmark:** Lisbjerg (10), Silkeborg (11).

Рис. 1. Область дослідження: Литва: Каунас – Ажуолінас (1), Міцкявічюс долина (2), Амаліай (3), Ботанічний сад (4), Жалаякальніс (5); Вільнюс – Балсіай (6), VU Ботанічний сад (7); Паневежис – Скаїстакалніс парк (8); Маріямполє – Пускелніай (9); Данія: Лісберґ (10), Сількеборґ (11).

Table 1. Detailed information about collection sites of *Arion lusitanicus*

Таблиця 1. Детальна інформація про колекцію сайтів *Arion lusitanicus*

No.	Location	Coordinates	Data	Number of specimens	Presence of <i>A. rufus</i>
1.	Kaunas, Ķhuolynas	54°53' 52"N, 23° 56' 59"E	2015 07 22	22	yes
2.	Kaunas, Mickeviius valley	54° 53'50"N, 23° 56' 56"E	2015 07 22	11	yes
3.	Kaunas, Amaliai	54°53' 54"N, 24°0'13"E	2015 07 23	10	no
4.	Kaunas, VDU Botanical garden	54° 52' 24"N, 23° 54' 49"E	2015 07 22	42	no
5.	Kaunas, Ķhaliakalnis	54° 54' 37"N, 23° 54' 36"E	2015 07 09	11	yes
6.	Vilnius, Balsiai	54° 47' 4"N, 25° 21' 39"E	2014 07 14 2015 08 03	19 18	no
7.	Vilnius, VU Botanical garden	54° 44' 5"N, 25° 24' 26"E	2015 07 15 2015 08 05	28 20	no
8.	Panevėžys, Skaistakalnis park	55° 43' 57"N, 24° 22' 28"E	2015 08 20	25	no
9.	Marijampolė, Puskelniai	54° 36' 6"N, 23° 23' 2"E	2015 09 30	13	no
10.	Denmark, Lisbjerg	56°13'17"N 10°10'08"E	2014 08 19	12	no
11.	Denmark, Silkeborg	56°09'20"N 9°33'24"E	2014 08 20	32	yes

Table 2. Morphometric differences between *A. lusitanicus* from Lithuania (LT) and Denmark (DE)

Таблиця 2. Морфометричні відмінності між *A. lusitanicus* з Литви (LT) і Данії (DE)

Character	Average \pm SD		Min		Max	
	LT	DE	LT	DE	LT	DE
Body size, cm	4.5 \pm 1.2	–	2.1	–	9.6	–
Body width, cm	1.4 \pm 0.3	–	0.7	–	2.5	–
Body weight, g	3.9 \pm 2.3	–	1.0	–	11.8	–
Oviduct length, cm	0.9 \pm 0.3	0.9 \pm 1.2	0.3	0.6	1.8	1.2
Epiphallus length, cm	1.3 \pm 0.5	1.3 \pm 0.2	0.4	0.8	2.4	1.7
Vas deferens length, cm	1.6 \pm 0.5	1.7 \pm 0.4	0.4	1.0	3.2	2.3
Bursa copulatrix length, cm	0.6 \pm 0.3	1.0 \pm 0.3	0.1	0.6	1.8	2.1
Ligula length, cm	0.7 \pm 0.2	0.7 \pm 0.2	0.3	0.5	1.6	1.0

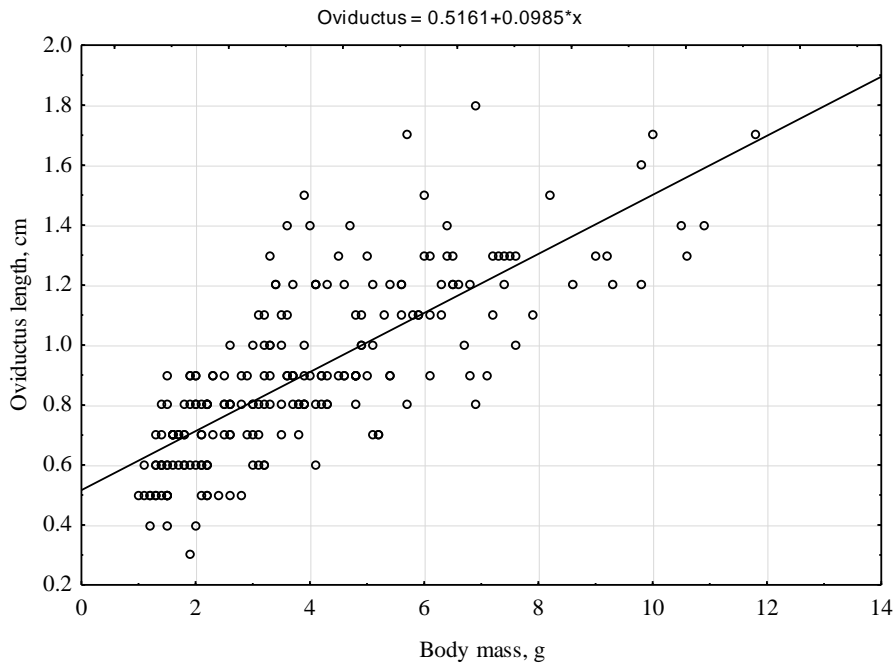


Fig. 2. A scatter plot of oviduct length (cm) against body mass (g) for 219 slugs. Spearman's correlation coefficient $R_S = 0.8$, $p < 0.05$.

Рис. 2. Залежність довжини овідукта (см) від маси тіла (г) для 219 слизнів. Коефіцієнт кореляції Спірмена $R_S = 0,8$, $p < 0,05$.

