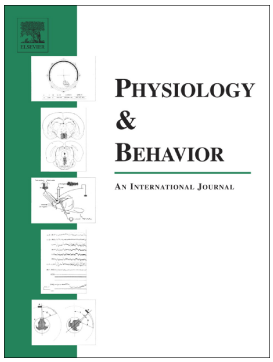

Evolutionary compromises to metabolic toxins: Ammonia and urea tolerance in Drosophila suzukii and Drosophila melanogaster

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Evolutionary compromises to metabolic toxins: ammonia and urea tolerance in *Drosophila suzukii* and *Drosophila melanogaster*.

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Abstract

The invasive pest *Drosophila suzukii* has evolved morphological and behavioural adaptations to lay eggs under the skin of fresh fruits. This results in severe damage to a wide range of small fruits. *Drosophila suzukii* females typically lay few eggs per fruit, preferring healthy fruits. Hence, larvae are exposed to a reduced amount of nitrogenous waste. Differently, the innocuous *Drosophila melanogaster* lays eggs on fermented fruits already infested by conspecifics, with larvae developing in a crowded environment with the accumulation of nitrogenous waste such as ammonia and urea. These compounds derive from nitrogen metabolism, protein degradation, and amino acids catabolism and are relatively toxic at high concentrations in an organism. The observed differences in oviposition site and larval ecological niche suggest that these species might differ in behavioural and physiological mechanisms used to cope with nitrogenous waste. We investigated how different concentrations of ammonia and urea affect oviposition and larval development in both species. Females and larvae of *D. suzukii* showed greater susceptibility to high concentrations of both compounds, with a dramatic decrease in the number of eggs laid and egg viability. Moreover, we tested the chemotactic response of third instar larvae to high concentrations of the compounds. Interestingly, ammonia resulted in a repulsive behaviour in respect of the control and urea groups. To better understand the pathways underlying these differences, we evaluated the effect on ornithine aminotransferase and glutathione-S-transferase, two enzymes involved in nitrogen metabolism and stress response that are expressed during larval development. Both ammonia and urea significantly reduced the expression of these enzymes in *D. suzukii* compared to *D. melanogaster*. This shows how the ecological shift of *D. suzukii* to fresh fruit is accompanied by less efficient detoxifying and excretory mechanisms, with important implications for evolutionary biology and applied research. Our data suggest that the ecological shift of *D. suzukii* to fresh fruit as oviposition substrate is accompanied by a reduced tolerance to metabolic toxins during larval development.

Keywords: *Drosophila suzukii*, *Drosophila melanogaster*, detoxification enzymes, nitrogenous waste, adaptation, pest species, larvae

1. Introduction

In the last decade, growing interest has emerged for the invasive pest *Drosophila suzukii*, a serious agricultural and economical threat [1, 2], as well as a model to investigate adaptation to novel ecological niche (*e.g.* [3-5]). This species is native to Asia and has invaded western countries, with a rapidly expanding range in America and Europe [6, 7]. Differently from other *Drosophila* species, which attack overripe and decaying fruits, females of *D. suzukii* lay eggs under the skin of fresh healthy fruits, through a peculiar serrated ovipositor [3]. Therefore, larval development and exposure to pathogens result in damage to a wide range of small and stone fruits [6, 8]. To date, most research on *D. suzukii* has focused on adults [3-5, 9-11], while little is known about larval adaptations, although the ecological niche of larvae in *D. suzukii* is quite unique. Our work focuses on the comparative analysis of larval behavioural and metabolic responses in the presence of nitrogenous waste and the related oviposition behaviour in adult females in *D. melanogaster* and *D. suzukii*.

The innocuous *D. melanogaster* lays eggs in rotten fruits and larvae develop in a crowded environment, rich in bacteria, mould, and yeast [12, 13]. Females of this species have a gregarious tendency in selecting the oviposition site and prefer to lay eggs where other larvae are present [14, 15]. High larval density combined with microorganism metabolic activity and protein-rich microbial community [16, 17] results in accumulation of nitrogen waste products such as ammonia and, at a lower extent, urea [18, 19]. Both are relatively toxic when concentrated in organism tissues [20, 21]. The concentration of ammonia in fruit flies vials can reach 30 mM [22], while ammonia environmental rich-sources may approach 100 mM [23, 24]. In *D. melanogaster*, high concentrations of dietary urea and ammonia have been associated with a decrease in the number of eggs laid per female (e.g. [25, 26]), a decline in egg-to-adult viability, as well as an increase in developmental time [22, 27]. Due to the limited mobility of larvae [28, 29], behavioural avoidance cannot prevent larval exposure to environmental toxins accumulating in the food, but physiological mechanisms help larvae to cope with toxic compounds [30,

31]. *Drosophila melanogaster* populations reared under crowded larval conditions exhibit greater competitive ability [22, 32] and increased resistance to both urea and ammonia [22, 33]. While the response of *D. melanogaster* to high levels of urea and ammonia has already been studied [22, 33], little is known about the effects in *D. suzukii*. This species occupies a unique ecological niche compared to other drosophilids, since larval development occurs in fresh fruits [6] rich in water [34] and relatively poor in microorganisms, due to the skin barrier [35]. Moreover, females of *D. suzukii* tend to lay few eggs per fruit [36, 37], resulting in a moderate larval density and, as a consequence, a low level of waste products. Therefore, we hypothesised to observe differences between *D. melanogaster* and *D. suzukii* in behavioural and physiological responses to nitrogenous waste products, as a potential effect of adaptation to different ecological niches.

To evaluate tolerance capacity for nitrogenous compounds in *D. suzukii* compared to *D. melanogaster*, we investigated the effect of different concentrations of urea and ammonia on female oviposition behaviour, under no-choice and choice conditions, and on larval development in both species. We further studied potential differences in the expression of ornithine aminotransferase and glutathione-S-transferase, two enzymes involved in metabolic and detoxifying pathways. Ornithine aminotransferase is highly expressed in third instar larvae of *D. melanogaster*, playing a crucial role in amino acids metabolism and nitrogen homeostasis [38, 39]. Glutathione-S-transferase is involved in insect resistance to endogenous and xenobiotic compounds and in protection against oxidative stress [40, 41]. It is known to be expressed in the larval midgut of *D. melanogaster* [42].

