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The short-chain fatty acids as potential protective agents against *Callosobruchus maculatus* infestation

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ABSTRACT

The cowpea weevil, *Callosobruchus maculatus,* is one of the most common pests of stored legumes. Its occurrence adversely affects the quality of stored beans, making them unfit for consumption, resulting in substantial financial losses. The aim of this study was to investigate the potential insecticidal properties of the volatile fatty acids (VFAs) (C1 − C5) and their influence on the insect's physiology and behavior. All VFAs in concentrations equal to 4 μl and 8 μl showed fumigant toxicity significantly higher from the control. The strongest effect was observed in the case of propionic and valeric acid in volume of 4 μl and 8 μl, where mortality was close to 100%. Except for butyric acid, all acids showed a significant repellent effect. Additionally, all VFAs significantly decreased the number of infested beans and influenced the locomotor activity. Of all tested acids only the formic acid did not affect the oxygen consumption of the insects. As the studied VFAs have noteworthy properties against *C. maculatus*, they could be considered as promising agents in new strategies for stored products pest management.

1. Introduction

The cowpea weevil, *Callosobruchus maculatus* (F.) (Coleoptera: Chrysomelidae), is a significant pest of legumes such as mung beans, *Vigna radiata* ((L.) Wilczek), cowpea, *Vigna unguiculata* ((L.) Walp.) and adzuki beans, *Vigna angularis* (Wild.) (Beck et al., 2013). The adult females lay eggs on the surface of the beans from which larvae hatch and bore into the bean. Inside the bean, the larva feeds and completes pupation. The described way of reproduction causes considerable losses in the weight of the beans, which become unsuitable for consumption and production of sprouts. Due to the short generation time, *C. maculatus* can infest up to 99% of stored beans in 6 months (Seck, 1993; Singh et al., 1978).

Currently, various methods are utilized to manage the infestation of this species. The most used insecticides are phosphine and neurotoxic contact compounds. However, the continuous use of these insecticides has led to the development of resistance by many major stored product insect species. While an older fumigant, methyl bromide, has been phased out due to its negative effect on the ozone layer. (Athanassiou et al., 2015; Daglish et al., 2018). For those reasons, alternative control means are necessary.

The aforementioned downsides and high non-target toxicity led researchers to evaluate novel substitutes. The main interest focuses primarily on natural insecticides, such as plant essential oils, plant extracts, and inert dusts (Krzyżowski et al., 2019). One of the promising groups of chemical compounds that could provide safer and cost-effective means of management of stored products pests are volatile fatty acids (VFAs) also known as short-chain fatty acids (defined as carboxylic acid with 1–5 carbon atoms). VFAs are widespread compounds in the natural environment as they are end products of microbial fermentation of cellulose and hemicellulose (Douglas, 2015). Moreover, VFAs are widely utilized by various species as semiochemicals. As such, they could provide information on environmental quality and, population density (Weaver et al., 1990). Additionally, most VFAs (formic, acetic, propionic) are generally recognized as safe (GRAS) by the American Food and Drug Administration and approved as additives for food production. Formic and propionic acids are commonly used as fungicides and food preservatives. Moreover, propionic acid also works as a bactericidal agent added to drinking water for livestock and poultry, a use which is registered by the U.S. Environmental Protection Agency.

VFAs, especially propionic acid, are attracting increasing attention as a potential insecticides and repellent agents in the protection of stored products. Although the body of literature concerning this topic seems to be still in development, the available results are

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highly promising. Propionic acid was reported as a promising fumigation agent against wheat weevil, *Sitophilus granarius* (L.), and rice weevil, *Sitophilus oryzae* (L.), acting both as an insecticide and repellent (Germinara et al., 2007). Furthermore, it was suggested that propionic acid could be utilized as an additive in manufacturing packaging for cereal storage as a preventive measure against the infestation by the aforementioned species (Germinara et al., 2010). Butyric, propionic, and valeric acids were reported to exhibit a repellent effect against mealworm beetle, *Tenebrio molitor* (L.) larvae (Weaver et al., 1990). All VFAs, except valeric, possess strong repellent properties against lesser mealworm, *Alphitobius diaperinus* (Panzer), and exposition to VFAs vapors significantly repelled *A. diaperinus* but also altered the overall character of its locomotor activity (Baran et al., 2018).

