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BEER AS ATTRACTANT FOR *ARION VULGARIS* MOQUIN-TANDON, 1885 (GASTROPODA: PULMONATA: ARIONIDAE)

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Abstract: *Arion vulgaris* Moquin-Tandon, an invasive slug, causes losses in crops all over Europe. Small farmers commonly use beer traps to control this pest. Our aim was to check which components of the volatile fraction of beer were important as slug attractants. Five beer brands available at the Polish market: Żubr, Warka Full, Karpackie Pils, Żywiec and Leżajsk Full, were used in the experiments. We examined the slugs' behaviour in the laboratory, using a star-shaped olfactometer (two-hour tests), and in the field (three-day tests with traps). Particular beer brands attracted the slugs to various extent, depending on the composition of their volatile fraction: the number of slugs caught in the traps was positively correlated with the content of decanoic acid and negatively with that of acrylic acid N-hydroxysuccinimide ester and CO_2.

Key words: *Arion vulgaris*, beer, attractant, pest, trap

INTRODUCTION

Arion vulgaris Moquin-Tandon, 1885 (= *A. lusitanicus* auct. non Mabille), the slug species originating from south-western Europe, probably S.W. France (Chevallier 1974), and till recently misidentified as *A. lusitanicus* Mabille, 1968 (Pfenninger et al. 2014, Hatteland et al. 2015), started expanding its range in Europe in the mid-twentieth century (Schmid 1970, Păpureanu et al. 2014). Its especially intensive expansion was observed in the 1970s (Chevallier 1972, Hagnell et al. 2006b, Kozłowski & Kozłowski 2011). In Poland (environs of Albigowa, province Podkarpackie), the species appeared probably in the late 1980s (Pisarek 2006, Kozłowski et al. 2010, Jaskulska et al. 2011). Its first, fully confirmed occurrence was observed a few years later (Kozłowski & Kornobis 1994). At present, *A. vulgaris* occurs throughout the country (Kozłowski 2007). Genetic studies indicate that the species' population in Poland is a result of multiple introductions from different sources (Kałuski et al. 2009, 2011).

A. vulgaris is a particularly serious pest, not only because it occupies a wide range of habitats: fields, gardens, allotments, parks or cemeteries (Kałuski et al. 2009), and causes significant losses in crops of vegetables, cereals, and oilseed rape (Jaskulska et al. 2011), but also because it can develop resistance to molluscicides (Sulzberger 1996). The slug can self-fertilise (Hagnell et al. 2006a, Engelke et al. 2011), displaces some of the native species (Kałuski et al. 2010) and hybridises with them (Foltz et al. 1982, Hagnell et al. 2003, Allgaier 2015, Hatteland et al. 2015). As a result it is difficult to identify; identification is based on the reproductive anatomy (Quinteiro et al. 2005, Păpureanu et al. 2014) and/or molecular characters (Pfenninger et al. 2014, Hatteland et al. 2015).

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At present, the number of molluscicides to choose from is limited; in Poland the twenty formulas authorised for use contain only two active substances (http://www.minrol.gov.pl/pol/Informacje-branzowe/Wyszukiwarka-srodkow-ochrony-roslin/%28action%29/search), and their environmental toxicity is commonly known (Rae et al. 2009). That is why some owners of small farms or gardens use traps with attractants, for example beer, to control the pest (Hagnell et al. 2006b, Dankowska 2011).

Since the effectiveness of beer as slug attractant had already been partially proved (Dankowska 2011, Piechowicz et al. 2014), in this study we decided to focus on determining which components of the volatile fraction of different beer brands could possibly play the role of attractants for *A. vulgaris*.

MATERIAL AND METHODS

ANIMALS

In the experiments we used adult *A. vulgaris* caught in the park surrounding the Institute of Applied Biotechnology and Basic Sciences, University of Rzeszów. No means of chemical plant protection had been used in the park for one year before the slug collecting. No chemicals which could interfere with the slug's olfactory perception of the volatile fraction of the tested beer brands had been used before the experiment.

BEER AS ATTRACTANT

The attractiveness of five popular brands of beer for slugs was tested in the laboratory and in the field (Table 1). The volatile fraction of the beer brands was subject to chromatographic analysis.

Laboratory tests were performed in the Institute of Applied Biotechnology and Basic Sciences, University of Rzeszów, from July to October 2010 and 2013. Modified olfactometer OLF0001 (constructed by Andrzej Zienkiewicz Zakład Remontowo Montażowy Aparatury Laboratoryjnej, Toruń, Poland) was used for the analysis the olfactory preference of the slugs (Fig. 1).