2. Materials and methods

2.1. Insect strains and rearing

We used adult flies of *Drosophila melanogaster* from a mixed population of 50 lines of the DGRP [43], a collection of inbred isofemale lines originally collected in Raleigh, US [44]. Lines were obtained

from the Bloomington Drosophila Stock Center (Indiana University, Bloomington, US) and represent a spectrum of naturally occurring genetic variation. The same isofemale lines were tested in all treatment groups. The *D. suzukii* flies used in this study were originally collected in the Trentino area, Italy, and maintained under the same laboratory conditions as the *D. melanogaster* population for several generations. All flies were raised on a standard Drosophila diet (see Appendix S1), at 25 ± 1°C, with 65 ± 1% relative humidity, and with a light:dark cycle of 14:10 h.

2.2. Chemicals

To reproduce nitrogenous waste products, we used ammonium chloride (NH_4Cl, purity≥99.5, Carl Roth, Karlsruhe, Germany) and urea (ACS reagent, purity ≥99-100.5%, Sigma-Aldrich, Milan, Italy). Antimicrobial agents as propionic acid (Carlo Erba Reagents, Milan, Italy) and methyl 4-hydroxybenzoate (purity ≥99%, Acros Organics, Milan, Italy) were added to the standard food medium after it had cooled down to 70°C.

Ammonium chloride and urea (pH ~5.5) were added to the standard food medium after it had cooled down to 48°C. In order to homogenize the mixture, it was rapidly stirred with a magnetic stirrer and dispensed into polypropylene vials (25 × 95 mm) or Ø 90 mm Petri dishes. In larval behavioural test, we used urea and ammonium hydroxide (28-30%, Sigma-Aldrich, Milan, Italy).

2.3. Behavioural and fitness tests

2.3.1. Oviposition behaviour and larval development in a no-choice assay

For both species, newly eclosed flies were transferred to fresh food vials and were maintained under standard conditions until tested. Under mild CO_2 anaesthesia, females (5-6 days old) of about the same size were individually assigned to vials with 5 ml of standard diet and one of the following supplements: urea at 25 mM (UL=urea low concentration), 250 mM (UH=urea high concentration),

ammonium chloride at 25 mM (AL=ammonia low concentration), 250 mM (AH=ammonia high concentration), or no supplements (CTRL). A pinch of active yeast was sprinkled on the food to stimulate oviposition. After 24 h, females were removed from the vials and eggs were counted under an optical microscope. In this assay, we assessed the oviposition behaviour via the number of eggs laid within 24 hours.

One day later, the presence of larvae and their conditions (alive/dead, $1^{st}/2^{nd}$ instar) were recorded under an optical microscope. Vials were checked every day at the same hour, at the beginning of pupation pupae were counted by careful visual inspection, and number and times of pupation were recorded twice per day, at 10 a.m. and 5 p.m., for five days. The larval developmental time was defined as the number of hours between hatching to pupation. From the beginning of adult emergence, flies were collected, using CO_2 anaesthesia, and the number of adults and their sex was recorded for each vial. The experiment was replicated 6 times for a total sample size of 32 females (*n*: 6, 6, 5, 5, 5, 5) for each treatment group (CTRL, UL, UH, AL, AH) and for each species. Oviposition behaviour was assessed on the 32 females, while larval development (time to pupation and number of pupae) and viability (eggs-to-pupae and egg-to-adults) were evaluated on 25 of the assessed vials (*n*: 5, 5, 5, 5, 5) for each treatment group and species.

2.3.2. Oviposition behaviour in a choice assay

Oviposition was tested both under no-choice and dual choice conditions to control for interaction between environmental cues and treatment [45, 46]. Flies were tested in a cage where both a control and an experimental medium were provided. The oviposition substrates consisted of Ø 90 mm Petri dishes filled with 20 ml of standard food (CTRL), or with standard food and one of the following supplements: urea at 25 mM (UL) or 250 mM (UH), or ammonium chloride at 25 mM (AL) or 250 mM (AH). Sprinkles of active yeast were added to the food to stimulate oviposition.

Freshly eclosed flies were transferred to fresh food vials and were maintained at standard conditions until tested. Ten females (5-6 days old) of each species were collected, using CO_2 anaesthesia, and transferred to a bug dorm insect-rearing cage (30×30×30 cm, BugDorm-1, MegaView Science Taichung, Taiwan) with the control Petri dish in one corner, and the Petri dish with supplemented food at the opposite corner. After 24 hours, the Petri dishes were collected and the eggs counted under an optical microscope. In this assay, we assessed oviposition via the number of eggs laid in 24 hours. The experiment included 10 replicates for each condition.

2.3.3. Larval chemotaxis assay

To investigate the olfactory response to nitrogenous waste products, third instar larvae of both species were selected and tested in a chemotaxis assay by exposing them to 250 mM of urea (UH), 250 mM of ammonium hydroxide (AH), or deionized water as a control (CTRL). Ammonia is highly volatile (2160 mm Hg at 25 °C), while urea is a low volatile compound ($1.2\ 10^{-5}$ mm Hg at 25 °C). For this reason no difference was expected between UH and the control group. Newly eclosed females of *D. melanogaster* and *D. suzukii* were individually transferred to new fresh food fly vials to lay eggs. After 24 h, females were removed and the vials were maintained under standard conditions. Chemical stimuli (20 µl), diluted in deionized water, were pipetted onto a filter paper placed inside a plastic cap located at the very edge of a Petri dish (Ø 90 mm) filled with 20 ml of nutritive agar (supplemented with 6% sucrose). Petri dishes were placed over a printed grid in a closed apparatus, to avoid external contamination. Single third instar larva were collected from the fly vials, washed in deionized water, individually placed in the centre of the plate, and their locomotor movement was video-recorded for 5 minutes. We tested three larvae for each vial, one for every odour condition (urea, ammonia, control), for a total of 22-25 larvae in each treatment. Assays were conducted in Petri dishes with closed lid and animals were tested within a few seconds of odour application. A new Petri dish was used for each

larva. Larval motor activity was tracked with EthoVision Pro tracking software (Noldus, Wageningen, Netherlands) at a sampling rate of 6 frames/s to evaluate the mean distance of the larva from the odour stimuli, the total distance covered, and the time spent in proximity to the odour stimuli. The arena and the zones of interest were marked and defined for the analysis (see Fig. S3). The plate was divided in three zones: A in proximity to the stimuli, B –intermediate, C – at distance (see Fig. S3). The plastic cap containing the filter paper with the stimuli was marked as odour.