In the presented study, the potential activity of VFAs as an insecticide against *C. maculatus* was investigated. The influence of the aforementioned substances on physiological and behavioral parameters was examined using respiratory rate, mortality, and locomotor activity assays. Additionally, the repellent effect of the tested acids was measured using a setup - a continuous flow olfactometer consisting of the rectangular chamber with oppositely positioned inlets delivering constant flow of pure air and air containing tested VFAs, originally described by Baran et al. (2018), specifically adapted for the experiments on cowpea weevil.

2. Materials and methods

2.1. Culture conditions

In all assessments, unsexed, adult individuals of the *C. maculatus* were used. The insects were reared on mung beans (*Vigna radiata*) in constant conditions of 30 ± 1 °C, 50% relative humidity, and the photoperiodic regime of 12/12h light/dark.

2.2. Used substances

Analytical grade, undiluted volatile fatty acids used in the presented research (formic, acetic, propionic, butyric, and valeric acid) were obtained from POCH S.A., Poland. In all the experiments, pure, undiluted acids were used.

2.3. Fumigation mortality

Mortality was assessed in four replications per volume (selected on the basis of previous pilot studies) of each acid (1 μl, 2 μl, 4 μl, 8 μl). Each replication consisted of ten unsexed insects that were put into 50 ml non-hermetic, plastic containers with tight-fitting lids. Tested acid (ultrapure water in the control group) was applied on the cotton pad attached to the cover of the container. Dead beetles were counted and removed after 24 and 48 h. Insects were considered dead when no movement for 1h was observed. The bioassays were conducted in constant conditions, i.e., 30 ± 1 °C, 50% relative humidity.

2.4. Contact mortality

Contact mortality for each acid was assessed in five replications, each consisting of ten unsexed insects. On each insect, 0.5 μl of undiluted acid (ultrapure water in the control group) was applied topically, using a micropipette (Eppendorf Research® Plus 0.1–2.5 μL manual pipette). Insects were subsequently placed into Petri dishes (140 mm diameter, LabTek) lined with a filter paper disk (Whatman N°1). Dead beetles were counted and removed after 24 and 48 h. The bioassays were conducted in constant conditions, i.e., 30 ± 1 °C, 50% relative humidity.

2.5. Egg-laying

For each VFA, three groups of ten females (one week old) were placed into the 50 ml plastic container with 18g of uninfested mung beans. For each replication, 2 μl of the tested acid (volume selected on the basis of results of the fumigant mortality assay) was applied on the cotton pad attached to the lid of the container. The number of eggs per bean was counted after all insects were considered dead. The bioassays were conducted in constant conditions, i.e., 30 ± 1 °C, 50% relative humidity.

2.6. Oxygen consumption

An oxygen consumption test was conducted in six replications per acid, using a SiLab data acquisition unit and oxygen sensor (sampling rate: 1/sec) tightly fitted into the 50 ml Falcon tube. For each experiment, ten unsexed individuals were put into airtight tubes containing 15g of mung beans. To avoid the contact of the insects with the tested acid, cotton wool wetted with acid was placed in custom-made containers (3D printed using photocurable resin) mounted on the bottom of Falcon tubes, and 4 μl of the tested acid (ultrapure water in the control group) was applied to each container. Measurements were started immediately after putting insects into the Falcon tube and lasted for 1h. The bioassays were conducted in constant conditions, i.e., 30 ± 1 °C, 50% relative humidity.

2.7. Repellency

The repellency test was conducted according to the method of Baran et al. (2018), with modifications. The bioassays were conducted in constant conditions, i.e., 30 ± 1 °C, 50% relative humidity. For every acid, 40 beetles were used. Each insect was placed separately in the rectangular chamber (3 mm height, 15 mm width, 160 mm long) made of clear Lucite with a constant flow of humidified air from one side and humidified air with the tested odor from the opposite. The inlet air for the odor side was pumped through the glass tube with cotton wool and 4 μl of the tested acid, while for the opposite site through cotton wool with 4 μl of ultrapure water. The airflow was kept at 10 L/h. The homogeneous, red background light was provided by transilluminator placed underneath the experimental setup. The insects were able to explore the chambers freely, and their movement was recorded with a Microsoft LifeCam Studio and AMCap software. Recordings lasted for 10 min at 15 fps frame rate with 640 × 860 px resolution. The obtained data was analyzed in the same way as in the article by Baran et al. (2018), allowing to calculate a preference index (PI). The test chamber was divided into six fictive compartments to which values were assigned (Fig. 1). The time spent in the given compartment was multiplied by its value, and results were summated for the entire duration of the video. The final value (PI) indicates the strength of the preference for either side (in the presented experiment, a negative value would indicate the repellent effect of tested substance while a positive value would indicate an attractant effect).

