

1 **Evolutionary compromises to environmental toxins: ammonia and urea tolerance in**  
2 *Drosophila suzukii* and *Drosophila melanogaster*.

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15 aminotransferase, nitrogenous waste, evolutionary adaptation, pest species

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## 25 **Summary**

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27 The invasive species *Drosophila suzukii* has evolved morphological and behavioral adaptations to lay  
28 eggs under the skin of fresh fruits. This results in severe damage of a wide range of small and stone  
29 fruits, thus making this species a serious agricultural and economical threat.

30 *Drosophila suzukii* females typically lay few eggs per fruit, preferring not infested fruits. Hence  
31 larvae are exposed to a reduced amount of nitrogenous waste products. On the contrary, the innocuous  
32 *Drosophila melanogaster* lays eggs on fermented fruits already infested by conspecifics, with larvae  
33 developing in a crowded environment characterized by accumulation of nitrogenous waste such as  
34 ammonia and urea. Given these differences in oviposition and larval ecological niche, we expected  
35 different behavioral and physiological mechanisms in the two species to cope with nitrogenous waste.

36 We investigated the impact of different concentrations of ammonia and urea on fecundity and larval  
37 development in both species. Females and larvae of *D. suzukii* showed a greater sensitivity to high  
38 concentration of both compounds, with a dramatic decrease in fecundity and egg viability.

39 To better understand the pathways underlying these differences, we evaluated the effect on ornithine  
40 aminotransferase and glutathione-S-transferase, two enzymes involved in nitrogen metabolism and  
41 stress response that are expressed during larval development. Under ammonia and urea exposure, the  
42 expression of these enzymes was significantly reduced in *D. suzukii*.

43 The fact that *D. suzukii*'s shift from rotten to fresh fruit as oviposition and larval substrate resulted in  
44 less efficient detoxifying and excretory mechanisms represents a potential approach for its control.  
45 Fecundity and larval development are in fact dramatically impaired by nitrogen waste products. These  
46 findings can help in planning effective strategies of sustainable pest management that targets both  
47 females and larvae.

## 48 **Introduction**

49 In the last decade, growing concern has turned on the invasive pest *Drosophila suzukii*, a serious  
50 agricultural and economical threat (Bolda, Goodhue & Zalom 2010; De Ros *et al.* 2012). This species  
51 is native to Asia and has recently invaded western countries, with rapidly expanding range in America  
52 and Europe (Rota-Stabelli, Blaxter & Anfora 2013; Walsh *et al.* 2011). Differently from other  
53 *Drosophila* species, that attack overripe and decaying fruits, females of *D. suzukii* lay eggs under the  
54 skin of fresh fruits, through a serrated ovipositor. Therefore, larval development and exposure to  
55 pathogens result in damage of a wide range of small and stone fruits (Goodhue *et al.* 2011; Rota-  
56 Stabelli, Blaxter & Anfora 2013). To date, most research on *D. suzukii* has focused on adults (Crava *et*  
57 *al.* 2016; Hamby & Becher 2016; Gong *et al.* 2016; Keeseey, Knaden & Hansson 2015; Rossi-Stacconi  
58 *et al.* 2016), with little attention to larvae. A more comprehensive understanding of the ecology and  
59 biology of this species is important to develop management strategies and to successfully minimize its  
60 spread and impact (Cini 2012; Dreves 2011).

61 The innocuous *Drosophila melanogaster* lays eggs in rotten fruits and larvae develop in a crowded  
62 environment, rich in bacteria, mold, and yeast (Becher *et al.* 2012; Zhu, Park & Baker 2003). Females  
63 of this species have a gregarious tendency in selecting the oviposition site and prefer to lay eggs  
64 where other larvae are present (Sarin & Dukas 2009; del Solar & Palomino 1966). High larval density  
65 combined with microorganism metabolic activity and protein-rich microbial community (Begon 1982;  
66 Chandler, Eisen & Kopp 2012) result in accumulation of nitrogen waste products such as ammonia  
67 and, at lower extent, urea (Botella *et al.* 1985; Mueller 1995), that are relatively toxic when  
68 concentrated in organism tissues (David *et al.* 1999; Maas, Seibel & Walsh 2012). In *D.*  
69 *melanogaster*, high concentrations of dietary urea and ammonia have been associated with a decrease  
70 in female fecundity (e.g. Joshi *et al.* 1997, Min *et al.* 2013), a decline in egg-to-adult viability, as well  
71 as an increase in developmental time (Borash *et al.* 2000; Shiotsugu *et al.* 1997). Due to the limited