2.4. Semiquantitative analysis of the expression of genes coding for metabolic and detoxifying enzymes

Expression of ornithine aminotransferase (OAT), glutathione-S-transferase D2 (gstD2), and D4 (gstD4) was assayed by RT-PCR in third instar larvae that had developed in standard food (CTRL) or in standard food with one of the following supplements: urea at 25 mM (UL) or 250 mM (UH), or ammonium chloride at 25 mM (AL) or 250 mM (AH).

A total sample size of about 15 larvae per treatment group was collected. We had no samples from the AH group because no larvae developed to the third instar. Total RNA was extracted from samples using a TRIreagent:chloroform (Sigma-Aldrich, Milan, Italy) protocol, performed according to the manufacturer's instructions. RNA samples were quantified using a Nanodrop spectrophotometer (ThermoFisher Scientific, Waltham, US) and reverse-transcribed to cDNA using the Revert Aid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, US) with specific primers (Table S1). PCR products were analysed by gel electrophoresis in a 1% ethidium bromide-stained agarose gel. Gel documentation was collected using a "Gel Doc XR", digitally evaluated with "Quantity One" (Bio-Rad Lab., Milan, Italy) and normalized to the corresponding signals for tubulin.

2.5. Statistical analysis

For each species, non-parametric data related to egg number, number of alive larvae, eggs-to-pupae and eggs-to-adults viability, and sex-ratio were analysed by a Kruskal-Wallis test. To compare the effect between species, the number of eggs was normalized relative to the control and analysed with a Mann-Whitney test. Data related to larval developmental time (from hatching to pupation) were averaged for each vial and tested by a one-way analysis of covariance (ANCOVA) with Treatment as factor and number of eggs as covariate. A Bonferroni correction was applied when post hoc multiple comparisons were performed.

The number of eggs laid in the preference test was analysed with a Wilcoxon signed-rank test to compare conditions (CTRL versus Treatment). In the larval chemotaxis assay, differences between conditions for the total distance covered and time spent in the proximity of the stimuli were tested by a Kruskal-Wallis test. Differences in mean distance from the odour stimuli were tested with a one-way analysis of variance (ANOVA). A Bonferroni correction was applied when post hoc multiple comparisons were performed. Densitometry for enzymes expression was tested by a Kruskal-Wallis test. A *p*-value of less than 0.05 was considered significant. All statistical analyses were carried out using SPSS version 17 (IBM, Armonk, US).

3. Results

3.1. Behavioural and fitness tests

3.1.1. Oviposition behaviour and larval development in no-choice assay

No significant difference in oviposition behaviour was observed at a low concentration of ammonia and urea with respect to the control. At high concentration, ammonia (AH) reduced significantly the number of eggs in *D. melanogaster* ($\chi^2(4)=22.89$, $p<0.001$, see Fig. 1A). In *D. suzukii*, high concentrations of both urea (UH) and ammonia (AH) decreased the number of eggs ($\chi^2(4)=70.77$, $p<0.001$, see Fig. 1B).

When comparing the effects between species, *D. suzukii* females showed a greater sensitivity to the treatment (AH: U=195, p<0.001; UH: U=237, p<0.001, see Fig. S1).

Twenty-four hours after the start of hatching, first and second instar larvae were present in all treatment groups except for AH of *D. suzukii* (*D. melanogaster*: $\chi^2(4)=5.31$, p=0.25; *D. suzukii*: $\chi^2(4)=57.5$, p<0.001, Fig. 2A, B). Under this condition, larvae died soon after hatching, and many of them were observed on the wall of the vial, suggesting a possible escaping behaviour.

In *D. melanogaster*, larval development was affected by exposure to high concentration of urea (140±3 h) and ammonia (130±2 h) with respect to the control (106±2 h), with a significant delay in pupation (Treatment: $F(4,119)=68.24$, p<0.001, see Fig. S2).

