Ann. Anim. Sci., Vol. 16, No. 2 (2016) 463–476 *DOI: 10.1515/aoas-2016-0005*

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THE SMELL OF BEER AS A FACTOR AFFECTING THE EMISSION OF CARBON DIOXIDE BY *ARION LUSITANICUS* AUCT. NON-MABILLE

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Abstract

More and more frequently beer is used as an attractant in traps to eliminate the slug *Arion lusitanicus* auct. non-Mabille. The smell of beer is not indifferent to animals. Hence it is highly probable that it affects the physiological processes in the slug's body. The aim of our study was to examine whether the smell can induce changes in respiration activity (measured as CO_2 emission) of adult individuals of *Arion lusitanicus*. The results showed that all the tested brands of beer caused an increase in CO_2 emission. Furthermore, in all the samples of studied brands of beer, this increase in CO_2 emission correlated negatively to the content of the following compounds: acrylic acid N–hydroxysuccinimide ester, decanoic acid, (9Z,12Z)–9,12–octadecadienoic acid, 2–(acetyloxy)–1–[(acetyloxy)methyl]ethyl ester, bicyclo[4,1,0]heptane and ethyl caprylate.

Key words: *Arion lusitanicus*, beer, CO_2 emission, respirometry

The sense of smell is exploited by slugs to actively seek sexual partners (Takeichi et al., 2007) and to detect predators (Armsworth, 2005; Dalesman et al., 2006). Sometimes it is used for the evaluation of the environmental attractiveness (Toda et al., 2000; Ungless, 2001; Atkinson, 2003). In many cases slugs show an innate pref-

erence to some smells and an aversion to the others (Smith, 1977; Willows, 1978), which may be modified due to the ability of using the olfactory learning by some of them (Ungless, 2001; Yamagishi et al., 2008). More and more frequently, the preference of some smells by slugs is utilized in plant protection, being the alternative method to control slugs using the synthetic molluscicides, expensive, sometimes not very effective, and acting on the environment (Hagnell et al., 2006; Jankowska and Wilk, 2009; Kozłowski et al., 2009; Baker et al., 2012). One of the numerous ways of protecting plants, more and more commonly used to eliminate invasive slugs on plantations are beer traps (Hagnell et al., 2006; Dankowska, 2011; Piechowicz et al., 2014). Beer is characterized by the presence of volatile chemical compounds, attractive to the slugs (Cranshaw, 1997), and carbon dioxide (CO_2), the factors that modify chemoreception. According to the literature (Gouveia, 1999), changes in physiological parameters can be the response to environmental cues, such as the presence of the smell of food.

The aim of the research was to check if the smell of beer, as food attractant for slugs, would change CO_2 emission by *Arion lusitanicus* auct. non-Mabille (or *Arion vulgaris* Moquin-Tandon 1885) individuals. We have made an attempt to indicate, which component of the volatile fraction of the selected brands of beer can be a factor responsible for changes in the respiration of slugs.

Material and methods

Material

Experiments were carried out on adult slugs *A. lusitanicus*, caught in the historic park belonging to the Institute of Applied Biotechnology and Basic Sciences, University of Rzeszów. The affiliation of the animal to the species was determined based on anatomical characteristics of the reproductive system. For the year before the collection of slugs, none of the treatments using plant protection products, has been performed, as well as no other chemicals that may influence the respiratory system of tested animals, have been used. In total, 49 individuals of *A. lusitanicus* (40 in the research + 9 to determine the effect of experimental conditions on the animal weight), were used in the experiments.

Beer

We tested five brands of beer popular in Poland:

Żubr – extract content: 12% by weight, ethanol content: 6% by volume. Brewery: Kompania Piwowarska S.A.;

Warka – extract content: 12.5% by weight, ethanol content: 5.7% by volume. Brewery: Grupa Żywiec S.A.;

Karpackie Pils – extract content: 10% by weight, ethanol content: 4.0% by volume. Brewery: Van Pur S.A.;

Żywiec – extract content: 12.5% by weight, ethanol content: 5.6% by volume. Brewery: Grupa Żywiec S.A.;

Leżajsk Full – extract content: 12% by weight, ethanol content: 5.5% by volume. Brewery: Grupa Żywiec S.A.