72 mobility of larvae (Durisko *et al.* 2014; Philippe *et al.* 2016), behavioral avoidance cannot prevent  
73 larval exposure to environmental toxins accumulating in the food, but physiological mechanisms help  
74 larvae to cope with toxic compounds (Heinstra *et al.* 1989; Wilson 2001). *D. melanogaster*  
75 populations reared under crowded larval conditions develop greater competitive ability (Borash *et al.*  
76 2000; Sarangi *et al.* 2016) and increased resistance to both urea and ammonia (Borash *et al.* 2000;  
77 Etienne, Fortunat & Pierce 2001). While the response of *D. melanogaster* to high levels of urea and  
78 ammonia has been studied (Borash *et al.* 2000; Etienne, Fortunat & Pierce 2001), little is known on  
79 the effects in *D. sukuzii*. This species occupies a unique ecological niche compared to other  
80 drosophilids, since larval development occurs in fresh fruits (Rota-Stabelli, Blaxter & Anfora 2013),  
81 rich in water (Ishida, Koizumi & Kano 1994) and relatively poor in microorganisms, due to the skin  
82 barrier (Tournas & Katsoudas 2005). Moreover, females of *D. sukuzii* tend to lay few eggs per fruit  
83 (Burrack *et al.* 2013; Yu, Zalom & Hamby 2013), resulting in a moderate larval density and, as a  
84 consequence, a low level of waste products. Thus, we hypothesised to observe differences in the  
85 behavioral and physiological responses of *D. melanogaster* and *D. sukuzii* to nitrogenous waste  
86 products, as an effect of adaptation to different ecological niches  
87 To evaluate tolerance capacity for nitrogenous compounds in *D. sukuzii* compared to *D.*  
88 *melanogaster*, we investigated the effect of different concentrations of urea and ammonia on female  
89 fecundity, under no-choice and choice conditions, and on larval development in both species. We  
90 further studied potential differences in the expression of ornithine aminotransferase and glutathione-S-  
91 transferase, enzymes involved in metabolic and detoxifying pathways. Ornithine aminotransferase is  
92 an enzyme highly expressed in third instar larvae of *D. melanogaster*, playing a crucial role in amino  
93 acids metabolism and nitrogen homeostasis (Ventura *et al.* 2008; Yoshida, Juni & Hori 1997).  
94 Glutathione-S-transferase is an enzyme involved in insect resistance to endogenous and xenobiotic

95 compounds and in protection against oxidative stress (Hamby *et al.* 2013; Perry, Batterham & Daborn  
96 2011), which is expressed in the larval midgut of *D. melanogaster* (Li *et al.* 2008).

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## 98 **Materials and methods**

### 99 *Insect strains and rearing*

100 We used adult flies of *Drosophila melanogaster* from 50 lines of the DGRP population (Mackay *et al.*  
101 2012), a collection of inbred isofemale lines originally collected in Raleigh, US (Ayroles *et al.* 2009).  
102 Lines were obtained from the Bloomington Drosophila Stock Center (Indiana University,  
103 Bloomington, US) and represent a spectrum of natural occurring genetic variation. The same  
104 isofemale lines were tested in all treatment groups. The *Drosophila suzukii* flies used in this study  
105 were originally collected in the Trentino area, Italy, and maintained under the same laboratory  
106 conditions of the *Drosophila melanogaster* population for several generations. All flies were raised on  
107 a standard Drosophila diet (see Appendix 1. in Supporting Information) at  $25 \pm 1$  °C with  $65 \pm 1\%$   
108 relative humidity with a light:dark cycle of 14:10 h.