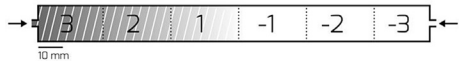

Fig. 1. The compartmentalisation of the test chamber and odor gradient. Adapted after Baran et al. (2018).

2.8. Open field test

To assess the influence of the VFAs on the *C. maculatus* movement, the open field test paradigm was utilized. The paradigm was adapted for *C. maculatus* by using 100 mm glass Petri dishes lined with filter paper disk (Whatman N°1) as the arenas. On each paper filter, 4 μl of the tested substance was applied. For every VFA, ten unsexed insects were placed in each Petri dish in four replications. The rest of the measurement was carried out as described in the repellency procedure (Microsoft LifeCam Studio camera, AMCap software, 15 fps, 640 × 860 px resolution). The recording lasted for 50 min, but because of the difference in the behavioral reaction of the insects, the videos were analyzed as five separate intervals. The first 10 min are the immediate influence of the VFAs on insects. The remaining four intervals (40 min) were analyzed to assess the basic activity level of *C. maculatus* treated with tested acids. The analysis of insects' movement was performed with SwissTrack 4 software (Lochmatter et al., 2008) and in R environment with a trajr (McLean and Skowron, 2018) package to calculate the traveled distance and immobility time. The bioassays were conducted in constant conditions, i.e., 30 ± 1 °C, 50% relative humidity.

2.9. Statistical analysis

Statistical analysis was conducted using GraphPad Prism v6.00 software for Windows. For all the obtained data, the normality test was performed (Shapiro-Wilk normality test and standard deviation similarity Brown-Forsythe test). For the analysis of oxygen consumption, locomotor activity, oviposition rate, and fumigant toxicity, ANOVA (Tukey test, $P < 0.05$) was used. Analysis of histograms on number of eggs per bean was conducted using Chi-square test ($P < 0.05$). The repellent effect was analyzed with the use of a nonparametric test - Kruskal-Wallis with multiple comparisons ($P < 0.05$).

3. Results

3.1. Fumigation mortality

In both 24 and 48 h mortality assays, the ascending concentrations of the three acids (butyric, propionic and valeric) caused increased mortality of the beetles. In the case of propionic, the increase occurred in 4 μl and 8 μl, while in the case of butyric acid only in the highest (8 μl) volume. After 24 h, all the groups, except 1 μl and 2 μl of formic acid, were statistically different from the control group (Fig. 2). In the case of 48 h exposure/mortality test, only the group treated with 1 μl formic acid did not differ significantly from the control group (Fig. 3).

3.2. Contact mortality

In contact mortality assay after 24 h, 100% mortality was observed in groups treated with propionic, butyric, and valeric acids (Fig. 4). In contrast, in the case of formic and acetic acids, mortality increased only slightly after 48 h in comparison to 24 h (Fig. 5).

3.3. Egg-laying

The observed average number of eggs in groups treated with formic, butyric, and valeric acids was significantly lower in comparison with the control group (Fig. 6). In the control group, most of the beans had one or two eggs, while no beans with three laid eggs were observed. VFAs treatment significantly increased the proportion of beans with no eggs as well as decreased the number of beans with a single egg (Fig. 7).

3.4. Oxygen consumption

All treated groups except the formic acid exposed group differed significantly from the control group. The highest inhibition of oxygen consumption was observed in the case of the insects treated with propionic acid (Fig. 8).

3.5. Repellency

In the preference index test, propionic, formic and acetic acid turned out to be the most repellent VFAs. All acids, except butyric, were significantly repellent in comparison to the control group (Fig. 9).

