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COMPONENTS OF THE SMELL OF BEER AS ENTICING FACTOR FOR INVASIVE SLUGS *Arion lusitanicus* NON-MABILLE

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Abstract: The study on the smell preference to for six beer brands in invasive slug *Arion vulgaris* was carried out under field and laboratory conditions. The effect of beer smell on CO_2 emission was also estimated. Additionally, chromatographic determination (GC-MS) of volatile fraction of the tested beer brands was carried out. Chemical compounds responsible for the attractiveness of beer brands for the slugs were determined using statistical methods. The correlation analysis between the results of performed tests was made.

It was shown that components of beer volatile fraction, such as: t-muurolol, aristolene epoxide, decanoic acid, 9Z,12Z)-9,12-octadecadienoic acid 2-acetyloxy-1-(acetyloxymethyl)ethyl ester, t-cadinol and oleic acid have a positive effect, and γ-elemene and bicyclo[4,1,0]heptane,3,7,7 trimethyl have a negative effect on the attractiveness of beer smell for slugs. Respirometry tests showed an increase in CO_2 emission in slugs exposed to the smell of beer, however, it appeared impossible to indicate unambiguously which chemical compound could be responsible for the observed change in their physiological parameters. The increase in CO_2 emission by slugs *A. vulgaris* exposed to the smell of beer did not correlate with the results of their smell

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preference in the field and laboratory tests. On the other hand, only the results of the laboratory tests performed on 6 individuals well correlated with the results obtained during the preference field tests, which indicate, that estimation the slugs' preference may be limited to the laboratory tests.

Keywords: *Arion vulgaris*, attractants, olfactory preferences, beer, CO_2 emission

Introduction

Since the last few decades, *Arion vulgaris* (Moquin-Tandon, 1855), up to now referred as *A. lusitanicus* Mabille 1868 [1, 2], has spread from South-West Europe to almost the whole continent [3].

Slugs, also *Arion vulgaris,* are perfectly adapted to new environments. They are able to reproduce by self-fertilization [4] and to interbreed with other snail species, creating hybrids [5]. They forage mainly on young shoots of plants induce not only significant crop losses, destroying seedlings and growth cones, and reducing the assimilation area of leaves, but also they cause contamination of host plants by their mucus, eggs and excrements [6]. They are also vectors of parasites of the nervous system [7]. Altogether, this threatens a biodiversity by an extinction of native species of snails [8] and destruction of eggs [9].

The harmfulness of *A. vulgaris* is intensified also by the fact that some insecticides, used commonly for the crop protection, may act as food attractants for this species [10]. Moreover, nowadays, an access to molluscicides, also the environmentally sensitive factors [11], is small (currently 31 formulations are authorized for use in Poland, containing only two active substances: metaldehyde and iron(III) phosphate [12]. All these factors make that alternative methods to reduce losses caused by invasive slugs are being sought [13–15].

Our field and laboratory tests were aimed to determine, which of beer volatile compounds is responsible for its attractiveness for the slug *A. vulgaris*. Additionally, we tested whether the smell of beer could cause an increase in CO_2 emission by slugs, and, whether the level of such increase could be adequate to the number of slugs that selected the smell of the given beer brand. Finally, using statistical methods, we determined whether the results of olfactometry tests in field and laboratory conditions and changes in the slug respirometry correlate to each other, hence whether they can be potentially used vicariously.

Materials and methods

Animals

Experiments were performed on adult slugs caught in the park belonging to the Institute of Biotechnology, University of Rzeszow, from July to October 2012 and 2013. The species identification was based on the reproductive anatomy. In total, 398 individuals of *A. vulgaris* were used during the laboratory tests (300 for the olfactometry tests on 6 individuals, 50 for the olfactometry tests on single individuals,

and 48 for the respirometry tests). 520 adult *Arion vulgaris* individuals were caught in the traps during the field tests.

For two weeks before the laboratory experiments, adult *A. vulgaris* were kept in rearing chambers Bolarus S-500S/P with controller Delta, at 25.0 ± 1.0 °C, L:D 12:12 and RH 80 ±5 %. Animals had ad lib access to water and food (lettuce from organic farming). Forty eight hours before the measurement, animals were placed in chambers of 180 cm^3, without an access to food, but with an access to water. They were weighed just before the measurement.

The used beer brands

The attractiveness of 6 beer brands for slugs was tested in the laboratory and in their natural environment (Table 1). Beer brands usually sold in tins were used. Beer used to the test belonged to the products from the lower and middle price segment. All were purchased in stores.

Table 1

Beer brands used in field and laboratory tests

Beer brand	Declared ethanol content v/v [%]	Declared extract content [%]	Producer
Coberg Premium	3.0	—	Fuhrmann Sp. z o.o., Polczyn Zdroj, Poland
Donagger Strong	6.6	—	Van Pur S.A., Rakszawa, Poland
Tesco	4.0	—	Zaklady Piwowarskie Glubczyce S.A., Glubczyce, Poland
DeHelder Lager Premium	5.0	11.4	Van Pur S.A., Rakszawa, Poland
Rastiger Jasne Pelne	4.8	—	Fuhrmann Sp. z o.o., Polczyn Zdroj, Poland
Perla Export	5.6	11.5	Browary Lubelskie S.A, Lublin, Poland.

Analysis of beer volatiles

To identify the chemical compounds present in beer volatile fractions a gas chromatograph Varian 450 equipped with MS detector 240 and the column Varian VS-5MS 30 m × 0.25 mm × 0.25 μm was used. Headspace Solid-Phase Microextraction (HS-SPME) fiber-PDMS 100 μm (microns) was used.

Two cm^3 of a given beer was poured into a 5 cm^3 glass flask, and then its volatile fraction was absorbed by HS-SPME, for 30 min at 37 °C. and the HS-SPME Holder with the analyte was placed into a gas chromatograph dispenser at 200 °C, under helium flow of 1 cm^3/min, and splitless injection mode. The desorption process was carried out for 5 min and analytes were assayed at the following temperature program: 5 min isotherm at 50 °C, 50–250 °C with 10 °C/min temperature gradient, 10 min isotherm at

250 °C, 250–300 °C with 20°C/min temperature gradient, 10 min isotherm at 300 °C. Gas flow rate (He) was 1 cm^3/min MS detector settings: scan mode 50–500 m/z. Integration process was made based on the normalisation peak without the internal pattern, because in HS-SPME method, the content of compounds depends on the partial pressure of compartments. In the presented study a total number of 37 compounds was identified (Table 2), mostly from terpene and terpene oxygen-containing derivatives group. Due to a large number of patterns necessary for determination the chemical content of beer volatile fraction, only the libraries of National Institute of Standards and Technology (NIST) 08 database were used. Minimal probability for the confirmation of chemical structure of the compound was 50 %.