109

### 110 *Chemicals*

111 Ammonium chloride (NH<sub>4</sub>Cl; purity $\geq$ 99.5%) was purchased from Carl Roth (Karlsruhe, Germany);  
112 urea (ACS reagent 99-100.5%) was purchased from Sigma-Aldrich (St. Louis, USA). Propionic acid  
113 was obtained from Carlo Erba Reagents (Milan, Italy) and methyl 4-hydroxybenzoate (99%) was  
114 purchased from Acros Organics (Morris Plains, US).

115 Ammonium chloride and urea (pH  $\sim$ 5.5) were added to the standard food medium, after it had cooled  
116 down to 48 °C, and antimicrobial agents were added to the food. In order to homogenize the mixture,  
117 the supplemented media was placed upon a magnetic stirring apparatus, which rapidly stirred the  
118 mixture as it was dispensed into polypropylene vials (25 x 95 mm) or Ø 90 mm petri dishes.

119 *Female fecundity and larval development in a no-choice assay*

120 Newly eclosed flies were transferred to fresh food vials, and were maintained under standard  
121 conditions until tested. Females (5-6 days old) of about the same size were individually assigned,  
122 using light CO<sub>2</sub> anesthesia, to a vial with 5 ml of standard diet with one of the following supplements:  
123 urea at 25 mM (UL=urea low concentration) or 250 mM (UH=urea high concentration) or ammonium  
124 chloride at 25 mM (AL=ammonia low concentration) or 250 mM (AH=ammonia high concentration),  
125 or no supplements added to the food (CTRL). A pinch of active yeast was sprinkled on the food to  
126 stimulate oviposition. After 24 h, females were removed from the vials and eggs counted under an  
127 optical microscope. In this assay we quantified fecundity as the number of eggs laid within 24 hours.  
128 One day later, presence of larvae and their conditions (alive/dead, 1<sup>st</sup>/2<sup>nd</sup> instar) were recorded. After  
129 the first pupa appeared, pupae were counted and number and times of pupation were recorded twice  
130 per day, at 10 a.m. and 5 p.m., for the following five days. As development was expected to be slower  
131 on both ammonia- and urea-supplemented foods, the scores continued during the following days. The  
132 larval developmental time has been considered as the number of hours occurring from hatching to  
133 pupation. From the beginning of adult emergence, flies were collected, using CO<sub>2</sub> anesthesia, and the  
134 number of adults and their sex were recorded for each vial. Data were collected every morning (10  
135 a.m.) and checks ceased when in a period of 48 h no more flies had emerged from a given population.  
136 The experiment was replicated 6 times for a total sample size of 32 females for each treatment group.  
137 Larval development (time to pupation and number of pupae) and viability (eggs-to-pupae and egg-to-  
138 adults) were evaluated on 25 vials for each treatment group.

139

140 *Female fecundity in a choice assay*

141 Oviposition behavior was tested both under no-choice and dual choice conditions to control for  
142 interaction between environmental cues and treatment (Lihoreau et al. 2016; Sheeba, Madhyastha &

143 Joshi 1998). Flies were tested in a cage where both a control and an experimental medium were  
144 provided. The oviposition substrates consisted of Ø 90 mm petri dishes filled with 20 ml of standard  
145 food (CTRL), or with standard food and one of the following supplements: urea at 25 mM (UL) or  
146 250 mM (UH), or ammonium chloride at 25 mM (AL) or 250 mM (AH). Sprinkles of active yeast  
147 were added to the food to stimulate oviposition.

148 Freshly eclosed flies were transferred to fresh food vials and were maintained at standard conditions  
149 until tested. Ten females (5-6 days old) of each species were collected, using CO<sub>2</sub> anesthesia, and  
150 transferred to a bug dorm insect-rearing cage (30x30x30 cm; BugDorm-1, MegaView Science  
151 Taichung, Taiwan) with the control petri dish in one corner, and the petri dish with supplemented  
152 food on the opposite side. After 24 hours, the petri dishes were collected and the eggs counted under  
153 an optical microscope. In this assay we quantified fecundity as the number of eggs laid in 24 hours.  
154 The experiment included 10 replicates for each condition.