3.6. Locomotor activity in open field test

During the bioassay testing, the locomotor response of the *C. maculatus* to the VFAs vapors in the phase of initial activity (first interval-10 min, the exploratory behavior is altered by the novelty of the environment) the groups treated with the VFAs, except the valeric acid, had significantly elevated locomotor activity Fig. 10. In

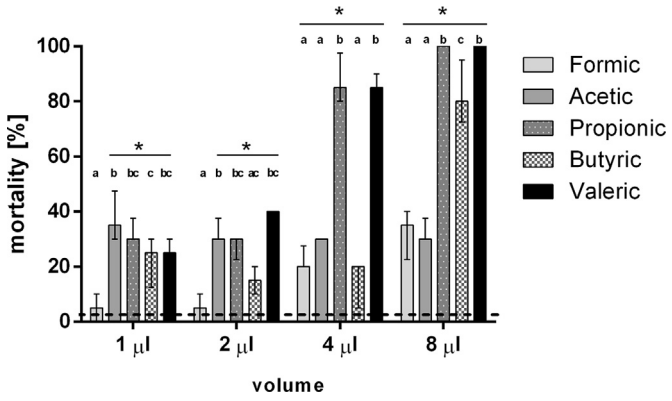

Fig. 2. Mortality of *C. maculatus* in fumigant toxicity assay after 24h of treatment. Bars represent the medians and quartiles. The dashed line indicates the mortality level for the control group. The same letters indicate statistically homogenous groups for particular volume and * indicates groups different from the control. N = 4, Kruskal-Wallis test with multiple comparisons, $P < 0.05$.

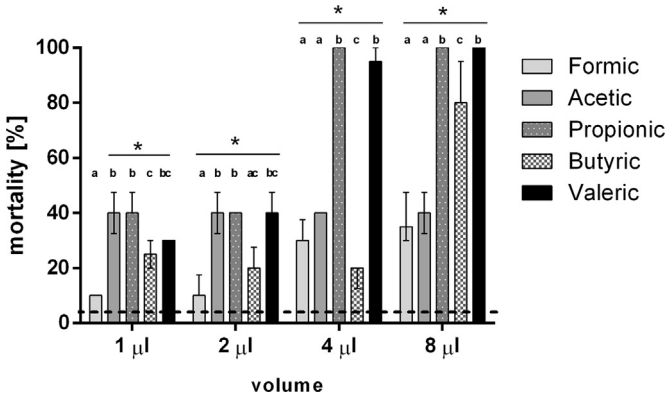

Fig. 3. Mortality of *C. maculatus* in fumigant toxicity assay after 48h of treatment. Bars represent the medians and quartiles. The dashed line indicates the mortality level for the control group. Letters indicate statistically homogenous groups for particular volume and * indicates groups different from the control. N = 4, Kruskal-Wallis test with multiple comparisons, $P < 0.05$.

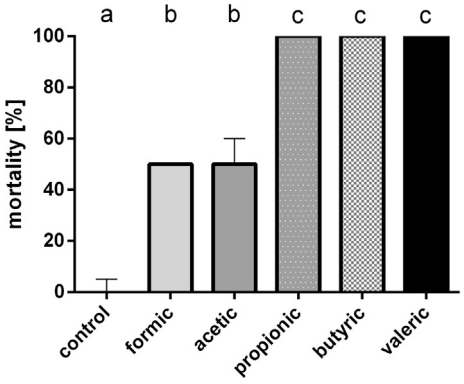

Fig. 4. Mortality of *C. maculatus* in contact toxicity assay after 24h of treatment with VFAs (medians and quartiles). Letters indicate statistically homogenous groups. N = 5, Kruskal-Wallis test with multiple comparisons $P < 0.05$ (treatment effect: K–W value 28,07, $P < 0.0001$).

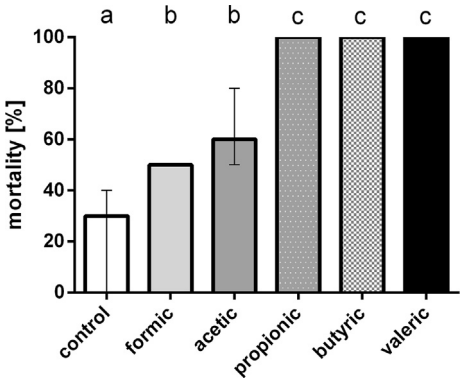

Fig. 5. Mortality of *C. maculatus* in contact toxicity assay after 48h of treatment with VFAs (medians and quartiles). Letters indicate statistically homogenous groups. N = 5, Kruskal-Wallis with multiple comparisons, $P < 0.05$ (treatment effect: K–W value 28,50, $P < 0,0001$).