Olfactory preference

Examinations of the behavioral responses of slugs to chemical compounds present in a volatile fraction of a given beer were carried out in laboratory conditions, in the morning (6.30 ± 15 min), i.e. at a diurnal initiation of the slugs activity, using six-radial

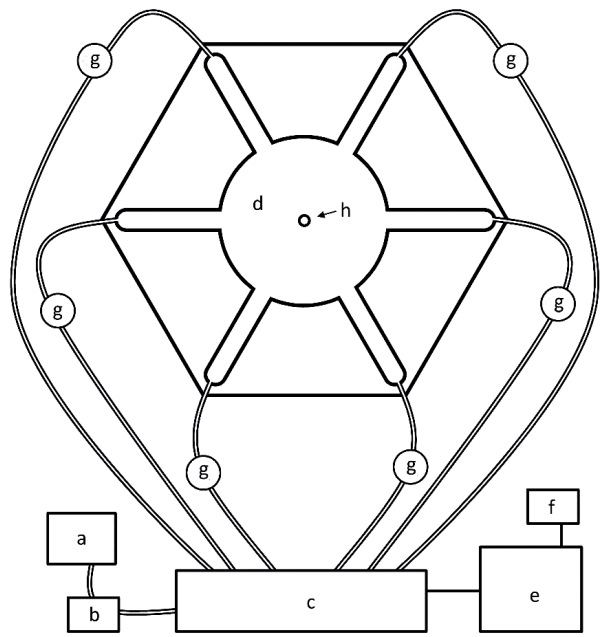

Fig. 1. Scheme of the olfactometer: a – container with synthetic air (producer: AirLiquide Poland); b – set of carbon filters (producer: Qubit Systems Inc., Canada); c – eight-channel flow meter G245 (producer: Qubit Systems Inc., Canada); d – teflon arena of the olfactometer, including a space where the animals were placed, and 6 arms, into whose distal parts gas was directed; the whole system was covered with a glass plate (producer: Andrzej Zienkiewicz, Zaklad Remontowo Montazowy Aparatury Laboratoryjnej, Poland); e – working station with 'Olfak' software to operate the rotameter (producer: Andrzej Zienkiewicz, Zaklad Remontowo Montazowy Aparatury Laboratoryjnej, Poland) and 'BioVid' (producer: FerroSoftware, Poland) for visual registration; f – setup for visual registration; g – glass flasks with samples of beer; h – the outflow of air

olfactometer OLF0001 (constructed by: A. Zienkiewicz, Zaklad Remontowo-Montazowy Aparatury Laboratoryjnej, Poland).

Synthetic air (21 % O_2 in 79 % N_2) (AirLiquide, Poland) of purity 2.0, additionally purified by a carbon filter, was directed using a rotameter and set of tubes Bev-A-Line IV (USPlasticCorp, USA) to six arms of the olfactometer (each of them 0.03 m in width and 0.17 m of length, with gas inlet situated at the opposite end than the located centrally arena for introducing the animals, 0.25 m in diameter). Gas was pumped to each channel at a rate 100 ±5 cm^3/min. Before introducing to the olfactometer, the gas flowed additionally through the flask of 50 cm^3 (10 mm above the surface of the liquid), where the 20 cm^3 samples of studied brands of beer were poured. The outlet of the gas carrying the volatile fraction of beer was located at the upper part of the vessel. The beer sample was introduced into the experimental setup. During each the measurement, the sample was randomly directed into its given arm. (In order to most accurate removal of physical and chemical traces of snails and the smell of beer, after each test the olfactometer area was cleaned successively with water, acetone and petroleum ether).

The study involved observation and recording the selection of olfactometer arm with an smell released by beer of different brand by slugs.

Animals were wetted (water at a temperature of 20 °C) and located at a beaded olfactometer arena in order to rise their activity. Similarly, in order to eliminate water seeking behaviour, an olfactometer arena was accurately covered by a tight layer of water.

Registering the placement of A. vulgaris individuals in the olfactometer arena took place only once, after 120 min of their stay in the olfactometer. This time was determined experimentally, as such, when the elevated terrain exploration by animals was not observed, and the arena desiccation, which could have affect the animal preferences, was not visible. Experiments were performed on groups consisting of six individuals (50 repetitions, in total 300 slugs) and on individuals placed separately in the apparatus (50 repetitions). Only the individuals with marked motility were chosen for the experiments. After the assumed period of animal stay in the middle of the olfactometer arena, none of them has been seen, who might have remained there. A 350 slugs of the species A. vulgaris were used (tests, in which not all the slugs chose channel with the smell and these, when animals were on the border between the arena and the individual olfactometer arms were not analysed).

The field tests of the preference

Field tests were conducted in the park surrounding the Institute of Applied Biotechnology and Basic Sciences in Werynia (province Podkarpackie, Poland). Sets of traps (each of them consisting of 6 traps), were spaced at four randomly selected locations along the irrigation channels. In that habitat the highest activity of *A. vulgaris* was observed. In each localization the traps were placed at a distance of 2 m from the channel's shore; the distances between traps were 15–20 m. Particular beer brands were poured into the traps in random way (only one beer brand in each localization). The

tests were performed in eight terms (totally 32 repetitions), each using new traps and new portion of beer.

Bottles made up of polyterephtalane ethyl (PET) of 1.5 dm^3 volume were used to prepare traps for the slug collection, to evaluate the attractiveness of different beer samples, (according to the method described by Hagnell et al. [16]). The upper part of each bottle was cut at 0.120 ±0.005 m. It was reversed and fastened again to its bottom part. Traps were buried into the ground in the way that their upper part would be placed at a ground level to prevent damaging the slug's leg by their sharp edges. 125 cm^3 of beer was poured into each trap. After three days, traps were transferred into the laboratory where the species affiliation of the captured slugs was identified by analyzing the anatomy of their reproductive system [17]. As well, the animals were counted. Only animals that belonged to *A. vulgaris* were taken to analysis.

Sampling the smell of beer for the respirometric tests

Samples of the smell of beer for each animal were collected from the separate cans. Just after opening the can, two 20 cm^3 analytical portions of beer (one for the experimental survey and one for the blind study) were taken and poured into 50 cm^3 glass flasks. The 10 cm^3 volume sterile syringe KD-JECT III type with 'luer lock' (KD Medical, USA), was placed at the upper part of the flask, so that the tip of the syringe was located exactly 10 mm above the surface of beer, and then the under pressure was created by blocking a syringe plunger at a level 10 cm^3 of the volume. After that, in order to equalize the concentration of the individual components of the gas phase, the set was placed into a laboratory incubator 400 Memmert IPP (Memmert GmbH, Germany) at 37.0 °C for 30 min.