155

156 *Semiquantitative analysis of the expression of genes coding for metabolic and detoxifying enzymes*

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

161 A total sample size of about 15 larvae per treatment group was collected. We had no samples from the  
162 AH group, because no larvae arrived at the third instar stage. Total RNA extracted from samples  
163 using a TRIreagent:chloroform (Sigma-Aldrich, St. Louis, US) extraction, performed according to the  
164 manufacturer's instructions. RNA samples were quantified using a Nanodrop spectrophotometer  
165 (ThermoFisher Scientific, Waltham, US) and reverse-transcribed to cDNA using the Revert Aid First  
166 Strand cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, US) with specific primers (table

167 S1.). PCR products were analysed by gel electrophoresis in a 1% ethidium bromide-stained agarose  
168 gel. Gel documentation was collected using a “Gel Doc XR”, digitally evaluated with “Quantity One”  
169 (Bio-Rad Lab., Milano, Italy) and normalized to the correspondent signals for tubulin.

170

### 171 *Statistical analysis*

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

179 The number of eggs laid in the preference test was analyzed with a Wilcoxon signed-rank test to  
180 compare conditions (CTRL versus Treatment). A *p*-value of less than 0.05 was considered significant.

181 All statistical analyses were carried out using SPSS version 17 (IBM, Armonk, US).

182

## 183 **Results**

### 184 *Female fecundity and larval development in no-choice assay*

185 No significant difference in female fecundity was observed at low concentration of ammonia and urea  
186 with respect to the control. At high concentration, ammonia (AH) reduced significantly the number of  
187 eggs in *D. melanogaster* ( $\chi^2(4)=22.89$ ,  $p<0.001$ , see Fig. 1a). However, high concentrations of both  
188 urea (UH) and ammonia (AH) decreased female fecundity in *D. sukukii* ( $\chi^2(4)=70.77$ ,  $p<0.001$ , see  
189 Fig. 1b). When comparing the effects between species, *D. sukukii* females showed a greater sensitivity  
190 to the treatment (AH:  $U=195$ ,  $p<0.001$ ; UH:  $U=237$ ,  $p<0.001$ , see Appendix 1).

191 Twenty-four hours after the start of hatching, first and second instar larvae were present in all  
192 treatment groups except for AH of *D. suzukii* (Fig. 2a, b). In this condition, larvae died soon after  
193 hatching, and many of them were observed on the wall of the vial, suggesting a possible escaping  
194 behavior. In *D. melanogaster*, larval development was affected by exposure to high concentration of  
195 urea ( $140 \pm 3$  hours) and ammonia ( $130 \pm 2$  hours) with respect to the control ( $106 \pm 2$  hours), with a  
196 significant delay in pupation (Treatment:  $F(4,119)=68.24$ ,  $p<0.001$ ). These durations did not depend  
197 on the number of eggs (Treatment\*Number of eggs:  $F(4,119)=1.54$ ,  $p=0.19$ , see Appendix 2).  
198 However, when considering eggs-to-pupae viability, a significant decrease was observed only in UH  
199 ( $\chi^2(4)=25.76$ ,  $p=0.01$ , see Fig. 3a), while no difference was found under ammonia exposure. In *D.*  
200 *suzukii*, the effect of the treatment was even stronger, with no pupae in the AH group, and only a few  
201 pupae under high concentration of urea ( $\chi^2(4)=78.16$ ,  $p<0.001$ , see Fig 3b), accompanied by a visible  
202 delay.

203 Finally, pupariation and emergence of adult flies was strongly impaired by a high concentration of  
204 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  
205 Fig. 3c, d), while eggs-to-adults viability was not significantly affected by high concentration of  
206 ammonia in *D. melanogaster* (see Fig. 3c). No difference in adult sex ratio was observed among  
207 groups (*D. melanogaster*:  $\chi^2(4)=1.69$ ,  $p=0.8$ ; *D. suzukii*:  $\chi^2(2)=1.72$   $p=0.4$ ).