Synthetic air, additionally purified by a carbon filter, was directed using the rotameter and a set of tubes Bev-A-Line IV (producer: USPlasticCorp, USA) into five of six arms of the olfactometer (the sixth arm was closed during the tests). Gas was pumped into each channel at the rate of 100 ± 5 ml/min. Before introducing into the olfactometer, the gas flowed through a flask of 50 ml (1 cm above the surface of the liquid), where a 20 ml sample of the studied beer brand was placed. The outlet of the gas carrying the

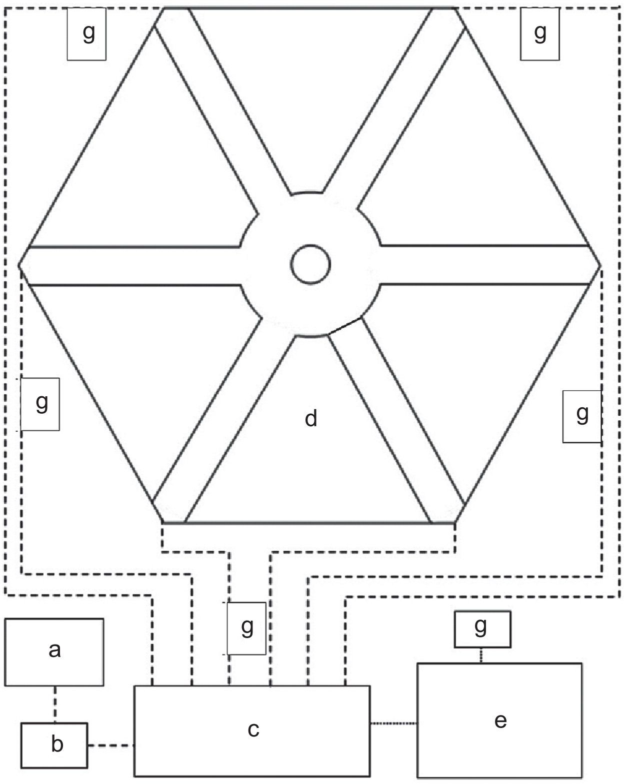

Fig. 1. Schematic representation of the olfactometer. The olfactometer consisted of: a – container with synthetic air (producer: AirLiquide Poland), b – set of carbon filters (producer: Qubit Systems, Canada), c – eight-channel flow meter G245 (producer: Qubit Systems, Canada), d – teflon arena of the olfactometer, including a space where the animals were placed, and six arms, into whose distal parts gas was directed; the whole system was covered with a glass plate (producer: Andrzej Zienkiewicz Zakład Remontowo Montażowy Aparatury Laboratoryjnej, Poland), e – working station with 'Olfak' software to operate the rotameter (producer: Andrzej Zienkiewicz, Zakład Remontowo Montażowy Aparatury Laboratoryjnej, Poland) and 'BioVid' (producer: FerroSoftware Poland) for visual registration, f – setup for visual registration, g – glass flasks with samples

Table 1. Beer brands used in the study

No.	Brand	Declared alcohol content (% v/v)	Declared extract content (% v/v)	Brewery
1	Żubr	6.0	12.0	Kompania Piwowarska S.A.
2	Warka Full	5.7	12.5	Grupa Żywiec S.A.
3	Karpackie Pils	4.0	10.0	Van Pur S.A.
4	Żywiec	5.6	12.5	Grupa Żywiec S.A.
5	Leżajsk Full	5.5	12.0	Grupa Żywiec S.A.

volatile fraction of beer was located at the upper part of the vessel. Thus prepared sample was introduced into the distal part of the olfactometer arm. During each measurement the sample was randomly directed into one of the olfactometer channels.

Selection of the olfactometer arm with the smell of a given beer brand by the slugs was recorded. The position of the slugs in the olfactometer arena was recorded after 120 minutes of their stay in the olfactometer. Each time five individuals were placed in the arena (only vigorously moving slugs were chosen for the experiment). After the 120 minute period of the animals' stay in the middle of the olfactometer arena, none of them was seen to remain there. A total of 50 replicates of the tests were performed, using a total of 250 individuals of *A. vulgaris*.

Field tests were carried out in the park surrounding the Institute of Applied Biotechnology and Basic Sciences in Werynia (province Podkarpackie, Poland). Traps were placed at the following locations: 1. within 2 m of the drainage ditch, 2. in the middle of the park, and 3. on a small meadow.