Equipment

The study on CO_2 emission by *A. lusitanicus* was carried out using a set Multichannel Gas Exchange System (MCGES; Qubit Systems Inc.) with CO_2 detector S157 2000 ppm CO_2 Analyzer (Figure 1). Synthetic air, of purity 5.0, was a source of breathing gas in the system. Gases used during the respirometric measurements had a humidity of 0%. Experimental chambers, with animals inside, were placed into a modified incubator Q-Cell model ERC0750. The content of CO_2 above the surface of beer was determined by CO_2 detector Telaire 7001. Headspace-Solid Phase Microextraction (HS-SPME) was proposed for the assessment of volatile compound content, utilizing polydimethylsiloxane (PDMS) – 100 µm fibre. Chromatographic analysis was carried out using Varian 450 GC chromatograph coupled with 240-MS mass spectrometer. The separation of analytes was made on the Varian VF-5 ms column (0.25 mm × 30 m × 0.25 µm). The resulting chromatograms and mass spectra were analysed using Varian MS Workstation Version 6.9.1 (Varian, Inc.) and NIST 08 Standard Reference Database software.

Methods

Incubation of the animals

For two weeks before the experiment, adult slugs *A. lusitanicus* 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%. Animals had unrestricted access to water and food (lettuce from organic farming). Forty-eight hours before the measurement, animals were placed into the chambers of 180 mL, without access to food and with access to water. Immediately before the measurement, they were weighed and placed into the chambers. During the two days of acclimation in experimental conditions and during the actual measurement period their body mass decreased about 4.2%. (Measurements were performed on the group of 9 animals other than those used in the respirometric tests).

Sampling the smell of beer

Samples of the smell of beer for each animal were collected from the separate cans. Just after opening the can, two 20 mL analytical portions of beer (one for the experimental surveys and one for the blind study) were taken and poured into 50 mL glass flasks. The syringe of 10 mL volume, 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 underpressure was created by blocking a syringe plunger at a level 10 mL 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 at 37.0°C for 30 min.

Measuring the CO_2 content in the air above the surface of beer was performed by placing 400 mL of the tested beer into a glass vessel, 1 litre capacity. CO_2 detector Telaire 7001 was placed 5 cm above the surface of beer, and the whole container was sealed. The whole system was placed into a laboratory incubator 400 Memmert IPP at 37.0 ± 0.5°C, and then, after 30 min., a reading of CO_2 content in the air above the surface of beer was made. Each assay was performed in triplicate for a given brand of beer, whereas for each repetition, new packaging of beer was used.

Respirometry

Previously weighed animals were placed into the respirometric chambers (volume 80 ml ø 3.2 cm, length 9.95 cm), in the incubator Q-Cell ERC0750 at 25.0±0.5°C, in constant darkness. During the experiments, animals had an opportunity to make small movements in the range allowed by the chamber wall (test overall gas exchange). In the system, the gas flow at a rate of 100 mL/min. was run in a cycle: 16.5 min. – the flow through the chamber with the animal inside it, and 3.5 min. – the reference flow. In such conditions animals were acclimated to the new environment for 240 min. After the time of such acclimation, the registration of CO_2 emission was initiated using the C950-MCGES version 3.8.7 software. It lasted 30 minutes (control measurement), and then a gaseous phase, taken up from the surface of beer, was introduced into the chamber using a syringe. The smell was introduced into the experimental setup to one of arms of the injector located outside the incubator, ahead of the incubation chambers, in order to minimize the influence of mechanical (shaking), and physical (light, temperature fluctuation) factors. The measurements were continued for a further 48 minutes. (Four hours of acclimation of animals to the respirometric chamber conditions cost them a 2.19±1.02% decrease in body mass). During the measurement 1.14±0.29% loss in body mass took place. Measurements were performed on a group of 9 animals other than those used in the respirometric tests. Before the study 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, in order to eliminate the measurement error, due to the presence of CO_2 coming from the samples of beer. The final result (with the slug disposed in the system) was reduced by the results of the blind study. The effect of each studied beer brand was analysed on eight animals.