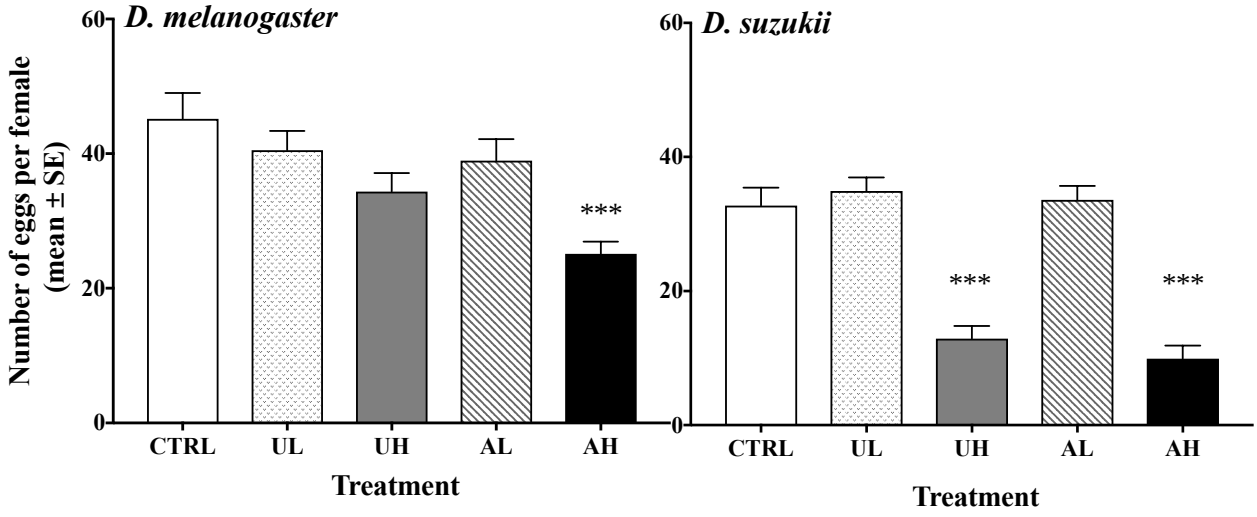

Figure 1. Eggs laid during a 24-hour period by single females exposed to standard food and standard food supplemented with urea or ammonia. CTRL: standard food; UL: standard food with 25 mM of urea; UH: standard food with 250 mM of urea; AL: standard food with 25 mM of ammonium chloride; AH: standard food with 250 mM of ammonium chloride. Mean ± standard error (SE) are shown, ***p<0.001. The experiment was replicated 6 times for a total sample size of 32 females (n: 6, 6, 5, 5, 5, 5) for each treatment group (CTRL, UL, UH, AL, AH) and for each species.

These durations did not depend on the number of eggs (Treatment*Number of eggs: $F(4,119)=1.54$, $p=0.19$). However, when considering eggs-to-pupae viability, a significant decrease was observed only in UH ($\chi^2(4)=25.76$, $p=0.01$, see Fig. 3A), while no difference was found under ammonia exposure. In *D. suzukii*, the effect of the treatment was even stronger, with no pupae in the AH group, and only a few pupae under high concentration of urea ($\chi^2(4)=78.16$, $p<0.001$, see Fig 3B), accompanied by a temporal delay.

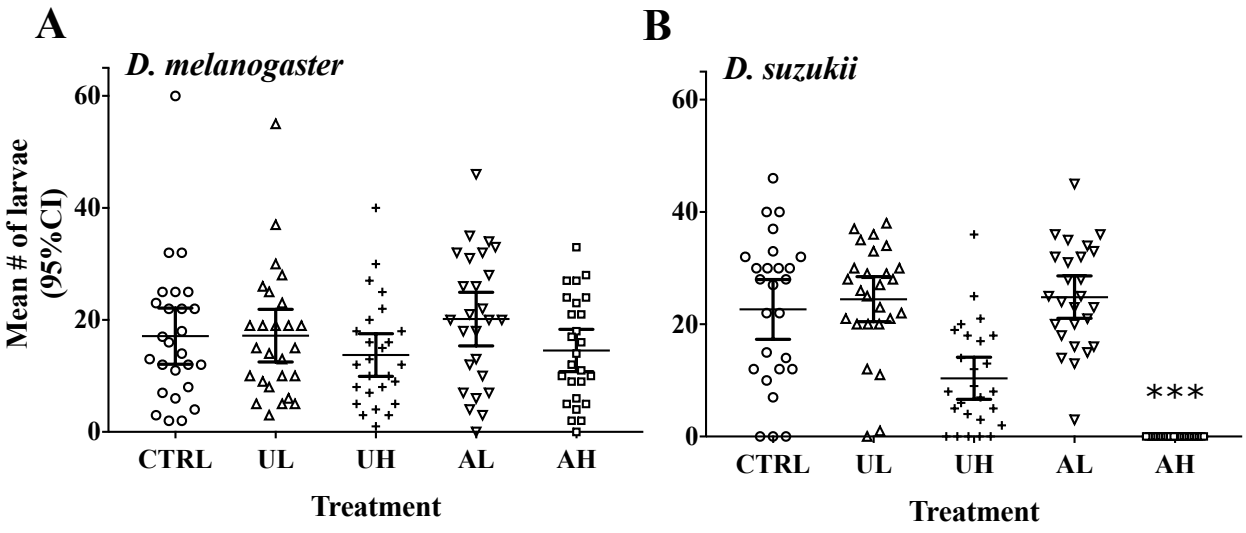

Figure 2. Estimated number of alive larvae 24 hours after hatching in standard food and in standard food supplemented with urea or ammonia. CTRL: standard food; UL: standard food with 25 mM of urea; UH: standard food with 250 mM of urea; AL: standard food with 25 mM of ammonium chloride; AH: standard food with 250 mM of ammonium chloride. Horizontal bars indicate mean and 95% confidence interval, ***$p<0.001$. The experiment was replicated 5 times (n: 6, 5, 5, 5, 5) for a total of 26 vials for each treatment group (CTRL, UL, UH, AL, AH).

Finally, pupation and emergence of adult flies were strongly impaired by a high concentration of urea in both species (*D. melanogaster*: $\chi^2(4)=60.19$, $p<0.001$; *D. suzukii*: $\chi^2(3)=48.94$, $p<0.001$, see

Fig. 3C, D), while eggs-to-adults viability was not significantly affected by high concentration of ammonia in *D. melanogaster* (see Fig. 3C). No difference in adult sex ratio was observed among groups (*D. melanogaster*: $\chi^2(4)=1.69$, $p=0.8$; *D. suzukii*: $\chi^2(2)=1.72$ $p=0.4$).