The respirometric tests

The study on CO_2 emission by *A. vulgaris* was carried out using the set Multichannel Gas Exchange System (MCGES) (Qubit Systems Inc., Canada) with CO_2 detector S157 2000 ppm CO_2 Analyzer. Breathing gas used in experimental setup was Synthetic air, of purity 2.0 (Air Liquide, Poland). Gases used during the respirometric measurements had a humidity of 0 %. Experimental chambers, with animals inside, were placed into the incubator Q – Cell model ERC0750 (Pol-Lab, Poland).

Previously weighted animals were placed into the respirometric chambers (volume 80 cm^3, ∅ 0.032 m, length 0.0995 m), in the incubator Q – Cell ERC0750 at 25.0 ± 0.5 °C, in constant darkness. During the experiments, animals could make small movements in the range allowed only by the chamber wall (test overall gas exchange). In the system, the gas, flowed at a rate of 100 cm^3/min, running in a cycle: 16.5 min – the flow through the chamber with the animal inside it, and 3.5 min – the reference flow. Initially, animals were acclimated to new environment for 240 min. After the acclimation period, the registration of CO_2 emission was initiated using the C950 – MCGES version 3.8.7 software. It took 30 min (blind measurement), and then the gaseous phase, taken up from the surface of beer, was introduced into the chamber

using a syringe. In order to minimize the influence of mechanical (shaking), and physical (light, temperature fluctuation) factors the smell was introduced into the experimental setup, to the injector located outside the incubator, ahead of the incubation chambers. The measurements were continued for a further 48 min.

In order to eliminate the measurement error, resulting from the presence of CO_2 in beer samples, before the test using animals, the blind study was performed. It consisted of the introduction into the system (without the slug disposed therein, but in analogous way as in the test with a slug) one of the two prepared gas samples. The final result of each measurement (with the slug disposed in the system) was reduced by the corresponding result of the blind study. The effect of each studied beer brand was analyzed on eight animals.

Responses to the smell of beer were calculated according to the formula:

$$R_{(\%)} = \frac{z}{c} \cdot 100 \tag{1}$$

where: $R_{(\%)}$ is CO_2 emission in relation to the blind (trial) measurement, z corresponds to CO_2 emission after the exposure to the smell of beer and C is CO_2 emission in control tests.

Statistical analysis

The used beer brands were ranked in terms of their attractiveness to slugs (the mean frequency of choices by slugs) in the field and laboratory tests, and according to mean relative concentration of volatile substances. Then, the relationship between those two variables was analysed using the Spearman rank correlation test ($P < 0.05$).

The Bernoulli scheme was used to check whether in the laboratory tests the slugs chose randomly the volatile fractions of beer with given chemical composition. The experiment of placing 6 slugs, which could potentially choose between 6 cells, was run in 50 replicates. The success was that 0, 1, 2, 3, 4, 5, or 6 slugs that would choose given beer brand and for each beer brand we could get the most probable number of choices. In order to determine whether the choice was random, the test of comparing the fractions was used.

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 and Friedman Test.

Results

Volatile fractions of the studied beer brands differed in chemical composition, but the differences were not significant, but these differences were so insignificant that in statistical terms beer correlated with the chemical composition (Table 2).

The composition, and the ranking of the individual components are shown in Table 3. From the compounds occurring in beer volatile fraction 49.9 to 84.3 % could be identified.

Table 2

Matrix of beer brands versus their chemical composition (Spearman rank correlation test)

Beer brand	Perla Eksport	DeHelder Lager Premium	Rastiger Jasne Pelne	Tesco	Donagger Strong	Coberg Premium
Perla Eksport	1.00	—	—	—	—	—
DeHelder Lager Premium	0.85*	1.00	—	—	—	—
Rastiger Jasne Pelne	0.93*	0.92*	1.00	—	—	—
Tesco	0.97*	0.78*	0.90*	1.00	—	—
Donagger Strong	0.84*	0.97*	0.97*	0.81*	1.00	—
Coberg Premium	0.86*	0.88*	0.94*	0.86*	0.91*	1.00

* – significant correlations

Table 3

The average content ± standard deviation [%] of individual chemical compounds in the volatile fraction of beer of the studied brands and the ranking of beer brands in terms of those substances content (in brackets)

Component of the volatile fraction	Perla Eksport	DeHelder Lager Premium	Rastiger Jasne Pelne	Tesco	Donagger Strong	Coberg Premium
3-Methylbut-1-yl ethanoate-	11.0 ± 0.1 (4)	1.7 ± 1.5 (6)	14.1 ± 1.3 (1)	13.8 ± 1.9 (2)	10.1 ± 1.2 (5)	11.8 ± 0.9 (3)
Acrylic acid N-hydroxysuccinimide	12.1 ± 2.1 (5)	10.2 ± 1.9 (6)	16.7 ± 0.8 (2)	15.4 ± 0.9 (3)	18.3 ± 0.9 (1)	13.0 ± 0.9 (4)
Cis-3-nonen-1-ol	3.2 ± 0.3 (2)	3.5 ± 0.3 (1)	0.9 ± 0.3 (5)	1.0 ± 0.1 (4)	2.11 ± 0.1 (3)	0.4 ± 0.0 (6)
Phenethyl alcohol	6.4 ± 0.8 (1)	5.2 ± 0.8 (3)	3.4 ± 0.5 (4)	5.7 ± 1.4 (2)	0.8 ± 0.2 (6)	3.0 ± 0.5 (5)
Octanoic acid	0.2 ± 0.1 (1)	0.0 ± 0.0 (4.5)	0.0 ± 0.0 (4.5)	0.0 ± 0.0 (4.5)	0.1 ± 0.1 (2)	0.0 ± 0.0 (4.5)
Ethyl caprylate	0.0 ± 0.0 (2)	0.2 ± 0.0 (1)	0.0 ± 0.0 (5)	0.0 ± 0.0 (5)	0.0 ± 0.0 (5)	0.1 ± 0.0 (3)
Ethyl octanoate	15.4 ± 2.2 (5)	28.5 ± 0.5 (3)	31.0 ± 1.7 (2)	14.1 ± 1.8 (6)	44.9 ± 2.5 (1)	26.7 ± 2.0 (4)
Ethyl palmitate	0.0 ± 0.0 (4.5)	0.0 ± 0.0 (4.5)	0.1 ± 0.0 (2)	0.0 ± 0.0 (4.5)	0.0 ± 0.0-(4.5)	0.2 ± 0.2 (1)
2-Methoxy-4-vinylphenol	0.0 ± 0.0 (5)	0.0 ± 0.0 (5)	3.5 ± 0.3 (2)	0.3 ± 0.0 (3)	0.0 ± 0.0 (5)	8.1 ± 0.2 (1)
α-Caryophyllene	1.1 ± 0.0 (1)	0.8 ± 0.0 (3)	0.7 ± 0.0 (4)	0.3 ± 0.0 (6)	1.1 ± 0.0 (2)	0.7 ± 0.1 (5)
Lauric acid ethyl ester	0.0 ± 0.0 (4)	0.0 ± 0.0 (4)	0.0 ± 0.0 (4)	0.3 ± 0.0 (1)	0.0 ± 0.0 (4)	0.0 ± 0.0 (4)
2,6-Diisopropylnaphtalene	0.2 ± 0.2 (6)	0.3 ± 0.0 (5)	0.5 ± 0.2 (4)	1.0 ± 0.0 (1)	0.5 ± 0.0 (3)	0.5 ± 0.0 (2)
Phthalic acid isobutyl nonyl ester	0.0 ± 0.0 (4)	0.0 ± 0.0 (4)	0.0 ± 0.0 (4)	0.0 ± 0.0 (4)	0.0 ± 0.0 (4)	0.3 ± 0.1 (1)
Hexadecanoic acid ethyl ester	0.0 ± 0.0 (4.5)	0.01 ± 0.0 (2)	0.0 ± 0.0 (4.5)	0.0 ± 0.0 (4.5)	0.0 ± 0.0 (4.5)	8.5 ± 0.8 (1)
6,9-Octadecadiynoic acid, methyl ester	0.02 ± 0.0 (1)	0.0 ± 0.0 (2)	0.0 ± 0.0 (4.5)	0.0 ± 0.0 (4.5)	0.0 ± 0.0 (4.5)	0.0 ± 0.0 (4.5)
t-Muurolol[b*(1.0)**]	0.0 ± 0.0 (5)	0.0 ± 0.0 (5)	0.0 ± 0.0 (5)	0.01 ± 0.0 (3)	0.3 ± 0.0 (2)	0.5 ± 0.0 (1)
Aristolene epoxide[a(0.85), b(0.90)]	0.0 ± 0.0 (4.5)	0.0 ± 0.0 (4.5)	0.0 ± 0.0 (4.5)	0.0 ± 0.0 (4.5)	0.03 ± 0.0 (2)	0.1 ± 0.0 (1)
Bicyclo[4,1,0]heptane,3,7,7-trimethyl[b(–0.85)]	0.01 ± 0.0 (3)	0.01 ± 0.0 (3)	0.03 ± 0.1 (1)	0.01 ± 0.0 (3)	0.0 ± 0.0 (5.5)	0.0 ± 0.0 (5.5)