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

$$R_{(\%)} = \frac{z \times 100}{C}$$

where:

$R_{(\%)}$ is a relative CO_2 emission in relation to the control measurement,

z corresponds to CO_2 emission after the exposure to the smell of beer (mL CO_2/g b.m.×h),

C is CO_2 emissions in control tests (mL CO_2/g b.m. × h).

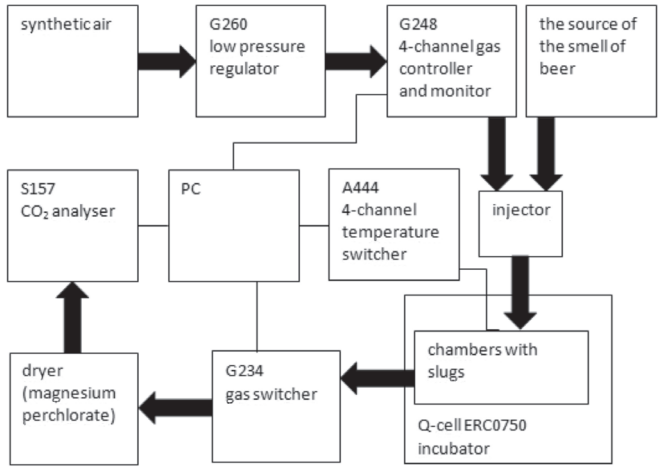

Figure 1. A diagram of the experimental setup for measuring CO_2 emission by animals

Chromatographic conditions

Beer (2 mL) was placed in a vial of 5 mL volume with a rubber top, and in order to adsorb analytes using HS-SPME method the fiber was exposed to the vapours above the liquid for 30 min. at 37°C, and then the chromatographic analysis was carried out. For this purpose SPME holder, with the analytes adsorbed on the fiber, was placed into a PTV injector at 200°C in splitless mode. The desorption process was carried out for 5 minutes and analytes were assayed using the following temperature program: 5 minutes isotherm at 50°C, 50–250°C with 10°C/min temperature gradient, 10 minutes isotherm at 250°C, 250–300°C with 20°C/min temperature gradient, 10 minutes isotherm at 300°C. Gas flow rate (He) was 1 mL/min. MS detector settings: scan mode 50–500 m/z.

Statistics

Cluster analysis, with the use of Ward and Taxicab metric, was applied to determine the similarities. In order to check out if the experimental groups were differentiated by distributions of results, non-parametric counterparts of variance analysis, such as U Mann-Whitney test and Kruskal-Wallis test, were used.

Results

At the first minute of the exposition to the smell of Żywiec Beer, the highest level of CO_2 emission by the animals was observed, while the lowest level was noted under the influence of Karpackie Pils, during 3 minutes of the exposition. Significant differences in CO_2 emission compared to the control persisted to 5 minutes of the exposure to the smell of Żubr and Karpackie Pils and up to 13 minutes in the case of Żywiec and Warka Full (Figure 2, Table 1).