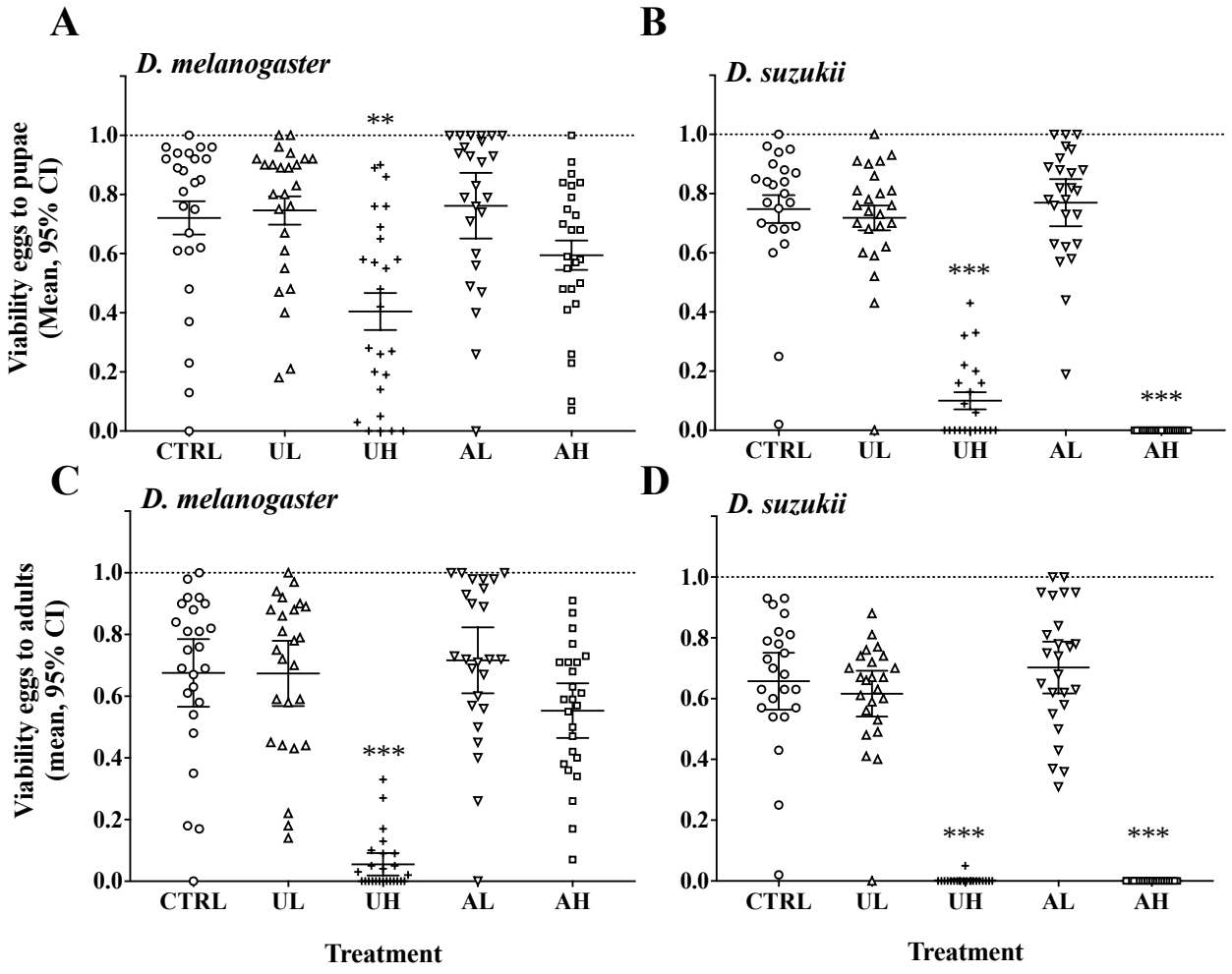

Figure 3. **Viability eggs-to-pupae (A, B) and eggs-to-adults (C, D) in standard food and standard food supplemented with dietary urea and ammonia**. CTRL: standard food; UL: standard food with 25 mM of urea; UH: standard food with 250 mM of urea; AL: standard food with 25 mM of ammonium chloride; AH: standard food with 250 mM of ammonium chloride. Horizontal bars indicate mean and 95% confidence interval. $^{**}p\leq0.01$, $^{***}p<0.001$. The experiment was replicated 5 times for a total of 25 vials per treatment group.

3.1.2. Female oviposition in a choice assay

Oviposition preference was tested in a choice assay between experimental substrates (supplemented with urea or ammonia) and control substrates. Females did not show significant egg laying preferences between control food and food supplemented with low concentration of urea (*D. melanogaster* Z(9)=-0.76, p=0.5, *D. suzukii* Z(9)=-1.78, p=0.08) and ammonia (*D. melanogaster* Z(9)=-1.07, p=0.3, *D. suzukii* Z(9)=-1.63, p=0.1, see Fig. 4A,B). When the concentration of ammonia was increased, females displayed a strong aversion and only about 11% of the eggs were laid in the AH substrate in *D. melanogaster* (Z(9)=-2.70, p<0.01, see Fig. 4A) and 9% in *D. suzukii* (Z(9)=-2.80, p<0.01, see Fig. 4B).