Table 3 contd.

Component of the volatile fraction	Perla Eksport	DeHelder Lager Premium	Rastiger Jasne Pelne	Tesco	Donagger Strong	Coberg Premium
2-Phenylethanol	0.0 ± 0.0 (4)	0.0 ± 0.0 (4)	0.02 ± 0.0 (1)	0.0 ± 0.0 (4)	0.0 ± 0.0 (4)	0.0 ± 0.0 (4)
Decanoic acid[b(0.82)]	0.2 ± 0.0 (6)	0.2 ± 0.0 (5)	0.2 ± 0.0 (3)	0.2 ± 0.0 (4)	0.3 ± 0.0 (2)	0.4 ± 0.0 (1)
(9Z,12Z)-9,12-octadecadienoic acid 2-acetyloxy-1-(acetyloxy-methyl)ethyl ester [a(0.85), b(0.90)]	0.0 ± 0.0 (4.5)	0.0 ± 0.0 (4.5)	0.0 ± 0.0 (4.5)	0.0 ± 0.0 (4.5)	0.1 ± 0.0 (2)	0.4 ± 0.0 (1)
1,5,5,8-Tetramethylcycloundeca-3,7-dien-1-ol	0.0 ± 0.0 (4)	0.0 ± 0.0 (4)	0.0 ± 0.0 (4)	0.0 ± 0.0 (4)	0.0 ± 0.0 (4)	0.8 ± 0.1 (1)
γ-Elemene[a(–0.84), b(–0.92)]	0.04 ± 0.0 (4)	0.1 ± 0.0 (1)	0.1 ± 0.0 (2)	0.04 ± 0.0 (3)	0.02 ± 0.0 (5)	0.01 ± 0.0 (6)
t-Cadinol[b(0.82)]	0.0 ± 0.0 (6)	0.2 ± 0.0 (3)	0.1 ± 0.0 (5)	0.1 ± 0.0 (4)	0.2 ± 0.0 (2)	0.3 ± 0.0 (1)
α-Eudesmol	0.0 ± 0.0 (5)	0.03 ± 0.0 (3)	0.2 ± 0.0 (2)	0.7 ± 0.2 (1)	0.0 ± 0.0 (5)	0.0 ± 0.0 (5)
Oleic acid[b(0.94)]	0.0 ± 0.0 (6)	3.1 ± 0.0 (4)	1.1 ± 0.1 (5)	4.5 ± 0.2 (3)	5.4 ± 0.4 (2)	7.1 ± 0.0 (1)
Cubenol	0.0 ± 0.0 (4.5)	0.01 ± 0.0 (2)	0.0 ± 0.0 (4.5)	0.011 ± 0.0 (1)	0.0 ± 0.0 (4.5)	0.0 ± 0.0 (4.5)
Ethyl palmitate	0.0 ± 0.0 (4.5)	0.0 ± 0.0 (4.5)	0.01 ± 0.0 (2)	0.02 ± 0.0 (1)	0.0 ± 0.0 (4.5)	0.0 ± 0.0 (4.5)
Sum	49.9	54.1	72.7	56.7	84.3	82.9

[a] Statistical correlation with tests on 6 individuals; [b] statistical correlation with tests on 1 individual, [(n)] correlation factor.

The Spearman rank correlation test showed the compatibility only for the laboratory tests (Table 7). T-muurolol, aristolene epoxide, oleic acid, decanoic acid, t-cadinol and oleic acid correlated positively, γ-elemene and bicyclo[4,1,0]heptane,3,7,7-trimethyl correlated negatively with the slugs smell preferences.

Olfactometry tests performed on the group of 6 individuals

Olfactometry tests performed on the group of 6 individuals showed that *A. vulgaris* prefers some beer brands than the other. Smells of Perla Eksport and DeHelder Premium Lager, as well as Rastiger Jasne Pelne, Tesco and Donagger Strong were preferred by animals in a similar manner. Another kind of preference constituted Coberg Premium (Fig. 2).