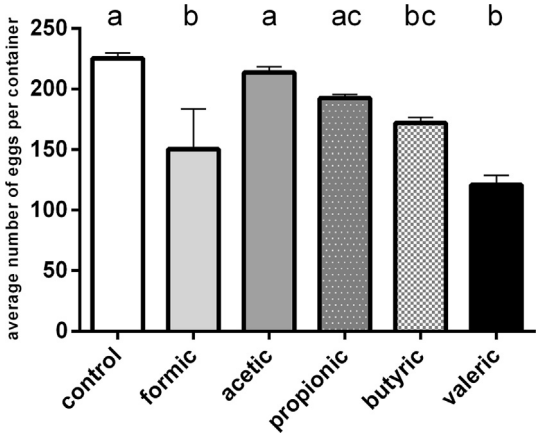

Fig. 6. The average (mean ±*SD*) number of eggs laid on the surface of the beans after fumigation with VFAs. Letters indicate statistically homogenous groups. N = 3, two-way ANOVA, Tukey's multiple comparisons test, $P < 0.05$ (treatment effect: F (Douglas, 2015; McLean and Skowron Volponi, 2018) = 22.67; $P < 0.0001$).

subsequent intervals, the movement of the insects treated with all the VFAs was inhibited (propionic and butyric acid – from the second interval, valeric acid – from the third interval, formic and acetic acid - in the last interval) and ceased entirely after the third interval in propionic and butyric acid-treated groups.

4. Discussion

The results presented in the manuscript contribute novel data to the field of *C. maculatus* management methods, and to the authors' best knowledge, there is no previous research assessing the influence of VFAs on the aforementioned insect. Hitherto, only a few articles reported the insecticidal activity of VFAs on insects, namely: *S. oryzae*, *S. granarius* (Germinara et al., 2007) yellow fever mosquito, *Aedes aegypti* (L.), southern house mosquito, *Culex quiquefasciatus* (Say) (Paulraj et al., 2011) and German cockroach, *Blattella germanica* (L.) (Sims et al., 2014). However, the presented study is the first one to report not only the mortality after the treatment but also physiological and behavioral parameters, such as oxygen consumption and locomotor activity.

In fumigation bioassays, all tested acids showed substantial toxic effect. The strongest influence was observed in groups treated with propionic and valeric acids, which, in 4 μl volume, turned out over two-fold more potent than other tested substances. In higher volume, (8 μl) butyric acid also caused very high mortality of tested insects. Sims et al. (2014) conducted a fumigant toxicity test on the *B. germanica*, using saturated fatty acids between C1 to C14 showing high toxicity of the entire range of C1 to C11 (including VFAs). Similarly to the presented data, the highest mortality was caused by propionic acid. Topical toxicity assays were conducted to investigate the effect of the direct contact of the insects with the tested substances. An evident trend is visible in the results where the toxicity increased with carbon chain length (Figs. 2 and 3). The same effect was reported by Tattersfeld and Gimingham (1927), who studied the toxic effect of the saturated fatty acids with 1–18 carbon atoms. In their research, the toxicity increased with the molecular weight. Additionally, similar observations were reported in the article by Shaaya and Pisarev (1990), where the toxic effect of the fatty acids was tested on pulse beetle, *Callosobruchus chinensis* (L.) (species phylogenetically closely related to *C. maculatus*). The tested acids were more effective as the length of the chain increased. This effect may be caused by increase of lipophilic character with the length of the VFA carbon chain, which potentially results in better penetration of cellular membranes (Prochazka, 2008).