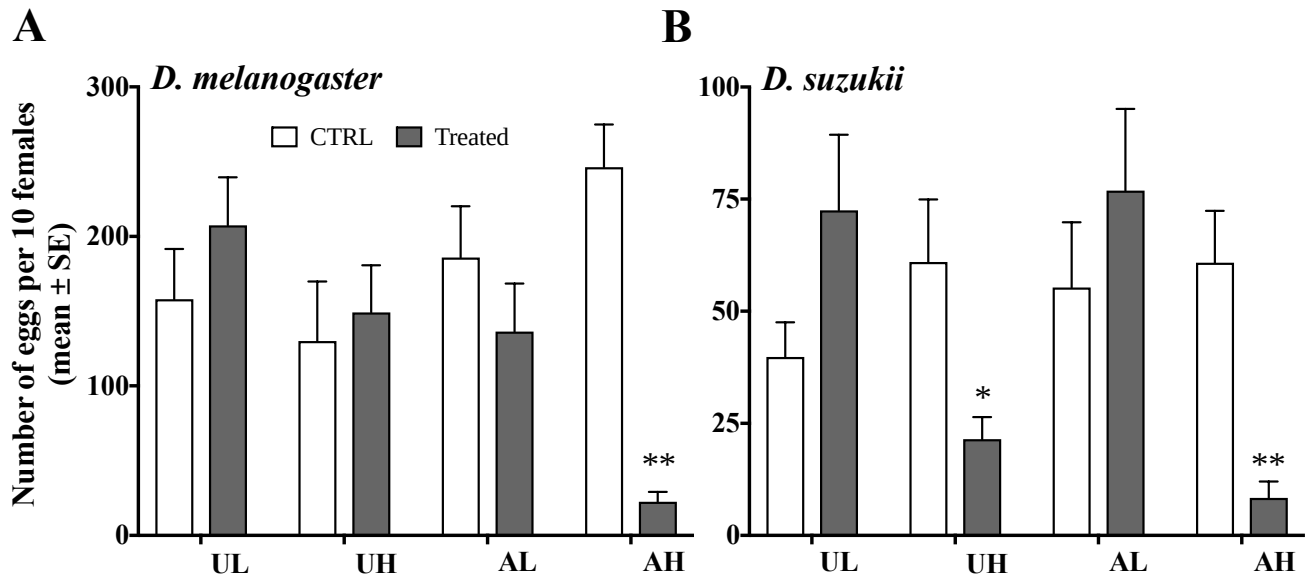

Figure 4. Oviposition site preference for standard food supplemented with dietary urea and ammonia (Treated) against non-supplemented standard food (CTRL). UL: 25 mM of urea; UH: 250 mM of urea; AL: 25 mM of ammonium chloride; AH: 250 mM of ammonium chloride. Mean ± standard error (SE) is shown. *p<0.5, **p<0.01. Each experiment tested 10 females and was replicated 10 times for a total sample size of 100 females for treatment.

Interestingly, oviposition preference was unaffected by high concentration of urea in *D. melanogaster* (53% of the eggs were laid in the UH substrate), whereas *D. suzukii* females laid significantly less in the UH site (29%) than in the urea-free medium ($Z(9)=-2.09$, $p<0.05$, see Fig. 4A,B).

3.1.3. Larval chemotaxis assay

No significant difference in the total distance covered was observed under exposure to high concentration of ammonia and urea with respect to the control in both species (*D. melanogaster*: $\chi^2(2)=1.24$, $p=0.54$; *D. suzukii*: $\chi^2(2)=1.61$, $p=0.44$, see Fig. 5E, F).

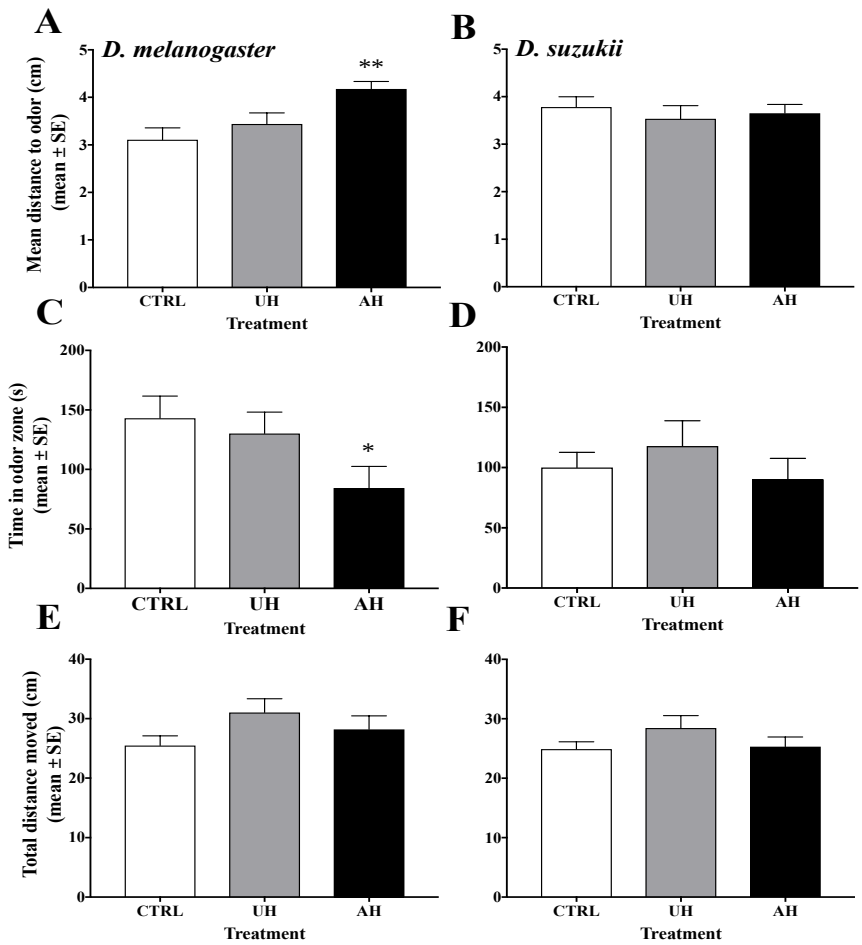

Figure 5. Chemotactic behaviour in *D. melanogaster* and *D. suzukii* during 5 minutes exposure to odour stimuli: CTRL, deionized water; UH, urea (250 mM); AH, ammonium hydroxide (250

mM). Mean distance to odour is calculated at the end of the experiment; time in odour zone is the total time spent in proximity to the stimuli (zone A) on 5 min test; the total distance covered is calculated on the total period. Data are collected at a sampling rate of 6 frames/s. Data show mean ± standard error (SE). ***** $p< 0.05$, ****** $p< 0.01$. n=22-25 larvae per treatment.

In *D. suzukii*, chemotactic behaviour was not affected by the treatment (Mean distance to odour: $F(2,72)=0.29$, $p=0.74$; Time in proximity to odour: $\chi^2(2)=1.05$, $p=0.59$, Fig. 5B, D), while *D. melanogaster* showed an aversive response to high level of ammonia with a greater mean distance from the odour ($F(2,63)=6.41$, $p<0.001$, see Fig. 5A) and less time spent in proximity to the odour ($\chi^2(2)=7.27$, $p<0.05$, see Fig. 5B).

3.2. Expression of genes coding for metabolic and detoxifying enzymes

Semi-quantitative RT-PCR analysis of the expression of OAT, gstD2 and gstD4 evidenced a different expression pattern between both species. Despite a common constitutive expression of the three genes observed in the control specimens, the expression of OAT, gstD2, and gstD4 resulted highly increased in *D. melanogaster* with respect to *D. suzukii* after exposure to ammonia and urea. In particular, a significant increase of the OAT was observed in *D. melanogaster* in the presence of urea, both at a low and a high concentration ($\chi^2(4)=12.01$, $p<0.05$), whereas no induction was evident in *D. suzukii* ($\chi^2(3)=6.59$, $p=0.07$; Figs. S4A, 6A).