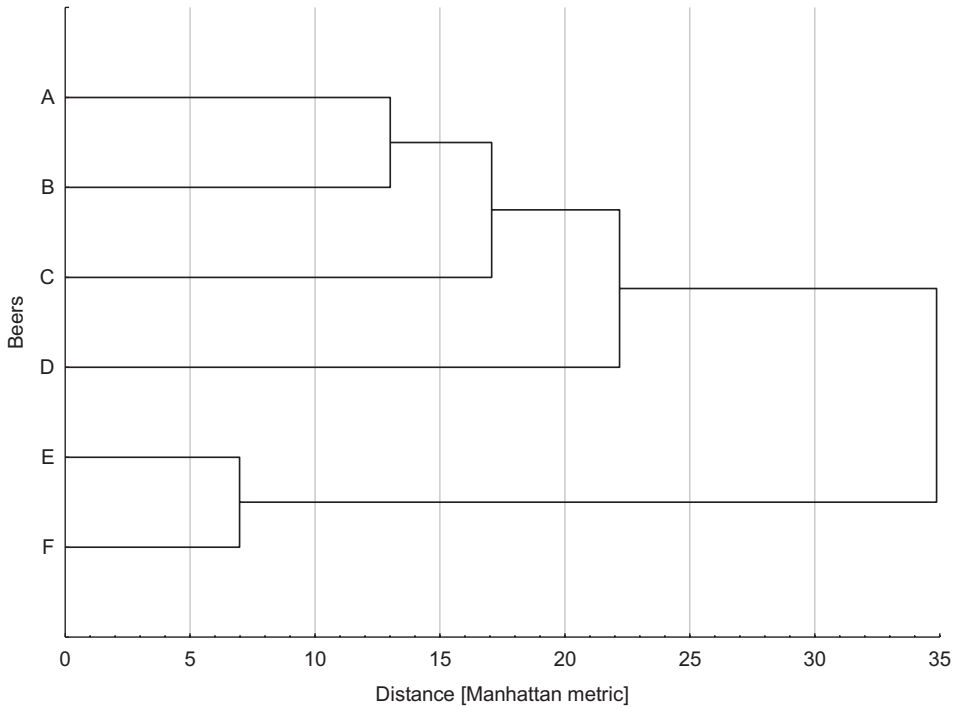

Fig. 2. Cluster analysis of the smell preference of: A – Coberg Premium; B – Rastiger Jasne Pelne; C – DeHelder Lager Premium; D – Donagger Strong; E – Tesco; F – Perla Eksport by *A. vulgaris* in the laboratory tests

Using the Bernoulli test and the test comparing fractions it was proven that the smell of Coberg Premium (111 choices) was selected by two slugs much more frequently than indicated by the most probable values (Table 4). Channels with the smell of this beer brand were favoured by the slugs. Two individuals, from the six tested in each repetition, chose Coberg Premium much more frequently (30 choices). Similar situation

occurred in the case of Donagger Strong, which was selected by 1 individual much more frequently than by groups of animals (25 choices), while Tesco, Rastiger Jasne Pelne (both 25 choices) and DeHelder Lager Premium (28 choices) were selected less frequently than it was suggested by the most probable values (Table 4).

Table 4

Average frequency of choices the smell of given beer brands by groups of 6 individuals *A. vulgaris* (Bernoulli scheme)

Number of choices	The most probable number of choices	Perla Eksport	DeHelder Lager Premium	Rastiger Jasne Pelne	Tesco	Donagger Strong	Coberg Premium
		Real number of choices					
0 choices	17	16	28*	25*	25*	12	0
1 choice	20	21	19	17	20	25*	5
2 choices	10	11	3*	8	5*	12	30*
3 choices	2	2	0	0	0	1	14
4 choices	0	0	0	0	0	0	1
5 choices	0	0	0	0	0	0	0
6 choices	0	0	0	0	0	0	0
Laboratory Ranking		3	6	4	5	2	1
Sum of choices		49	25	33	30	52	111

* The values that differ significantly from the random (most probable).

The Friedman test indicated differences in the smell preference of individual beer brands by the slugs ($\chi^2 = 89.3$; $p = 0.00$; the compatibility factor = 0.36).

Statistical analysis using ranks and Spearman rank correlation tests indicated that the presence of aristolene epoxide, (9Z,12Z)-9,12-octadecadienoic acid 2-acetyloxy-1-(acetyloxymethyl)ethyl ester and γ-Elemene in the volatile fraction of Coberg Premium and Donagger Strong was responsible for the tendency of their smell selection, while the presence of the first two compounds was noted only for most frequently selected beer brands, i.e. aristone epoxide in Coberg Premium (0.1 %) and in Donagger Strong (0.03 %), and (9Z,12Z)-9,12-octadecadienoic acid 2-acetyloxy-1-(acetyloxymethyl)-ethyl ester (0.4 % and 0.03 %, respectively) (Table 3), The amount of γ-Elemene correlated negatively with smell preference of the slugs. For beer brands selected most frequently by the slugs the percentage concentrations of those substances were the lowest (0.01 % and 0.02 %, respectively).

Olfactometric tests with 1 individual

Results of the actual preference of individual beer brands indicated that the smell of Coberg Premium was selected significantly more (17 choices) frequently than suggested by the most probable values, while the smell of Tesco, DeHelder Lager Premium,

Rastiger Jasne Pelne and Perla Eksport was preferred slightly less frequently (> 8) than suggested by the most probable values (Table 5).

Table 5

Average frequency of choices the smell of various beer brands by 1 individuals *A. vulgaris*, determined using the olfactometry

Beer brand	Most probable number of choices	Actual number of choices	Ranking
Perla Eksport	8	6	5
DeHelder Lager Premium	8	6	5
Rastiger Jasne Pelne	8	6	5
Tesco	8	7	3
Donagger Strong	8	8	2
Coberg Premium	8	17*	1

* The values that differ significantly from the random (most probable).

As it was in olfactometric tests on 6 individuals, statistical analysis using ranks and the Spearman rank correlation tests indicated that the presence of (9Z,12Z)-9,12-octadecadienoic acid 2-acetyloxy-1-(acetyloxymethyl)ethyl ester, and t-muurolol, decanoic acid, t-cadinol and oleic acid (for volastile fraction of Coberg Premium concentration up to 0.4 %, 0.5 %, 0.4 %, 0.3 % and 7.1 %, respectively) was responsible for the positive smell selection tendencies by slugs. Negative correlation for the preference of given beer smells was shown for the presence of compounds as: γ-elemene and bicyclo[4,1,0]heptane,3,7,7-trimethyl, which were absent in smell of more frequentlu selected beer brands.

Field tests of the preference

During the field tests, a total of 520 individuals *A. vulgaris* was caught into traps. The field tests indicated that similar number of animals chose the traps with Perla Export and Rastiger Jasne Pelne. Traps with DeHelder Lager Premium, Tesco and Donnager Strong were chosen in similar manner. Separate group were the traps filled with Coberg Premium (Fig. 3), which appeared to be most attractive for *A. vulgaris* (135 choices). The smallest number of animals chose the trap with Donager Strong (63 choices), which in olfaktometry tests were chosen a bit less frequently than the most popular Coberg Premium (Table 4).

Statistical analysis using ranks and the Spearman rank correlation test (Table 3) did not show any chemical factor that could significantly affect smell preferences of the studied animals. Compounds influencing on *A. vulgaris* smell preferences in the laboratory tests, i.e., aristolene epoxide, (9Z,12Z)-9,12-octadecadienoic acid 2-acetyloxy-1-(acetyloxymethyl)ethyl ester, decanoic acid, oleic acid, γ-elemene and many other components of the volatile fraction of the studied beer brands affected the slug behaviour also in the field tests.