Five traps (one for each beer brand) were placed at each location. They were home-made as described by HAGNELL et al. (2006b). Bottles made of ethyl polyterephtalane (PET) of 1.5 L–volume (producer GTX Hanex Plastic Sp. z o.o.), were used for the trap production. The upper part of the bottle was cut at 12 cm, reversed and fastened again to the bottom part of the bottle. Thus prepared traps were buried in the soil with their upper parts slightly below the soil surface, in order to prevent the slugs from injuring their soles on the sharp edges of the plastic. 125 ml of beer was poured into each trap. After three days, the traps were brought to the laboratory where the slugs were counted and identified based on their reproductive anatomy. The test was performed in ten replicates, each using new traps and new portions of beer.

Besides, on each day of the experiment, at 9.00, in all the locations approximate environmental parameters: insolation [lux], temperature [°C], and relative humidity [%], were recorded using a Meter Environmental Conditions DT 8820 (producer: CEM, UK).

CHROMATOGRAPHIC ANALYSIS

The analysis of the volatile fraction of the beer brands was done using a gas chromatograph Varian 450 equipped with an MS detector 240 and a column Varian VS-5MS 30 m × 0.25 mm × 0.25 μm. Headspace Solid-Phase Microextraction (HS-SPME) fibre-PDMS 100 μm (microns) was used. Qualitative analysis was performed using the NIST 08 database.

The sample of beer (2 ml) was poured into a 5 ml glass flask, and then its volatile fraction was sampled using HS-SPME, for 30 min. at 37°C. In order to avoid the influence of changes in atmospheric pressure on the gas pressure in the samples of volatile fraction, they were collected at the same time. The SPME Holder with the analyte adsorbed on the fibre was placed in a gas chromatograph dispenser at 200°C, under helium flow of 1 ml/min. and splitless injection mode. The desorption process was carried out for 5 minutes and the analytes were assayed using the following temperature programme: 50°C for the first 5 minutes → temperature increase at 10°C /min. to 250°C maintained for 10 minutes → temperature increase at 20°C /min. to 300°C maintained for 10 minutes. Peaks identification was carried out using the NIST 08 database.

STATISTICAL ANALYSIS

The beer brands were ranked according to their attractiveness to slugs (mean frequency of selection by slugs) in the field and laboratory tests, and according to the mean relative concentration of the volatile substances. Then, the relationship between the two variables was analysed using the Spearman rank correlation test.

The Bernoulli scheme was used to check if in the laboratory tests the slugs chose randomly among the volatile fractions of beer with particular chemical composition. The experiment of placing five slugs which could potentially choose among five cells was run in 50 replicates. The success was that 0, 1, 2, 3, 4, 5 slugs, respectively, would choose the given beer brand and for each beer brand we would get the most probable number of choices. In order to determine whether the choice was random, we used the test of comparing the fractions.

The similarity of beer brands in terms of attractiveness for slugs in the laboratory was examined using cluster analysis with Ward's method and Taxicab metric. The Kruskal-Wallis test was used to check if the attractiveness of beer for the slugs differed among the locations.

RESULTS

LABORATORY TESTS

The number of selections and ranking of the slug preferences for the individual beer brands are shown in Table 2. The olfactometer channels with the components of the volatile fraction of Leżajsk Full and Żubr were chosen much more frequently than the channels with the smell of other beer brands, and the smell of Warka Full was the least frequently chosen (Table 3).

The cluster analysis of the number of choices of the individual beer brands by the slugs confirmed that the components of the volatile fraction of Warka Full were attractive to the slugs, similarly as the smell of Żywiec, while Karpackie Pils, Żubr and Leżajsk Full were preferred with varying frequency (Fig. 2).

Table 2. Number and frequency of choices of olfactometer channel with smell of different beer brands by *A. vulgaris*

Beer brand	Number (frequency [%]) of slugs selecting the beer brand	Ranking based on laboratory tests
Żubr	56 (22.4)	2.0
Warka Full	33 (13.2)	5.0
Karpackie Pils	50 (20.0)	4.0
Żywiec	53 (21.2)	3.0
Leżajsk Full	58 (23.2)	1.0

FIELD TESTS

The number of individuals *A. vulgaris* caught in the traps with beer brands of different chemical composition of volatile fraction, the frequency of choices and ranking of the number of caught animals, are shown in Table 4. The greatest number of slugs was