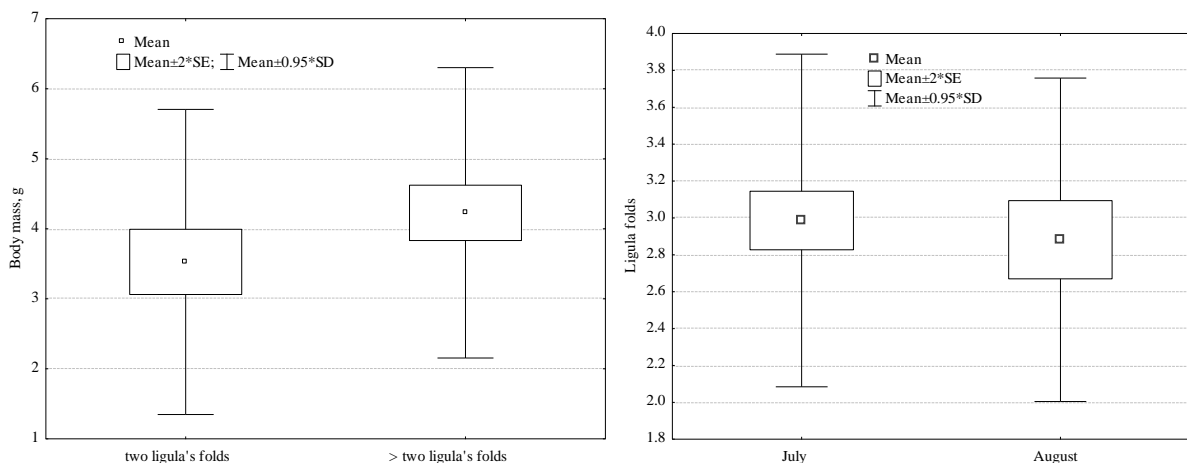


Fig. 3. A. *A. lusitanicus* ligula's folds variability within two months when majority of slugs were caught in Lithuania (left) and influence of body mass to number of ligula's folds ($p < 0.05$, right).

Рис. 3. Мінливість кількості складок лігули *A. lusitanicus* протягом двох місяців, коли було зібрано більшість слизнів у Литві (ліворуч), та вплив маси тіла на кількість складок лігули ($p < 0,05$, праворуч).

The less ligula's folds number of slugs from mixed populations suggests that either hybrids not present in mixed populations either this feature is not suitable for identification of hybrids.

But since variability of number of folds has been observed in pure *A. lusitanicus* populations we can conclude that ligula' form isn't sufficient character for identification of hybrids of *A. lusitanicus* and *A. rufus* as it varies depending on the size of slug and possible

is connected with copulation stage – is it slug before or after copulation. For verification of these data it is necessary to make analysis of mitochondrial and nuclear DNA. Moreover for summarizing similar conclusions about ligula's suitability for hybrid identification as Hatteland et al. made [17], it is necessary to obtain a statistically significant number of the second generation of real hybrids of *A. lusitanicus* and *A. rufus* if it possible under field conditions.

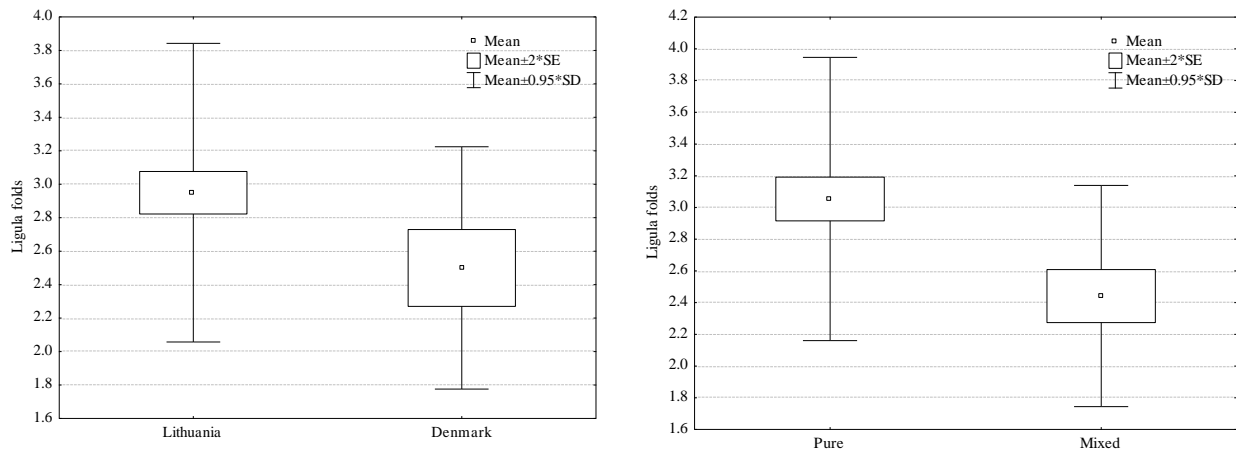


Fig. 4. *A. lusitanicus* ligulas' folds variability within two studied countries ($p < 0.05$, left) and within pure *A. lusitanicus* or mixed with *A. rufus* populations ($p < 0.05$, right).

Рис. 4. Мінливість кількості складок лігули *A. lusitanicus* у межах двох досліджених країн ($p < 0,05$, ліворуч) та у чистих і змішаних з *A. rufus* популяціях ($p < 0,05$, праворуч).

Conclusions

Our results show that ligula morphology of *A. lusitanicus* is insufficient feature for distinguishing hybrids between *A. lusitanicus* x *A. rufus* and pure *A. lusitanicus*.

Our results suggest that variations of ligula form and inside number of folds in *A. lusitanicus* seems to be determined both by size and reproduction stage (i.e. is it before or after copulation) factors.

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