Differently from this pattern, gstD2 resulted highly induced in the presence of ammonium at high concentration in *D. melanogaster* ($\chi^2(4)=13.06$, $p<0.001$), whereas no significant difference was detected at both concentrations of urea. In *D. suzukii* none of the treatments differed from control (Figs. S4B, 6B). Lastly, gstD4 showed an expression pattern similar to gstD2, with a significant induction under AH exposure in *D. melanogaster* ($\chi^2(4)=13.08$, $p<0.001$; Figs. S4C, 6C).

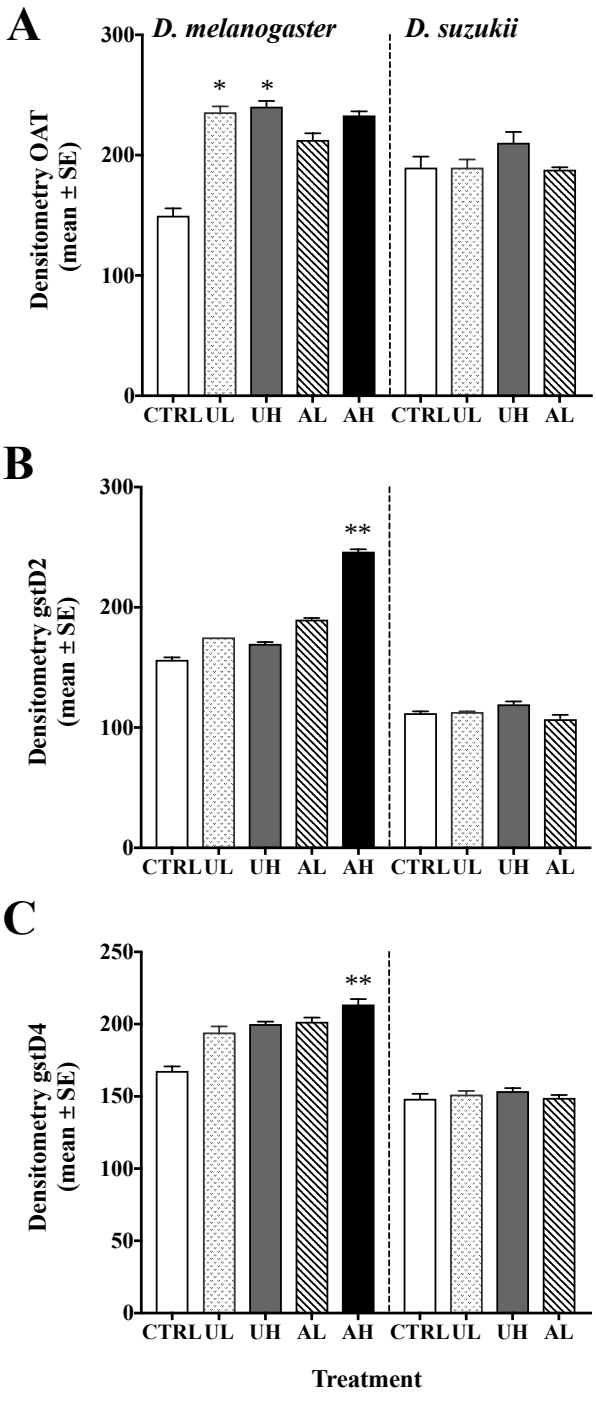

Figure 6. Semi-quantitative RT-PCR analysis of genes coding for OAT (A), gstD2 (B), and gstD4 (C) in third instar larvae exposed to standard food or food supplemented with urea and

ammonia. UL, 25 mM of urea; UH, 250 mM of urea; AL, 25 mM ammonium chloride, AH, 250 mM of ammonium chloride. Data show mean ± standard error (SE). *p<0.5, **p<0.01. N= 15 larvae.

4. Discussion

The ecological shift of *D. suzukii* from rotten to fresh fruit [3-5] makes the comparative investigation of behavioural and physiologic adaptation in adult females and larvae particularly interesting for basic and applied research. We compared the effect of low and high concentrations of urea and ammonia on oviposition behaviour and larval development in *D. melanogaster* and *D. suzukii*.

Our data show that both species are negatively affected by nitrogenous waste products, but significantly greater effects were observed in *D. suzukii*. When exposed to high concentrations of nitrogen compounds, oviposition was more negatively affected by ammonia than by urea in both species. However, while *D. melanogaster* experienced a 50% reduction in the number of eggs laid, in *D. suzukii* the number of eggs was reduced by 70%. While previous studies have documented the relevant role of ammonia as a sensory cue for female orientation and site selection in *D. melanogaster* [26, 47], we documented a greater physiological sensitivity of *D. suzukii* to ammonia compared to *D. melanogaster*. This outcome can be explained by a relaxation in selection for nitrogenous compounds tolerance in *D. suzukii* or by a trade-off between this trait and the adaptation to the ecological niche of fresh fruit.

The stronger response observed in *D. suzukii* could derive from a greater olfactory and/or gustatory sensitivity to ammonia (e.g. [47, 48]). In fact, the concentration of volatiles associated with different maturation stages can greatly affect olfactory choices in *D. melanogaster* (e.g. [13, 49]). Moreover, recent studies have shown how, during fruit maturation, changes in the composition and concentration of volatiles can provide different cues for *D. suzukii* and *D. melanogaster* [4, 5, 50]. Further studies

should clarify the role of sensory cues in determining the greater response observed in *D. suzukii* when exposed to ammonia.

In addition, we observed a significant difference between species also regarding a typically non-volatile compound as urea, resulting in a greater impact on *D. suzukii* compared to *D. melanogaster*. While the number of laid eggs was only slightly decreased in *D. melanogaster* (Joshi *et al.* [51] observed stronger effects at higher concentrations), *D. suzukii* shows a 60% decrease in the number of laid eggs. This suggests again a stronger repellent effect of nitrogenous waste for *D. suzukii* compared to *D. melanogaster*.