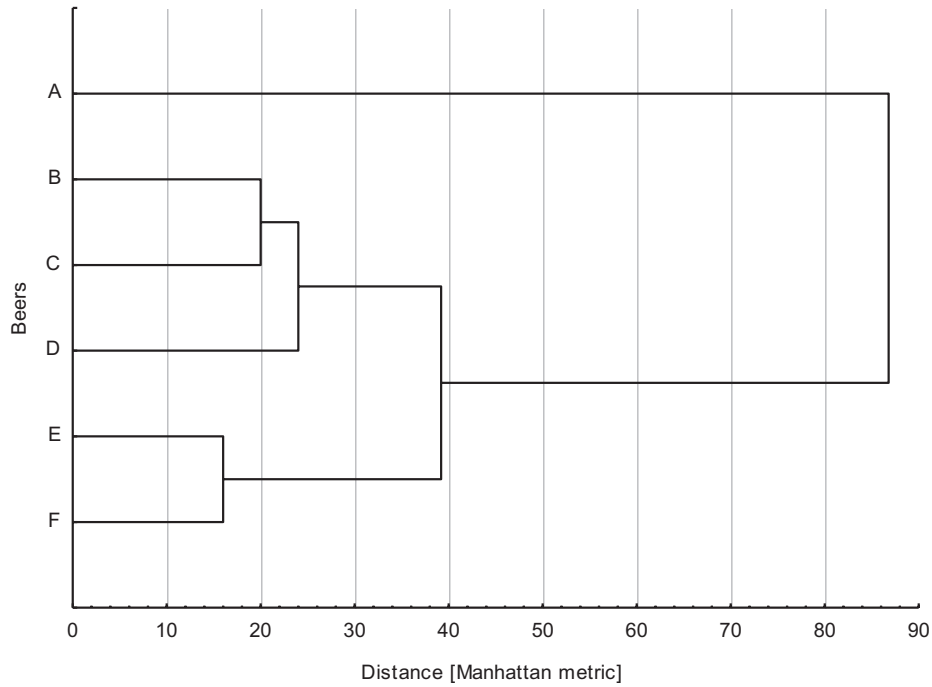

Fig. 3. Cluster analysis of the preference of smell of beer by *A. vulgaris* determined under the field conditions: A – Coberg Premium; B – Donagger Strong; C – DeHelder Lager Premium; D – Tesco; E – Rastiger Jasne Pelne; F – Perla Eksport

Table 6

The frequency of choices of traps with the smell of various beer brands by *A. vulgaris* determined in the field tests

Beer brand	Number of caught animals	Ranking the whole area
Perla Eksport	84	2
DeHelder Lager Premium	79	4
Rastiger Jasne Pelne	82	3
Tesco	77	5
Donagger Strong	63	6
Coberg Premium	135	1

CO_2 emission

Measurements of CO_2 emission by *A. vulgaris* indicated a transient, short-term increase in CO_2 emission in response to the smell of beer. The Cluster analysis indicated similar respiratory response of animals exposed to the smells of Perla Eksport, Tesco, Donagger strong and Coberg Premium. The other group were animals subjected to DeHelder Lager Premium and Rastiger Jasne Pelne (Fig. 4).

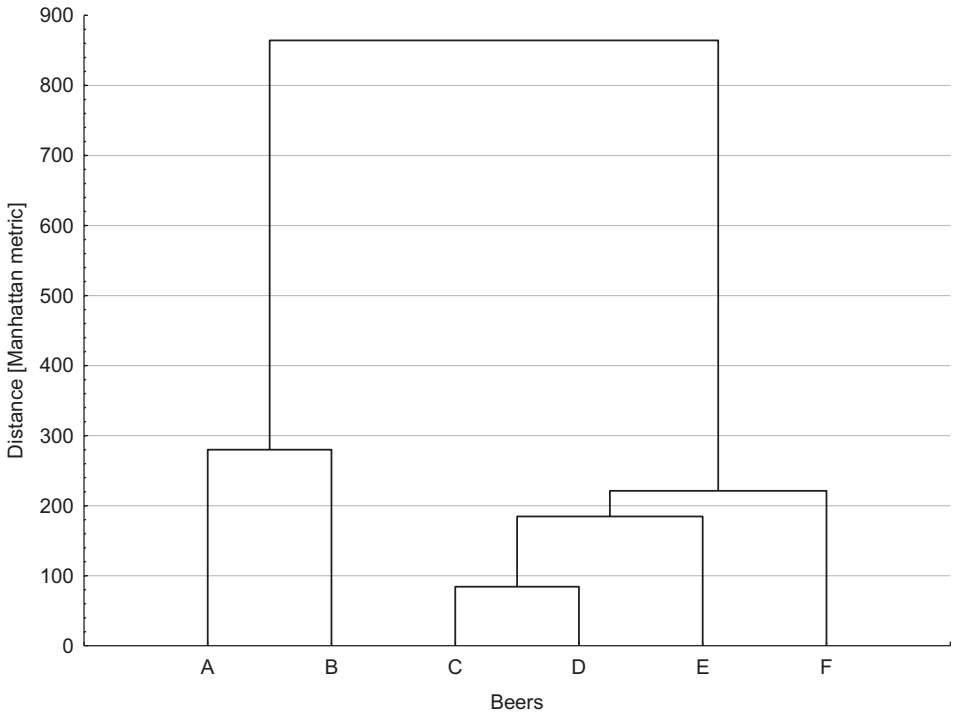

Fig. 4. Cluster analysis of an increase in CO_2 emission by *A. vulgaris* exposed to the smell of beer: A – Rastiger Jasne Pelne; B – DeHelder Lager Premium; C – Perla Eksport; D – Tesco; E – Donagger Strong; F – Coberg Premium

The highest level of CO_2 emission was observed in animals exposed to the smell of Donnager Strong, while the lowest after the exposure to the smell of DeHelder Lager Premium (Table 7).

Table 7

CO_2 emission after the exposure of *A. vulgaris* to the smell of beer in comparison to the control measurement

Beer brand	CO_2 emission in the control measurement	CO_2 emission after the exposure to the smell of beer	The increase in CO_2 emission	Ranking relative to the mean
	[cm^3 CO_2 / (g body mass · h)]		[%]	
Perla Eksport	0.14 ± 0.02	0.50 ± 0.01	356	3
DeHelder Lager Premium	0.15 ± 0.03	0.26 ± 0.02	173	6
Rastiger Jasne Pelne	0.18 ± 0.03	0.47 ± 0.03	263	5
Tesco	0.17 ± 0.03	0.58 ± 0.04	343	4
Donnager Strong	0.14 ± 0.04	0.55 ± 0.03	393	1
Coberg Premium	0.12 ± 0.03	0.44 ± 0.07	371	2

Statistical analysis using ranks and the Spearman rank correlation tests (Table 3) did not indicate any component of volatile fraction of the studied beer brands that were responsible for the observed increase in respirometry of slugs.