The impact of VFAs on oxygen consumption sharply differed between the tested acids. Acetic and valeric acids reduced consumption to about 50% and propionic to about 25% of the value observed in the control group. There are no similar analyses in the literature that would allow to compare them to the obtained results. At this stage, it is difficult to say whether the reduction of oxygen consumption is associated with triggering the defense mechanisms (such as the closing of the spiracles) or with a disturbance on metabolic processes related to respiration. The available results of the test on vertebrates indicate a multitarget mode of action, but they only apply to propionic acid (Frye et al., 2016). The observed changes are associated with metabolically universal processes characteristic for all animals. Frye et al. (2016) suggest that propionic acid modulates the action of mitochondria, and its higher concentrations lead to lower proton leak respiration. In addition, it also caused an increase in radical oxygen species at the inner mitochondrial membrane. These results allow linking the observed decrease in oxygen consumption in the groups treated with propionic acid with its potential impact on respiration processes.

All of the tested VFAs, except butyric, significantly repelled *C. maculatus*. The most repellent (the lowest observed PI) was the propionic acid. Germinara et al. (2007) presented the influence of different concentrations of propionic acid on the spatial preference

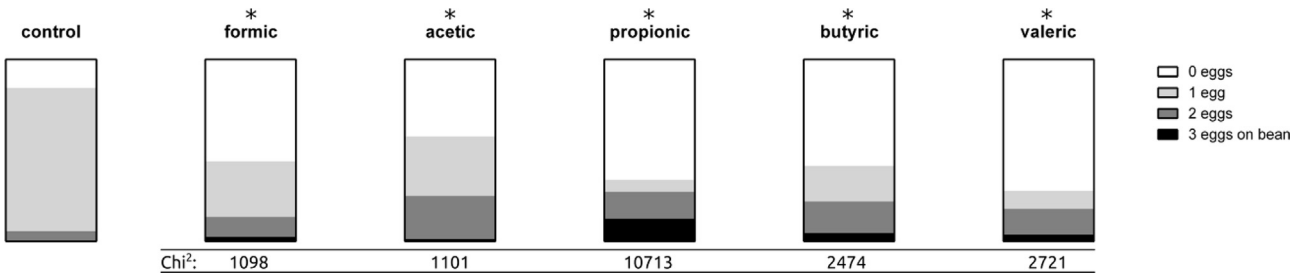

Fig. 7. Quantification of females' egg-laying after VFA treatment and distribution of number of eggs present on the bean surface. * indicates groups different from the control. Mean number of beans per treatment − 760, Chi-square test, $P < 0.0001$.

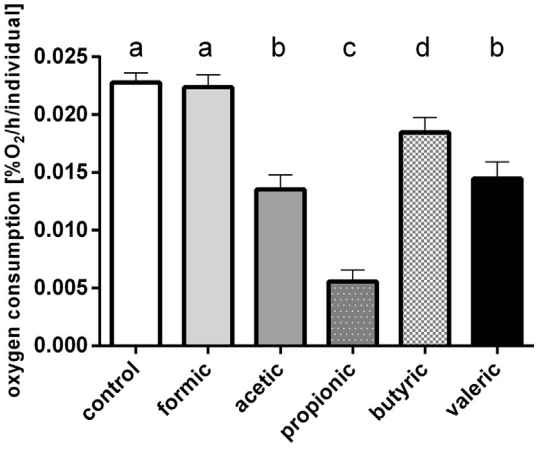

Fig. 8. Level of oxygen consumption (mean ±*SD*) by insects treated with 4 μl of different VFAs for 1h. Letters indicate statistically homogenous groups. N = 6, ANOVA, Tukey's multiple comparisons test, $P < 0.05$ (treatment effect: F (5, 30) = 187.4; $P < 0.0001$).

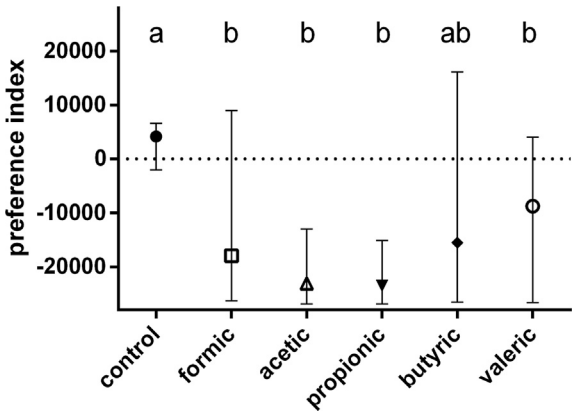

Fig. 9. Preference index (PI) for insects treated with tested VFAs. Median and quartiles values are presented. Letters indicate statistically homogenous groups. N = 40, Kruskal-Wallis with multiple comparisons, $P < 0.05$ (treatment effect: K–W value 31,78, $P < 0,0001$.