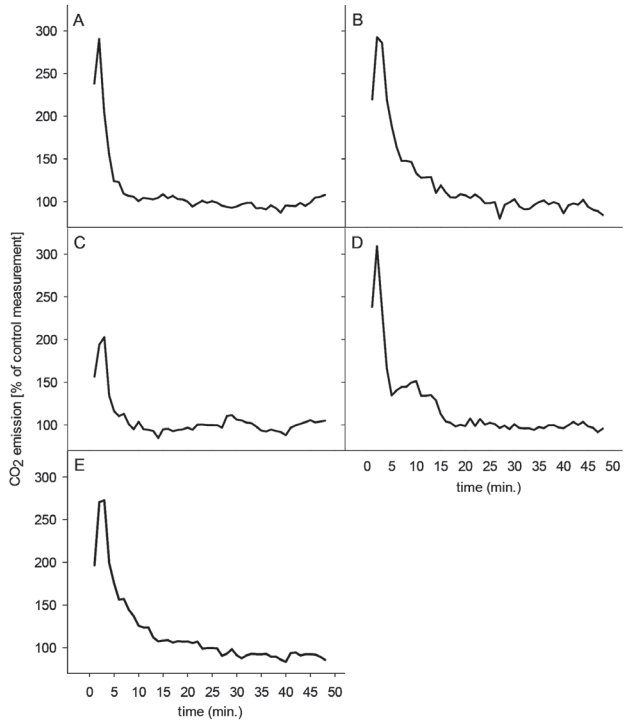

Figure 2. The CO_2 emission by *A. lusitanicus* exposed to the smell of beer in % of emission noted in the control measurement. A – Żubr, B – Warka Full, C – Karpackie Pils, D – Żywiec, E – Leżajsk Full

Table 1. The periods of statistically significant differences of CO_2 emission by *A. lusitanicus* subjected to the smell of beer, compared to the control measurement; Values of probability were added in parentheses

Żubr	Warka Full	Karpackie Pils	Żywiec	Leżajsk Full
1–4 min. (0.001) 5 min. (0.05)	1–9 min. (0.001) 10 min. (0.01) 11–13 min. (0.05)	1–4 min. (0.001) 5 min. (0.05)	1–4 min. (0.001) 5–8 min. (0.05) 9–10 min. (0.01) 13 min. (0.05)	1–9 min. (0.001) 10 min. (0.01) 11–12 min. (0.05)

Figure 2 shows CO_2 emission by adult *A. lusitanicus* exposed to the smell of beer expressed in % of the emission noted in control measurement. The obtained results indicated that the highest increase in CO_2 emission was induced by Żywiec Beer (control value: 0.2017±0.059 mL/g b.m×h), and consecutively, by Warka Full (0.1792±0.048 mL/g b.m×h), Żubr (0.1835±0.064 mL/g b.m×h), Leżajsk Full (0.1876±0.071 mL/g b.m×h) and Karpackie Pils (0.1972±0.036 mL/g b.m×h).

Table 2 presents a list of substances in volatile fraction of the studied brands of beer. These results indicated that they differed in the content of volatile fraction. The pronounced variability of chemical content between different batches of the same beer brand was also observed.

Table 2. Volatile substances, whose presence was noted in the studied brands of beer (the tests have been performed using the gas chromatograph Varian 450 GC, omitting the CO_2 concentration analysis made by the Telaire 7001 apparatus)

Chemical name	Żubr	Warka Full	Karpackie Pils	Żywiec	Leżajsk Full
Acrylic acid N–hydroxysuccinimide ester	11.82±1.08	15.98±2.01	8.44±1.51	14.30±2.86	19.77±1.53
Cis–3–nonen–1–ol	1.69±0.21	0.45±0.00	0.85±0.17	2.77±0.20	2.22±0.10
Octanoic acid	-	-	-	-	0.1±0.00
Ethyl caprylate	0.02±0.00	0.12±0.01	2.83±0.00	-	0.20±0.05
Ethyl palmitate	-	0.24±0.12	-	-	-
2–methoxy–4–vinylphenol	7.36±0.57	-	-	4.58±0.40	3.20±0.00
Alpha–caryophyllene	1.67±0.20	1.06±0.00	0.63±0.02	1.10±0.08	0.63±0.14
Lauric acid ethyl ester	-	0.25±0.00	-	-	-
2,6–diisopropylnaphtalene	0.65±0.30	0.90±0.00	0.99±0.13	0.53±0.01	0.57±0.29
Phthalic acid, isobutyl nonyl ester	-	-	0.54±012	-	0.03±0.00
T–muurolol	0.40±0.00	0.32±0.01	-	-	-
Aristolene epoxide	0.19±0.06	-	-	-	-
Bicyclo[4,1,0]heptane	-	-	0.85±0.00	-	2.37±0.00
2–phenylethanol	-	-	-	0.12±0.01	-
Decanoic acid	0.40±0.00	0.32±0.03	0.44±0.22	0.37±0.02	0.31±0.03
(9Z,12Z)–9,12–octadecadienoic acid 2–acetyloxy–1–(acetyloxymethyl)ethyl ester	0.33±0.00	0.04±0.00	0.02±0.00	-	-
1,5,5,8–tetramethylcycloundeca–3,7–dien–1–ol	0.52±0.15	-		-	-
T–cadinol	0.33±0.33	0.18±0.03	0.38±0.00	0.17±0.03	-
Alpha–eudesmol	-	-	1.15±0.00	-	0.57±0.33
Oleic acid	9.95±2.51	-	4.78±0.50	3.14±0.37	1.4±1.01
CO_2	12.44±0.70	12.61±0.55	12.08±0.89	12.93±0.65	12.41±0.90