208

### 209 *Female fecundity in a choice assay*

210 Oviposition preference was tested in a choice assay between experimental substrates (supplemented  
211 with urea or ammonia) and control substrates. Females did not show significant egg laying  
212 preferences between control food and food supplemented with low concentration of urea (*D.*  
213 *melanogaster*  $Z(9)=-0.76$ ,  $p=0.5$ , *D. suzukii*  $Z(9)=-1.78$ ,  $p=0.08$ ) and ammonia (*D. melanogaster*  
214  $Z(9)=-1.07$ ,  $p=0.3$ , *D. suzukii*  $Z(9)=-1.63$ ,  $p=0.1$ , see Fig. 4a,b) When the concentration of ammonia

215 was increased, females displayed a strong aversion and only about 11% of the eggs were laid in the  
216 AH substrate in *D. melanogaster* ( $Z(9)=-2.70$ ,  $p<0.01$ , see Fig. 4a) and 9% in *D. sukukii* ( $Z(9)=-2.80$ ,  
217  $p<0.01$ , see Fig. 4b). Interestingly, oviposition preference was unaffected by high concentration of  
218 urea in *D. melanogaster* (53% of the eggs were laid in the UH substrate), whereas *D. sukukii* females  
219 laid significantly less in the UH site (29%) than in the urea-free medium ( $Z(9)=-2.09$ ,  $p<0.05$ , see Fig.  
220 4a,b).

221

### 222 *Expression of genes coding for metabolic and detoxifying enzymes*

223 Semi-quantitative RT-PCR analysis of the expression of OAT, gsdD2 and gsdD4 evidenced a different  
224 expression pattern in the two species. Indeed, despite a common constitutive expression of the three  
225 genes in both the species observed in the control specimens, the expression of OAT, gsdD2 and gsdD4  
226 resulted highly increased in *D. melanogaster* respect to *D. sukukii* after the exposure to ammonia and  
227 urea.

228 In particular, a significant increase of the OAT has been observed in *D. melanogaster* in the presence  
229 of urea, both at low and high concentration ( $\chi^2(4)=12.01$ ,  $p<0.05$ ), whereas no induction was evident  
230 in *D. sukukii* ( $\chi^2(3)=6.59$ ,  $p=0.07$ ; Figs. S3a, 5a). Differently from this pattern, gsdD2 resulted highly  
231 induced in the presence of ammonium at high concentration in *D. melanogaster* ( $\chi^2(4)=13.06$ ,  
232  $p<0.001$ ), whereas no significant difference has been detected at both concentrations of urea, neither  
233 for both treatments in *D. sukukii* (Figs. S3.b, 5b). Lastly, gsdD4 showed an expression pattern similar  
234 to gsdD2, with a significant induction under AH exposure in *D. melanogaster* ( $\chi^2(4)=13.08$ ,  $p<0.001$ ;  
235 Figs. S3.c, 5c).

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237

238

## 239 **Discussion**

240 We compared the effect of low and high concentrations of urea and ammonia on fecundity and larval  
241 development in *D. melanogaster* and *D. sukukii*. Our data show that fecundity is negatively affected in  
242 both species by nitrogenous waste products, but significantly greater effects have been observed in *D.*  
243 *sukukii*. When exposed to high concentrations of nitrogen compounds, female fecundity was more  
244 negatively affected by ammonia than by urea in both species, however, while *D. melanogaster*  
245 experienced a 50% fecundity reduction, *D. sukukii* fecundity was reduced by 70%.

246 Previous studies have documented the relevant role of ammonia as a sensory cue for female  
247 orientation and site selection in *D. melanogaster* (Delventhal *et al.* 2017; Min *et al.* 2013) and in other  
248 insect species (Bateman & Morton 1981; Kendra *et al.* 2005). Here, we have documented for the first  
249 time the greater sensitivity of *D. sukukii* pest to ammonia compared to *D. melanogaster*.