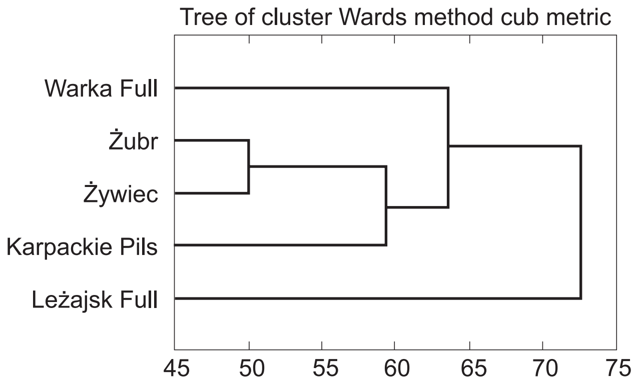

Fig. 2. Cluster analysis of the number of slug choices of olfactometer channels with volatile fraction of different beer brands

Table 3. Number of choices of smell of different beer brands in olfactometry tests, using Bernoulli scheme. Statistically significant differences marked with an asterisk

Choice	Most probable number of choices	Real number of choices and p-value				
		Żubr	Warka Full	Karpackie Pils	Żywiec	Leżajsk Full
No selection	16	19 (0.33)	26 (0.04)*	19 (0.33)	15 (0.44)	19 (0.33)
1 choice	20	12 (0.02)*	15 (0.25)	17 (0.35)	20 (1.00)	13 (0.03)*
2 choices	10	14 (0.05)*	9 (0.43)	9 (0.43)	12 (0.37)	10 (1.00)
3 choices	2	4 (0.27)	0 (0.15)	5 (0.20)	3 (0.37)	7 (0.02)*
4 choices	0	1 (0.23)	0 (1.00)	0 (1.00)	0 (1.00)	1 (0.23)
5 choices	0	0 (1.00)	0 (1.00)	0 (1.00)	0 (1.00)	0 (1.00)

Table 4. Number and frequency of individuals of *A. vulgaris* caught in traps with different beer brands and ranking of the number of trapped slugs

Location of the trap		Żubr	Warka Full	Karpackie Pils	Żywiec	Leżajsk Full
Drainage ditch	number of slugs selecting the brand	14	8	24	15	11
	frequency of choices [%]	19.4	11.1	33.3	20.8	15.3
	ranking	3.0	5.0	1.0	2.0	4.0
Park	number of slugs selecting the brand	1	0	3	0	1
	frequency of choices [%]	20.0	0.0	60.0	0.0	20.0
	ranking	2.5	4.5	1.0	4.5	2.5
Meadow	number of slugs selecting the brand	3	0	6	6	3
	frequency of choices [%]	16.7	0.0	33.3	33.3	16.7
	ranking	3.5	5.0	1.5	1.5	3.5
Total	number of choices	18	8	33	21	15
	frequency of choices [%]	18.9	8.4	34.7	22.1	15.8
	ranking	3.0	5.0	1.0	2.0	4.0

caught in the traps located near the drainage ditch. In all the locations the most numerous slugs were caught in the traps with Karpackie Pils, and the smallest number in the traps with Warka Full, while in the case of the traps placed in the middle of the park it was Żywiec. The Kruskal-Wallis test showed that for the traps with Warka Full and Leżajsk Full the distribution of the number of choices of the specific traps depended on their location (Table 5).

Table 5. Distribution of the number of choices of traps with different beer brands depending on trap location (Kruskal-Wallis test). Statistically significant differences marked with an asterisk

Beer brand	Chi^2	P
Żubr	3.35	0.19
Warka Full	9.12	0.01*
Karpackie Pils	4.01	0.13
Żywiec	5.67	0.06
Leżajsk Full	6.40	0.04*

Furthermore, the analyses of environmental conditions in all the locations showed that they differed in insolation, temperature and humidity (Table 6).

EFFECT OF CHEMICAL COMPOSITION OF BEER ON SLUG BEHAVIOUR

The chemical composition of volatile fraction of the beer brands was determined using gas chromatography with the MS detector and the HS-SPME method of sampling of volatile compounds. The results, together with the ranking based on the quantitative features, are shown in Table 7. The beer brands were ranked according to the preferences shown by the slugs in the laboratory studies and in different locations in the field, as well as according to the mean

Table 6. Environmental conditions at locations of field tests

	Drainage ditch	Park	Meadow
Lighting (lx ±standard deviation)	13,200 ± 6,289	5,423 ± 5,709	15,916 ± 14,147
Temperature (°C ±standard deviation)	25.8 ± 2.7	26.6 ± 4.9	26.3 ± 3.8
Humidity (% ±standard deviation)	63.3 ± 10.6	64.4 ± 13.0	65.32 ± 13.0