Using Ward's agglomeration method, Taxicab metric and Friedman tests, the statistic method of cluster analysis, it can be concluded that CO_2 emission by slugs *A. lusitanicus* exposed to the smell of Karpackie Pils Beer differed significantly from the others. These differences are given in Figures 3 and 4.

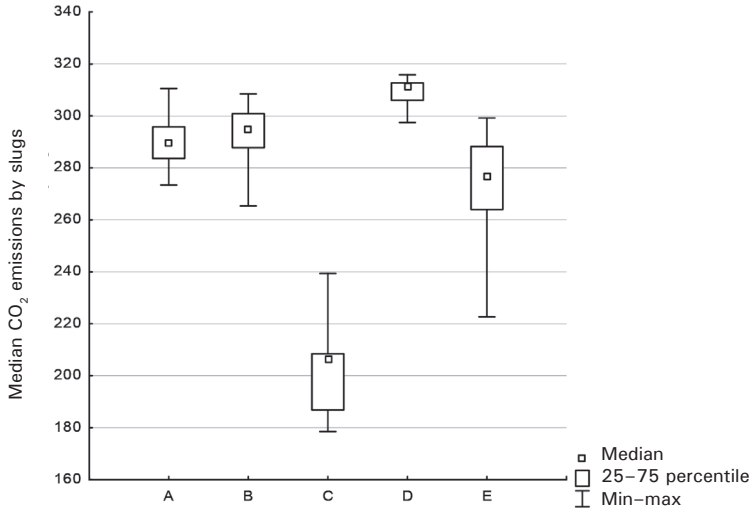

Figure 3. Chart median of CO_2 emission by slugs in % of the control. A – Żubr, B – Warka Full, C – Karpackie Pils, D – Żywiec, E – Leżajsk Full

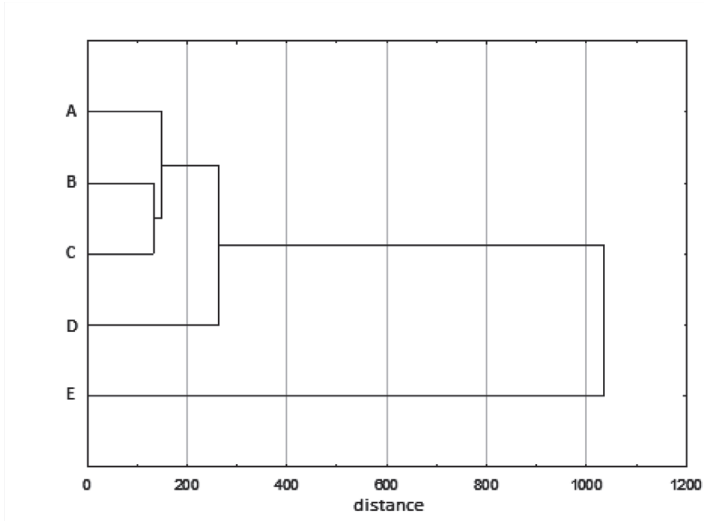

Figure 4. Similarities in the maximum CO_2 emission by slugs exposed to beer smell in % of the control. A – Żubr, B – Warka Full, C – Karpackie Pils, D – Żywiec, E – Leżajsk Full

Considering the relationship between CO_2 emission by slugs and the brand of beer, it can be noted that the significant, negative correlation in the case of slugs exposed to the smell of Karpackie Pils and the Leżajsk Full Beer ($r=-0.85$, $p=0.007$), occurs. That means that these brands of beer influence CO_2 emission by slugs exactly in the reverse manner.