The Friedman test indicated that after one-time exposure to the smell of given beer brand the relative CO_2 emission by *A. vulgaris* differed significantly ($\chi^2 = 35.6$; $p = 0.00$; compatibility coefficient = 0.79).

Correlation of tests

In an analogous manner the correlation analysis was carried out in the individual test types. Significant correlation was noted only when compared olfactometric tests on six individuals with the field tests (Table 8), although olfactometric tests on single individuals gave also similar results than those performed in groups of six individuals. Such result suggest that olfactometry tests on Groups of six slugs could replace the field tests.

Table 8

The results of analysis the correlation of various types of tests (Spearman rank correlation test)

Ranking	Olfactometry tests (6 individuals)	Olfactometry tests (1 individual)	Field tests	CO_2 emission tests
Olfactometry tests (6 individuals)	1.00	—	—	—
Olfactometry tests (1 individual)	0.70	1.00	—	—
Field tests	0.89*	0.64	1.00	—
CO_2 emission tests	0.37	–0.03	0.03	1.00

* Significant correlations.

Discussion

Results of the previous investigations confirmed, that *A. vulgaris* more frequently selected traps with the smell of beer than those with water [18, 19]. Therefore in the present study the aspect of beer as an attractant was omitted.

Our surveys showed that the tested beer brands only slightly (Table 2) differed from one another in the composition of their volatile fractions (Table 3), that could be affected by a complexity of technological process [20, 21], age, and storage conditions [22]. Among the studied beer brands, the most attractive for slugs appeared to be the smell of Coberg Premium (Table 4–6) – both in the laboratory tests performed with 1 or 6 individuals and in the field tests.

In the laboratory tests (Table 4 and 5) aristolene epoxide and (9Z,12Z)-9,12-octadecadienoic acid 2-acetyloxy-1-(acetyloxymethyl)ethyl ester (tests with 1 and 6 individuals), t-muurolol, decanoic acid, t-cadinol and oleic acid (test with 1 individual) correlated positively with the number of animals that selected individual beer brands, while γ-elemene (tests with 1 and 6 individuals), and bicyclo[4,1,0]heptane, 3,7,7-

trimethyl (test with 1 individual) correlated negatively. All above described substances could determine the observed range of smells (Table 8). It can't be excluded that not separate compounds, but their joint action on the animal receptors could be responsible for selections of *A. vulgaris.* Substances that would not have been identified could also play a role.

Based on the obtained volatile fraction compositions of the studied beer brands it was impossible to indicate specific chemical compound, which was responsible for the preferences for a given beer smell in the field tests. Additionally, the field tests specifies (the influence of the weather, living or nonliving elements of the environment which were trapped and, for example, reacted with beer constituents or altered the composition of the volatile fraction in a different way) caused that the results were not fully reliable.

Together with the study on the attractiveness of selected components of beer volatile fraction for *A. vulgaris* the respirometry tests were carried out. The results confirmed, that the smell of beer influenced CO_2 emissions by slugs (Fig. 4, Table 5). However, it was impossible to determine which component of beer volatile fraction was responsible for the observed physiological response (Table 8). This means that changes in CO_2 emission by the *A. vulgaris* exposure to the smell of beer, are caused not only by attractive or repellent action of the chemical components of the volatile fraction of individual beer brand. An increase in CO_2 emission alone is interesting, since in the evolution process of the snails, natural selection favoured organisms with the lowest metabolic rate [23]. Our results indicate that in spite of low general metabolic rate, much lower than it is observed for instance in insects, even practically not flying [24], snails are still able to increase periodically their metabolic rate even near fourfold, compared to the control level (Table 7) [25].

The increase in CO_2 emission by *A. vulgaris* could have been explained as organismal response to attractive beer components, however, it may not be excluded that it was a result of secondary release of CO_2 originating in the volatile fraction of beer to the haemolymph, because invertebrates have the storage capacity of this gas in their bodies [26]. The slugs exposed to the volatile fraction of beer could also switch their bodies to use the mechanisms of anaerobic metabolism, and then compensate for it by the intensification of processes of aerobic metabolism. This phenomenon has been observed in Molluscs by Brand and Riehlman [27].

The measured CO_2 emission level in *A. vulgaris* did not correlate in any case with used preference tests (Table 8). This indicates that, in order to determine the attractiveness of the smell of beer for *A. vulgaris*, the analyse of the smell preference of individual beer brands and the CO_2 emission cannot be used equally.

Small number of active substances permissible for fight with invasive slugs, but also activity of those animals elevated mainly during periods of precipitation and high humidity, when the use of molluscicides involves dissolving and migrating them from the places in which they are located, to the soil, all of this cause that combating the gastropoda pests is difficult and problematic. In such case using attractants and repellents, especially on small areas of domestic allotment gardens seems to be a possible alternative for using not very effective and environmentally harmful biocides. Beer as many other products of natural fermentation is such attractant for the invasive

slug *A. vulgaris.* Current research is a first step towards creating a new group of efficient and environmentally friendly snail attractants that can be used both in the production of traps and in attracting additives in the production of molluscicides.

Conclusions

1. Selected beer brands differed in composition of their volatile fractions.
2. 49.9–84.3 % of components of beer volatile fractions were identified with the minimal probability for the confirmation of chemical structure 50 %.
3. Olfactometry tests indicate that some components of beer volatile fraction, as: t-muurolol, aristolene epoxide, decanoic acid, 9Z,12Z)-9,12-octadecadienoic acid 2-acetyloxy-1-(acetyloxymethyl)ethyl ester, t-cadinol and oleic acid, increased their attractiveness for *A. vulgaris* while bicyclo[4,1,0]heptane,3,7,7-trimethyl and γ-elemene with an increase of concentration in the volatile fraction of beer decreased its attractiveness for *A. vulgaris*.
4. During the field tests it was impossible to determine factors responsible for larger attractiveness of smell of some beer brands.
5. Laboratory tests carried out on groups composed of 6 organisms and field tests proved well correlation of their preferences of beer smells.
6. Increase in the relative CO_2 emission by slugs exposed to beer smells was not correlated with the preference of the smell of beer in *A. vulgaris*.