Table 7. Mean content (% ±standard deviation) and ranking (in brackets) of components of volatile fraction of different beer brands

Chemical name [Common name]	Ranking of volatile substances (average values % ±standard deviation)				
	Żubr	Warka Full	Karpackie Pils	Żywiec	Leżajsk Full
Acrylic acid N-hydroxysuccinimide ester	11.8 ± 1.1 (4)	16.0 ± 2.0 (2)	8.4 ± 1.5 (5)	14.3 ± 2.9 (3)	19.8 ± 1.5 (1)
Cis-3-Nonen-1-ol	1.7 ± 0.2 (3)	0.5 ± 0.0 (5)	0.9 ± 0.2 (4)	2.8 ± 0.2 (1)	2.2 ± 0.1 (2)
Octanoic acid	0.0 ± 0.0 (3.5)	0.0 ± 0.0 (3.5)	0.0 ± 0.0 (3.5)	0.0 ± 0.0 (3.5)	0.1 ± 0.0 (1)
Ethyl caprylate	0.02 ± 0.0 (4)	0.1 ± 0.0 (3)	2.8 ± 0.0 (1)	0.0 ± 0.0 (5)	0.2 ± 0.1 (2)
Ethyl palmitate	3.5 ± 0.0 (0.0)	0.2 ± 0.1 (1)	0.0 ± 0.0 (3.5)	0.0 ± 0.0 (3.5)	0.0 ± 0.0 (3.5)
2-Methoxy-4-vinylphenol	7.4 ± 0.6 (1)	0.0 ± 0.0 (4.5)	0.0 ± 0.0 (4.5)	4.6 ± 0.4 (2)	3.2 ± 0.0 (3)
α-caryophyllene	1.7 ± 0.2 (1)	1.1 ± 0.0 (3)	0.6 ± 0.0 (4.5)	1.1 ± 0.1 (2)	0.6 ± 0.1 (4.5)
Lauric acid ethyl ester	0.0 ± 0.0 (3.5)	0.3 ± 0.0 (1)	0.0 ± 0.0 (3.5)	0.0 ± 0.0 (3.5)	0.0 ± 0.0 (3.5)
2.6-Diisopropylnaphtalene	0.7 ± 0.3 (3)	0.9 ± 0.0 (2)	1.0 ± 0.1 (1)	0.5 ± 0.0 (5)	0.6 ± 0.3 (4)
Phthalic acid. isobutyl nonyl ester	0.0 ± 0.0 (4)	0.0 ± 0.0 (4)	0.5 ± 0.00 (1)	0.0 ± 0.0 (4)	0.03 ± 0.0 (2)
t-Muurolol	0.4 ± 0.0 (1)	0.3 ± 0.0 (2)	0.0 ± 0.0 (4)	0.0 ± 0.0 (4)	0.0 ± 0.0 (4)
Aristolene epoxide	0.2 ± 0.1 (1)	0.0 ± 0.0 (3.5)	0.0 ± 0.0 (3.5)	0.0 ± 0.0 (3.5)	0.0 ± 0.0 (3.5)
Bicyclo[4.1.0]heptane.3.7.7-t	0.0 ± 0.0 (4)	0.0 ± 0.0 (4)	0.9 ± 0.0 (2)	0.0 ± 0.0 (4)	2.4 ± 0.0 (1)
2-Phenylethanol	0.0 ± 0.0 (3.5)	0.0 ± 0.0 (3.5)	0.0 ± 0.0 (3.5)	0.1 ± 0.0 (1)	0.0 ± 0.0 (3.5)
Decanoic acid	0.4 ± 0.0 (2)	0.3 ± 0.0 (4)	0.4 ± 0.2 (1)	0.4 ± 0.0 (3)	0.3 ± 0.0 (5)
(9Z.12Z)-9.12-Octadecadienoic acid 2-acetyloxy-1-(acetyloxymethyl)ethyl ester	0.3 ± 0.0 (2)	0.04 ± 0.0 (1)	0.02 ± 0.0 (3)	0.0 ± 0.0 (4.5)	0.0 ± 0.0 (4.5)
1.5.5.8-Tetramethylcycloundeca--3.7-dien-1-ol	0.5 ± 0.2 (1)	0.0 ± 0.0 (3.5)	0.0 ± 0.0 (3.5)	0.0 ± 0.0 (3.5)	0.0 ± 0.0 (3.5)
t-Cadinol	0.3 ± 0.3 (2)	0.2 ± 0.0 (3)	0.4 ± 0.0 (1)	0.2 ± 0.0 (4)	0.0 ± 0.0 (5)
α-Eudesmol	0.0 ± 0.0 (4)	0.0 ± 0.0 (4)	1.2 ± 0.0 (1)	0.0 ± 0.0 (4)	0.6 ± 0.3 (2)
Oleic acid	10.0 ± 2.5 (1)	0.0 ± 0.0 (5)	4.8 ± 0.5 (2)	3.1 ± 0.4 (3)	1.4 ± 1.0 (4)
CO_2	12.4 ± 0.7 (3)	12.6 ± 0.6 (2)	12.1 ± 0.9 (5)	12.9 ± 0.7 (1)	12.4 ± 12.4 (4)