250 The stronger response observed in *D. sukukii* could derive from a greater olfactory and/or gustatory  
251 sensitivity to ammonia (e.g. Delventhal *et al.* 2017; Menuz *et al.* 2014). In fact, the concentration of  
252 volatiles associated with different maturation stages can greatly affect olfactory choices in *D.*  
253 *melanogaster* (e.g. Versace *et al.* 2016; Zhu, Park & Baker 2003). Moreover, recent studies have  
254 shown how, during fruit maturation, changes occurring in the composition and concentration of  
255 volatiles can provide different cues for *D. sukukii* and *D. melanogaster* (Keeseey, Knaden & Hansson  
256 2015; Krause Pham & Ray 2015). Further studies should clarify the role of sensory cues in  
257 determining the greater sensitivity of *D. sukukii* to ammonia.

258 In addition, we observed a significant difference between species also regarding a typically non-  
259 volatile compound as urea, resulting in a greater reduction of fecundity in *D. sukukii* compared to *D.*  
260 *melanogaster*. While the number of laid eggs was only slightly decreased in *D. melanogaster* (Joshi *et*  
261 *al.* (1998) observed stronger effects at higher concentrations), *D. sukukii* experienced a 60% decrease

262 in the number of laid eggs. This suggests again a higher repellent effect by nitrogenous waste in *D.*  
263 *suzukii*.

264 Interestingly, we observed differences in fecundity of *D. melanogaster* under urea exposure between  
265 choice (UH; 53% of total number of eggs) and no-choice (UH; 44% of total CTRL and UH eggs)  
266 assays suggesting that the presence of more cues or choices can ameliorate the inhibitory effect of  
267 urea on fecundity. This modulation though was not found in *D. suzukii* (choice, UH; 29% of total  
268 number of eggs; no choice, UH; 28% of total CTRL and UH eggs). The presence of a high level of  
269 ammonia instead caused a greater reduction in fecundity in the choice assay (*D. melanogaster*, 11%;  
270 *D. suzukii*, 9%) than in the no-choice assay (*D. melanogaster*, 37%; *D. suzukii*, 24%) in both species.

271 Several studies have shown that *D. melanogaster* tends to hold eggs in absence of quality oviposition  
272 media (Joseph & Heberlein 2012; Schwarz, Durisko & Dukas 2014; Yang *et al.* 2008). However, *D.*  
273 *melanogaster* was found to lay eggs in substrate with potentially toxic chemicals when a harmless  
274 alternative is available (Azanchi, Kaun & Heberlein 2013).

275 We argue that the documented aversion of *D. suzukii* females for nitrogenous products might be an  
276 adaptive behavior to avoid substrates that can negatively affect larval fitness in this species more than  
277 in *D. melanogaster*. In fact, in *D. melanogaster* egg-laying behavior is influenced by the presence and  
278 density of larvae (Sarin & Dukas 2009; del Solar & Palomino 1966), and larval waste has been shown  
279 to modulate this effect (Aiken & Gibo 1979; Joshi *et al.* 1998). Along this line, we show that  
280 nitrogenous waste products affect *D. suzukii* larvae more negatively than *D. melanogaster* larvae. In  
281 our study, larval exposure to ammonia and urea resulted in high toxicity, showing a significant  
282 difference between species in the capacity to cope with the detrimental effects of these compounds. In  
283 *D. suzukii*, larvae were not able to survive in the presence of high concentration of ammonia, 100% of  
284 mortality was observed soon after hatching. On the other hand, larvae of *D. melanogaster* showed a  
285 delayed development, but viability remained comparable to the control. High levels of urea affected