Table 3. Differences in concentration of selected chemical components, between the selected beer brands (according to Kruskal-Wallis test)

Chemical name	Result of Kruskal-Wallis test or U Mann-Whitney test	P	P for multiple comparisons test	Detailed differences in CO_2 emission
Acrylic acid N–hydroxysuccinimide ester	K-W =12.23	0.015	Karpackie vs Leżajsk P=0.010	Karpackie < Leżajsk
Cis–3–nonen–1–ol	K-W =13.62	0.009	Warka vs Żywiec P=0.010	Warka < Żywiec
Octanoic acid			no differences	
Ethyl caprylate	K-W =10.68	0.013	Karpackie vs Żubr P=0.010	Karpackie > Żubr
Ethyl palmitate			no differences	
2–methoxy–4–vinylphenol	K-W =7.44	0.020	Żubr vs Leżajsk P=0.020	Żubr >Leżajsk
Alpha–caryophyllene	K-W =12.37	0.014	Żubr vs Leżajsk p=0.030	Żubr > Leżajsk
Lauric acid ethyl ester			no differences	
2.6–diisopropylnaphtalene				
Phthalic acid, isobutyl nonyl ester				
T–muurolol				
Aristolene epoxide				
Bicyclo[4,1,0]heptane	U M-W =1.98	0.040	Karpackie vs Leżajsk P=0.040	Karpackie < Leżajsk
2–phenylethanol			no differences	
Decanoic acid	K-W =12.77	0.012	Karpackie vs Leżajsk P=0.030	Karpackie > Leżajsk
(9Z,12Z)–9,12–octadecadienoic acid 2–acetyloxy–1–(acetyloxymethyl)ethyl ester	K-W=7.78	0.020	Warka vs Karpackie P=0.020	Warka > Karpackie
1,5,5,8–tetramethylcycloundeca–3,7–dien–1–ol			no differences	
T–cadinol				
Alpha–eudesmol				
Oleic acid	K-W =10.38	0.016	Żubr vs Leżajsk P=0.013	Żubr>Leżajsk
CO_2			no differences	

Since there were significant differences in CO_2 emission by slugs, depending on the brand of beer a question arose about their possible reasons. The most obvious cause was a various chemical content of them. Using Kruskal-Wallis test and Mann-Whitney U-test, it was established which of the selected chemical components (Table 3) differed in concentration, between the selected brands of beer.

Discussion

There is a lot of factors affecting the respiration activity and metabolic rate of invertebrate organisms, i.e.: locomotor activity and the structures linked with it, and with the functioning of the organism (Heinrich, 1979; Reinhold, 1999; Vogt et al., 2000; Davies and Blackwall, 2007), temperature (McMahon and Russel-Hunter, 1977; Poczopko, 1990; Sidorov, 2005; Marshall et al., 2011) and a level of acclimation to the special thermal conditions (Nielsen et al., 1999; Zotin and Ozernyuk, 2002; Sokolova and Pörtner, 2003), body mass (Heinrich and Heinrich, 1983; Levri and Lively, 1996; Todd, 1997; Mbata et al., 2000), the salt (Shumway, 1981), and calcium content in the environment (Dalesman and Lukowiak, 2010), oxygen and CO_2 concentrations in the air (Barnhart and McMahon, 1987; Erlichman and Leiter, 1997), the level of socialization of the species (Jaffe and Hebling-Beraldo, 1990, 1993), the place of origin of the species (McMahon and Russel-Hunter, 1977; Zotin and Ozernyuk, 2002), the presence of a considerable metabolically active tissue mass (Crnokrak and Roff, 2002), developmental stage (Mbata et al., 2000), caste and gender affiliation (Heinrich and Heinrich, 1983; Vogt and Appel, 1999; Shelton and Appel, 2001; Seuffert and Martin, 2010), health condition (Levri and Lively, 1996) or the presence of epibionts (Chan and Chan, 2005), and also the feeding behaviour (Hagvar and Ostbye, 1974; Heinrich, 1984), all these factors affect the respiratory activity of invertebrates.