concentration of various components (Tables 2–4, 7). The Spearman rank correlation test showed an increase in the preference for beer brands in the traps located in the drainage ditch with the increasing content of acrylic acid N-hydroxysuccinimide ester and decanoic acid ($r=0.9$; $p<0.05$), and a negative correlation between that preference in the traps located in the middle of the park and the content of CO_2 ($r=-0.95$; $p<0.05$).

DISCUSSION

The slugs fall into beer traps much more often than into water-containing traps (Hagnell et al. 2006b, Kappes et al. 2012, Piechowicz et al. 2014). There are thousands of beer brands in the world, and they can vary widely in their chemical composition. This was confirmed in our studies (Table 7). The differences in chemical composition between the beer brands resulted in different olfactory perception by and, consequently, attractiveness to the slugs (see also Perpète & Collin 2000, Clark et al. 2011).

Our aim was to check if the five beer brands tested might differ in their ability to attract *A. vulgaris*. We also attempted to identify the components of volatile fraction which might be responsible for inducing the slug's behavioural reaction.

During the olfactometry tests the olfactometer channel with the volatile fraction of Leżajsk Full was most frequently chosen by *A. vulgaris* (Table 2). In the field tests the greatest number of animals was caught in the traps with Karpackie Pils. In both kinds of tests the smallest proportion of slugs was attracted by the chemical components of Warka Full (Table 4).

The attractiveness of beer brands for slugs depends on the presence of specific chemicals. At least three ingredients of beer are currently known to affect the attractiveness for slugs: diacetyl (IUPAC name: butane-2,3-dione), acetoin (3-hydroxybutanone) and dihydroxyacetone (IUPAC name: 1,3-dihydroxypropan-2-one) (Cranshaw 1997). None of these substances was detected in the studied brands. Nevertheless, it seems that in the field tests the number of slugs caught in the traps was positively correlated with the content of decanoic acid but negatively with acrylic acid, N-hydroxysuccinimide ester and CO_2. The results of field tests are more difficult to interpret than the laboratory tests, because they may be influenced by additional environmental factors (e.g. ambient temperature or lighting), which in turn affect the rate of beer decomposition (Vanderhaegen et al. 2007, Suárez et al. 2011, Rodriguez-Bencomo et al. 2012). They can influence the chemical content of the air in the traps. All these factors can be responsible for the variation in the distribution of the number of trap choices by the slugs (Table 5). However, the observed variation in the frequency of choices of individual traps by the slugs (Table 4) indicates that the trap location has a greater effect on the number of trapped animals than on their preferences. On the other hand, the laboratory tests were carried out on groups of individuals, not on single slugs, and the slugs may have been moving along paths laid by their conspecifics. According to our observations, individuals placed separately on the olfactometer arena often do not show any motor activity, which would be desirable during such studies. Our previous study (Piechowicz et al. 2014) demonstrated that there were no significant differences in preferences for the smell of environment in the olfactometer between groups of *A. vulgaris*, and slugs placed there separately.

The laboratory and field tests confirmed that the chemical composition of Leżajsk Full and Żubr in the laboratory tests, and Karpackie Pils in the field tests attracted more individuals of *A. vulgaris* than the smells of the remaining beer brands. In both kinds of tests the smell of Warka Full appeared to be the least attractive. The field tests indicate that the presence of decanoic acid in the volatile fraction caused an increase, while acrylic acid, N-hydroxysuccinimide ester and CO_2 – a decrease in the number of trapped slugs. Such observations were impossible to confirm in the laboratory tests. The field studies also confirmed that the variable distribution of the number of choices of traps with specific beer brands by the slugs depended on the traps' location.

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