Interestingly, we observed differences in the oviposition behavior in *D. melanogaster* under urea exposure between choice and no-choice assays, indicating that the presence of more cues or alternative choices can ameliorate the inhibitory effect of urea on oviposition. In fact, females laid significantly less eggs in the UH group when compared with the control in the no-choice assay, while no difference was observed in presence and absence of urea in the choice assay. Differently, this modulation was not found in *D. suzukii*.. Several studies have shown that *D. melanogaster* tends to hold eggs in the absence of quality oviposition media [52-54], and one work has found that *D. melanogaster* lays eggs in substrates with potentially toxic chemicals when a harmless alternative is closely located [55].

The presence of a high level of ammonia, on the other hand, caused great reduction in eggs laid in both the choice assay, and the no-choice assay in both species.

We argue that the documented aversion of *D. suzukii* females for nitrogenous products might be an adaptation to avoid substrates that can negatively affect larval fitness in this species more than in *D. melanogaster*. In fact, in *D. melanogaster* egg-laying is influenced by the presence and density of larvae [14, 15], and larval waste has been shown to modulate this effect [51, 56]. Along this line, we show that nitrogenous waste products affect *D. suzukii* larvae more negatively than *D. melanogaster* larvae. In our study, larval exposure to ammonia and urea resulted in high toxicity, showing a

significant difference between species in the capacity to cope with the detrimental effects of these compounds. In *D. suzukii*, larvae were not able to survive in the presence of high concentration of ammonia. In fact, 100% of mortality was observed soon after hatching. On the other hand, larvae of *D. melanogaster* showed a delayed development, but viability remained comparable to the control. High levels of urea affected late larval stages in both species, influencing larval survival as well as developmental time and pupation process, but with a stronger detrimental effect in *D. suzukii*.

Differently from many toxic chemicals that attack a single or few targets [57, 58], ammonia and urea are able to impact the whole organism [20, 59, 60]. Strategies to resist and respond to the globally detrimental effects of these toxins include uptake reduction, detoxifying pathways, as well as efficient excretory mechanisms [61]. Interference with any of these processes could compromise an organism's survival [62, 63]. Adaptation to urea and ammonia results in decreased larval feeding rate and longer developmental time in *D. melanogaster* [18, 22]. This could explain our results at high levels of ammonia, where a significant increase in developmental time was associated with larval viability in *D. melanogaster*. Larvae of this species are able to detect many compounds and show strong odour-evoked chemotaxis [3, 64]. Similarly, in *D. melanogaster* we observed a clear aversive response to high levels of ammonia (Fig. 5), suggesting a larval capacity to adaptively react to exposure to toxic compounds. On the contrary, no significant chemotactic response was observed when larvae of *D. suzukii* were exposed to ammonia (Fig. 5), suggesting a larval inability to detect the compound or identify it as a toxin in this species. We argue that in *D. suzukii* compensation mechanisms failed, causing ammonia levels to rapidly increase, resulting in an acute intoxication. This hypothesis is supported by significant metabolic and detoxification differences observed between the two species. In fact, ornithine aminotransferase and glutathione-S-transferase gene expression increased under high levels of ammonia and urea in *D. melanogaster*, whereas no induction was apparent in *D. suzukii*. The ornithine aminotransferase enzyme plays a crucial role in amino acids metabolism and nitrogen

homeostasis [38, 39], while glutathione-S-transferase is highly expressed in response to xenobiotics and oxidative stress [40, 65]. Alteration in the expression of these enzymes is associated with inefficient detoxification and reduction of tolerance capacity to environmental stressors [65-68]. Consequently, the mortality observed in *D. suzukii* when first instar larvae were faced with a high level of ammonia in the medium could be related to these observed differences in gene expression.

Urea can interfere with important cell processes, act as protein denaturant, and reduce enzyme activity [60, 69, 70], resulting in developmental delay and larval stop [18, 27]. *Drosophila* larvae do not encounter high concentrations of urea in their environment [18, 71], nor are able to produce it [33, 72]. This scenario matches the less efficient physiological mechanisms to handle urea compared to ammonia. However, we observed a similar enzymatic response under urea and ammonia exposure, in agreement with previous studies describing the evolution of cross-tolerance between these stress traits [22, 73]. Developmental delay and reduction in feeding rate are adaptive strategies developed to reduce urea uptake in *D. melanogaster* larvae and favour toxin resistance [18, 22, 33]. Larvae of *D. suzukii* are characterized by a longer developmental time to reach the maximum size and to enter the pupal stage with respect to other species of the genus *Drosophila* and *D. melanogaster* in particular [74, 75]. A delay combined with urea alteration of protein synthesis [69] could strongly compromise eggs-to-pupae viability. This effect, combined with a lack of efficient detoxifying mechanisms confirmed by our study, explains well the incapacity *D. suzukii* to cope with high loads of urea.

The behavioural preference of *D. suzukii* for fresh fruits as oviposition substrate allows larvae to develop in an environment with small amounts of metabolic toxins. Our study shows that in the presence of high concentrations of urea and ammonia female oviposition is reduced, larval development is compromised, and detoxifying enzymes are less efficient. This outcome is compatible both with a relaxation of selective pressures on nitrogenous waste tolerance in *D. suzukii*, and with

negative selection induced by a trade-off with other traits. Further studies should clarify to which extent these scenarios contribute to the physiological, metabolic and behavioural specializations of *D. suzukii*.

Authors' Contributions

VB conceived the study and designed methodology; VB, AG, GB and MM collected the data; VB and AG and MM analysed the data; VB drafted the manuscript; VB, EV, AH and MM led the writing of the manuscript; EV and AH supervised the study. All authors contributed critically to the drafts and gave final approval for publication.

Competing interests

The authors declare that they do not have any conflict of interest.

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Highlights

- Susceptibility to nitrogenous compounds is greater in *D. suzukii* than in *D. melanogaster*
- Oviposition and larval survival are dramatically reduced by urea and ammonia in *D. suzukii*
- Larvae of *D. suzukii* show negative chemotaxis for ammonia volatiles
- Detoxifying enzymes (OAT and GSTs) are less efficient in *D. suzukii* larvae than in *D. melanogaster*