In addition to the above mentioned: the act of eating (Roces and Lighton, 1995; Gouveia et al., 2000; Bradley et al., 2003), becoming accustomed to the presence of food (Kovac and Stabentheiner, 1999), chemical factors that may be taken with food (Duncan and Newton, 2000; Hebling et al., 2000; Tęgowska et al., 2004 a, b; Notten et al., 2006; Kennedy et al., 2010; Seuffert and Martin, 2010), the energetic cost of obtaining food (Stabentheiner, 1996), energetic value of food (Salvucci and Crafts-Brandner, 2000), and its aroma (Gouveia, 1999), affect the respiratory activity of animals.

Our research showed that the smell of beer might induce similar results in all studied animals exposed to 48-h food deprivation, although in varying degree.

As it was already mentioned, beer, which is characterized by a complex and specific smell, is commonly known as the attractant, both in commercial and homemade traps, for the control of *A. lusitanicus* (Hagnell et al., 2006; Dankowska, 2011; Piechowicz et al., 2014). In terms of composition, it is a mixture of many chemical compounds, including the by-products generated in the process of the brewing. Currently it is assumed that such chemicals as butane–2,3–dione, propan–2–one or 1,3–dihydroxypropanone (Cranshaw, 1997) are responsible for the attractive smell of beer.

On the other hand, new methods of the detection of volatile compounds, for example chromatography associated with SPME, allow searching for other compounds modifying the physiological and behavioural activity of *A. lusitanicus*. We decided to conduct a preliminary assessment of the presence of volatile substances in the tested brands of beer, using the SPME technique (Table 2). Although, based on only few tested brands of beer, it is impossible to determine which of them, or their com-

binations present in beer, is responsible for CO_2 emission by *A. lusitanicus*. It can be stated that the chemical compounds detected in beer of the same brand are not repetitive in subsequent batches, at least in relation to its volatile fraction. This means that rather the substances commonly found in beer, than that, specific to the brand (Table 2) are responsible for the increase of CO_2 emission, observed in all analysed cases (Figure 2, Table 1). The tests used in our experiments do not allow indicating the factor responsible for a significant increase in the CO_2 emission, because only in the case of slugs exposed to Karpackie Beer, the lowest significant increase in CO_2 emission, compared with the other beer brands, was observed. We showed that there was a significant negative correlation in CO_2 emission between Karpackie and Leżajsk ($r=-0.85$, $p=0.007$). Our analyses revealed that Karpackie differed from the others in the content of such chemicals as: acrylic acid N–hydroxysuccinimide ester, decanoic acid, (9Z,12Z)–9,12–octadecadienoic acid, 2–(acetyloxy)–1–[(acetyloxy) methyl]ethyl ester, bicyclo[4,1,0]heptane and ethyl caprylate (Table 3, Figures 3 and 4).

Among the slugs, discontinuous gas exchange (DCG) is observed, based on the ability of these organisms to accumulate the significant amounts of CO_2 in their tissues. This accumulation depends mainly on metabolic activity of the organism and CO_2 level in the environment (Barnhart and McMahon, 1987). According to Brand and Mehlman (1953) periodic lack of oxygen can cause oxygen debt, which later manifests itself in increasing its consumption, resulting in significant increase in demand for O_2. This may secondarily lead to increased CO_2 emission, observed in our study (Figure 2, Table 1). Such a result may be induced by CO_2 – one of the volatile substances present in beer (Table 3), the more so that its perception by slugs must not depend directly on its concentration. It has been shown that CO_2 contained in beer has the capacity to modify the organoleptic properties of this beverage (Clark et al., 2011).

Our results indicate undoubtedly that the smell of beer causes physiological response of the respiratory system in *A. lusitanicus*, manifesting itself by an increase in CO_2 emission. It was impossible to demonstrate unambiguously the ingredient responsible for this effect, however, using statistical methods, it was proved that the following compounds: acrylic acid N–hydroxysuccinimide ester, decanoic acid, (9Z,12Z)–9,12–octadecadienoic acid, 2–(acetyloxy)–1–[(acetyloxy)methyl]ethyl ester, bicyclo[4,1,0]heptane and ethyl caprylate limit CO_2 emission.

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Received: 12 XI 2014
Accepted: 9 II 2016