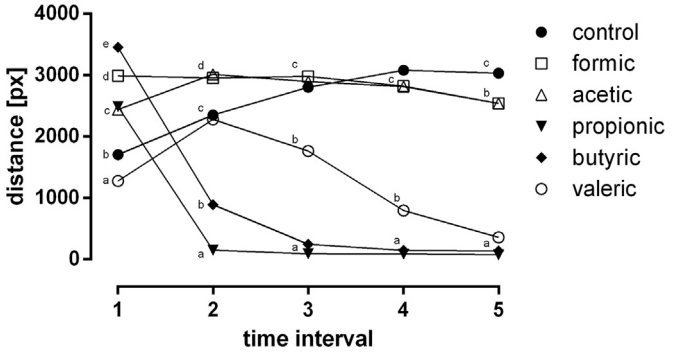

Fig. 10. Distance traveled by the adult insects treated with VFAs. Mean values in every interval are presented. Letters indicate statistically homogenous groups in a particular time interval (10 min). N = 4, ANOVA, Tukey's multiple comparisons test, $P < 0.05$ (treatment effect: F (Douglas, 2015; Sims et al., 2014) = 453,7, $P < 0,0001$, time effect F (4, 72) = 81,24, $P < 0,0001$, interaction effect F (20, 72) = 61,79, $P < 0,0001$).

of the *S. granarius* and *S. oryzae*. In Germinara's tests, insects were repelled by relatively small doses of propionic acid. A similar result was reported by Baran et al. (2018) on the effect of propionic acid on lesser mealworm, *Alphitobius diaperinus* (Panzer) - another species from the Coleoptera order, also considered as a stored products pest. The obtained results can support the hypothesis proposed in both of the aforementioned papers on a potential role of propionic acid as an infochemical used by insects in habitat choice. High concentrations of this compound may indicate ongoing decomposition of the substrate and, thus, its unsuitableness for oviposition (Xiros et al., 2019).

All the assays were conducted on adult *C. maculatus*, although considering the damage to stored legumes, it is caused exclusively by larval instars. Thus, to depict the potential of VFAs as a protection agent, the oviposition tests were conducted. The results indicate a significant effect of all the tested acids on oviposition - however, the average number of laid eggs differed significantly from the control group only after treatment with formic, butyric and valeric acid, although, all VFAs caused alterations in the egg-laying pattern. In all treated groups, the number of beans with no eggs is much higher than in the control group. Moreover, the insects were observed to lay three eggs per bean only in treated groups. The share of beans with three eggs per bean seems to correlate with the inhibition of locomotor activity. In the groups with the lowest locomotor activity (treated with propionic, butyric and valeric acids), the quantity of the beans with three eggs was significantly higher than in the other groups. Thus, it may indirectly indicate that females from treated groups explored fewer beans. This may be caused by sensory impairment or indicate the stimulation of egg-laying as well as a strong inhibition of locomotor activity.

The results of the open field test also revealed different reactions of insects to selected acids. The high activity of insects in groups exposed to formic and acetic acid clearly correlates to the relative lower toxicity of these VFAs. However, in the case of acids with higher toxicity (propionic, butyric and valeric acid), the activity decreases significantly over time. It is difficult to explain the mechanism of observed differences with the present level of knowledge.

The exact mechanism of the VFAs action on *C. maculatus* is yet to

be uncovered, although the presented results add to the growing data highlighting the potential infochemical role of VFAs in site selection. Considering all the above, VFAs, especially the propionic acid, present considerable potential as an agents against *C. maculatus* infestation, both as a repellent as well as an insecticide (contact and fumigant). Considering that the propionic acid is already commonly used in agriculture, extending its application as an insecticide would be both convenient and economically justified.

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Research involving human participants and/or animals

This article does not contain any studies with human participants or animals (vertebrates) performed by any of the authors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Michał Krzyżowski: Writing - original draft. **Jacek Francikowski:** Writing - original draft. **Bartosz Baran:** Writing - original draft. **Agnieszka Babczyńska:** Writing - original draft.

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