References

[1] Hatteland BA, Solhřy T, Schander C, Skage M, Proschwitz T, Noble LR. Malacologia. 2015;58:303-321. DOI: 10.4002/040.058.0210.
[2] Pfenninger M, Weigand A, Bálint M, Klussmann-Kolb A. Evol Appl. 2014;7:702-713. DOI: 10.1111/eva.12177.
[3] Kozłowski J, Kozłowski R. Folia Malacol. 2011;19:249-258. DOI: 10.2478/v10125-011-0005-8.
[4] Slotsbo S. Ecophysiology and life history of the slug, *Arion lusitanicus* [PhD Thesis]. Aarhus: Aarhus University; 2012. https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&ved=2ahUKEwjL85et-ZXiAhUBMuwKHbDRBSEQFjAAegQ IAhAB&url=http%3A%2F%2Fpure.au.dk%2Fportal%2Ffiles%2F52004438%2FPh.d._afhandling_Stine_Slotsbo.pdf &usg=AOvVaw2xMc_nZktqXoFaHyt6Ee8o
[5] Foltz DW, Ochman H, Jones JS, Evangelisti SM, Selander RK. Biol J Linnean Soc. 1982;17:225-241. DOI: 10.1111/j.1095-8312.1982.tb02018.x.
[6] Rae RG, Robertson JF, Wilson MJ. Crop Prot. 2009;28:765-773. DOI: 10.1016/j.cropro.2009.04.005.
[7] Majoros G, Fukar O, Farkas R. Vet Parasitol. 2010;174:351-354. DOI: 10.1016/j.vetpar.2010.09.012.
[8] Keller M, Kollmann J, Edwards PJ. Acta Oecol. 1999;20:109-118. DOI: 10.1016/S1146-609x(99)80023-x.
[9] Leniowski K, Węgrzyn E, Wojton A. Ethol Ecol Evol. 2013;25:70-81. DOI: 10.1080/03949370.2012.711369.
[10] Piechowicz B, Stawarczyk K, Stawarczyk M. Chem Didact Ecol Metrol. 2012;17:113-120. DOI: 10.2478/cdem-2013-0010.
[11] Purvis G, Bannon JW. Ann Appl Biol. 1992;121:215-223. DOI: 10.1111/j.1744-7348.1992.tb03453.x.
[12] Wyszukiwarka środków ochrony roślin (Search engine for plant protection products). http://www.minrol.gov.pl/Informacje-branzowe/ Wyszukiwarka-srodkow-ochrony-roslin
[13] Lindqvist I, Lindqvist B, Tiilikkala K. Agric Food Sci. 2010;19:1-12.
[14] Schüder I, Port G, Bennison J. Crop Prot. 2003;22:1033-1038. DOI: 10.1016/S0261-2194(03)00120-0.
[15] Laznik Z, Krizaj D, Trdan S. Span J Agric Res. 2011;9:894-900. DOI: 10.5424/sjar/20110903-412-10.

[16] Hagnell J, Schander C, Nilsson M, Ragnarsson J, Valstar H, Wollkopf AM, et al. Crop Prot. 2006;25:212-215. DOI: 10.1016/j.cropro.2005.04.008.
[17] Jaskulska M, Gawlak M, Kozłowski J. Morphological characters of copulatory organs and eggs of *Arion rufus* and *A. lusitanicus*. In: Pokryszko BM, Editor. The 29th Polish Malacological Seminar. Folia Malacol. 2013;21:183-203. DOI: 10.12657/folmal.021.022.
[18] Piechowicz B, Grodzicki P, Piechowicz I, Stawarczyk K. Chem Didact Ecol Metrol. 2014;1:119-125. DOI: 10.1515/cdem-2014-0011.
[19] Dankowska E. Folia Malacol. 2011;19:273-275. DOI: 10.2478/v10125-011-0012-9.
[20] Vanderhaegen B, Never H, Verachtert H, Derdelinckx G. Food Chem. 2006;95:357-38. DOI: 10.1016/j.foodchem.2005.01.006.
[21] Igyor MA, Ogbonna AC, Palmer GH. Proc Biochem. 2001;36:1039-1044. DOI: 10.1016/S0032-9592(00)00267-3.
[22] Saison D, Schutter DP, Delvaux F, Delvaux FR. J Chromatogr. A 2008;1190:342-349. DOI: 10.1016/j.chroma.2008.03.015.
[23] Artacho P, Nespolo RF. Evolution. 2009;63-4:1044-1050. DOI: 10.1111/j.1558-5646.2008.00603.x.
[24] Maliszewska J, Tęgowska E. J Central European Agricult. 2016;17(3):685-694. DOI: 10.5513/JCEA01/17.3.1763.
[25] Piechowicz B, Rębisz E, Grodzicki P, Zaręba L, Balawejder M, Pieniążek M, et al. Ann Anim Sci. 2016;16:463-476. DOI: 10.1515/aoas-2016-0005.
[26] Matthews PGD. J Comp Physiol B. 2018;188:195-210. DOI: 10.1007/s00360-017-1121-6.
[27] Brand T, Riehlman B. Biol Bull. 1953;104:301-312.

SKŁADNIKI FRAKCJI LOTNEJ PIWA JAKO ATRAKTANT DLA INWAZYJNEGO ŚLIMAKA *Arion lusitanicus* NON MABILLE

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Abstrakt: Streszczenie: Badanie preferencji zapachu sześciu marek piwa przez dorosłe osobniki inwazyjnego ślimaka *Arion vulgaris* przeprowadzono w warunkach polowych i laboratoryjnych. Oszacowano również wpływ frakcji lotnej badanych piw na emisję CO_2 przez ślimaki, jak również, wykorzystując metody chromatograficzne (GC-MS), oznaczono skład tej frakcji. Wpływ konkretnych substancji chemicznych na atrakcyjność zapachu marek piwa dla *A. vulgaris* określono przy użyciu metod statystycznych. Przeprowadzono również analizę korelacji pomiędzy poszczególnymi typami testów.

Uzyskane wyniki wskazują, że takie składniki lotnej frakcji piwa, jak: t-muurolol, epoksyd aristolenu, kwas dekanowy, ester 2-acetyloksy-1-(acetyloksymetylowy) kwasu (9Z, 12Z)-oktadekano-9,12-dienowego, t-cadinol i kwas oleinowy mają pozytywny, a elemen i 3,7,7-trimetylocyklo[4,1,0]heptan negatywny wpływ na atrakcyjność zapachu badanych piw dla *A. lusitanicus*. Testy respirometryczne wykazały wzrost emisji CO_2 przez ślimaki eksponowane na zapach piw. Nie udało się jednakże jednoznacznie wskazać, który ze składników frakcji lotnej napojów był odpowiedzialny za to zjawisko. Wzrost emisja CO_2 przez osobniki *A. vulgaris* eksponowane na zapach piwa nie korelował z wynikami preferencji konkretnych marek piwa w terenie oraz w badaniach laboratoryjnych. Wyniki wykonanych na grupach złożonych z 6 osobników testów preferencji zapachu przeprowadzonych w laboratorium dobrze korelowały z wynikami preferencji w warunkach terenowych, co może wskazywać, że oceniając preferencje węchowe ślimaków można ograniczyć się wyłącznie do testów laboratoryjnych.

Słowa kluczowe: *Arion vulgaris*, atraktanty, preferencje zapachu, piwo, emisja CO_2