286 late larval stages in both species, interfering with larval survival as well as developmental time and  
287 pupation process, but with a stronger detrimental effect in *D. suzukii*.  
288 Differently from many toxic chemicals that attack one or a few targets (Morton 1993; Russell *et al.*  
289 1990), ammonia and urea are able to impact the whole organism (Cagnon & Braissant 2007; David *et*  
290 *al.* 1999; Yancey & Somero 1979). For this reason, we observe mechanisms to respond and resist the  
291 globally detrimental effects of these toxins. Strategies can involve uptake reduction, detoxifying  
292 pathways, as well as efficient excretory mechanisms (O'Donnell & Donini 2017). Interference with  
293 any of these processes could compromise an organism' survival (Belloni & Scaraffia 2014; Scaraffia  
294 *et al.* 2005). Previous work showed that adaptation to urea and ammonia results in decreased larval  
295 feeding rate and longer developmental time in *D. melanogaster* (Borash *et al.* 2000; Botella *et al.*  
296 1985). This could explain our data at high levels of ammonia, where a significant increase in  
297 developmental time was associated with larval viability in *D. melanogaster*. However, in *D. suzukii*  
298 compensation mechanisms failed, causing ammonia levels to rapidly increase, resulting in an acute  
299 intoxication. This hypothesis is supported by significant metabolic and detoxification differences  
300 observed between the two species, where ornithine aminotransferase as well as glutathione-S-  
301 transferase gene expression increased under high levels of ammonia and urea in *D. melanogaster*,  
302 whereas no induction was evident in *D. suzukii*. The ornithine aminotransferase enzyme plays a  
303 crucial role in amino acids metabolism and nitrogen homeostasis (Ventura *et al.* 2008; Yoshida, Juni  
304 & Hori 1997), while glutathione-S-transferase is highly expressed in response to xenobiotics and  
305 oxidative stress (Hamby *et al.* 2013; Nguyen *et al.* 2016). Alteration in their expression can be  
306 associated with inefficient detoxification and reduction of tolerance capacity to environmental  
307 stressors (Claudianos *et al.* 2006; Nguyen *et al.* 2016; Passador-Gurgel *et al.* 2007; Seiler 2000). This  
308 explains the mortality observed in *D. suzukii* when first instar larvae were faced with high level of  
309 ammonia in the medium. Urea can interfere with important cell processes, act as protein denaturant,

310 and reduce enzyme activity (Somero & Yancey 1997; Yancey 1992; Yancey & Somero 1979),  
311 resulting in development delay and larval stop (Botella *et al.* 1985; Shiotsugu *et al.* 1997). *Drosophila*  
312 species are not familiar with this compound, due to the fact that they are not able to produce it,  
313 suggesting a lack of physiological mechanism to handle it. However, we observed a similar enzymatic  
314 response under urea and ammonia exposure, in agreement with previous studies describing the  
315 evolution of cross-tolerance between these stress traits (Borash *et al.* 2000; Borash & Shimada 2001).  
316 Developmental delay and reduction in feeding rate are adaptive strategies developed to reduce urea  
317 uptake in *D. melanogaster* larvae and favour toxin resistance (Borash *et al.* 2000; Botella *et al.* 1985;  
318 Etienne, Fortunat & Pierce 2001). Larvae of *D. sukukii* are characterized by a longer developmental  
319 time to reach the maximum size and to enter the pupal stage (Hamby *et al.* 2016; Wegman, Ainsley &  
320 Johnson 2010). A delay combined with urea alteration of protein synthesis (Somero & Yancey 1997)  
321 could strongly compromise eggs-to-pupae viability. This effect combined with a lack of efficient  
322 detoxifying mechanisms confirmed by our study, explains well the incapacity *D. sukukii* to cope with  
323 high load of urea.

324 In the wild, the adaptation of *D. sukukii* to fresh fruits as oviposition substrate, has allowed larvae to  
325 develop in a safer and healthier environment. However, our study shows how metabolic adaptations to  
326 fresh food have resulted in less efficient detoxifying and excretory mechanisms. Further studies are  
327 necessary to better understand the interaction between female fecundity and nitrogenous compounds  
328 for possible use of these chemicals as repellants or reproductive toxicants in natural conditions.  
329 Moreover, more attention needs to be directed to the larval stage in *D. sukukii*, which is highly  
330 affected by environmental variations and shows important adaptations to a diverse ecological niche  
331 with respect to *D. melanogaster*. Our findings, in fact, suggest the possibility to significantly  
332 compromise larval fitness and survival in the pest species via the exposure to environmental  
333 compounds.

334 **Authors' Contributions**

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

339

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544 **Figures**

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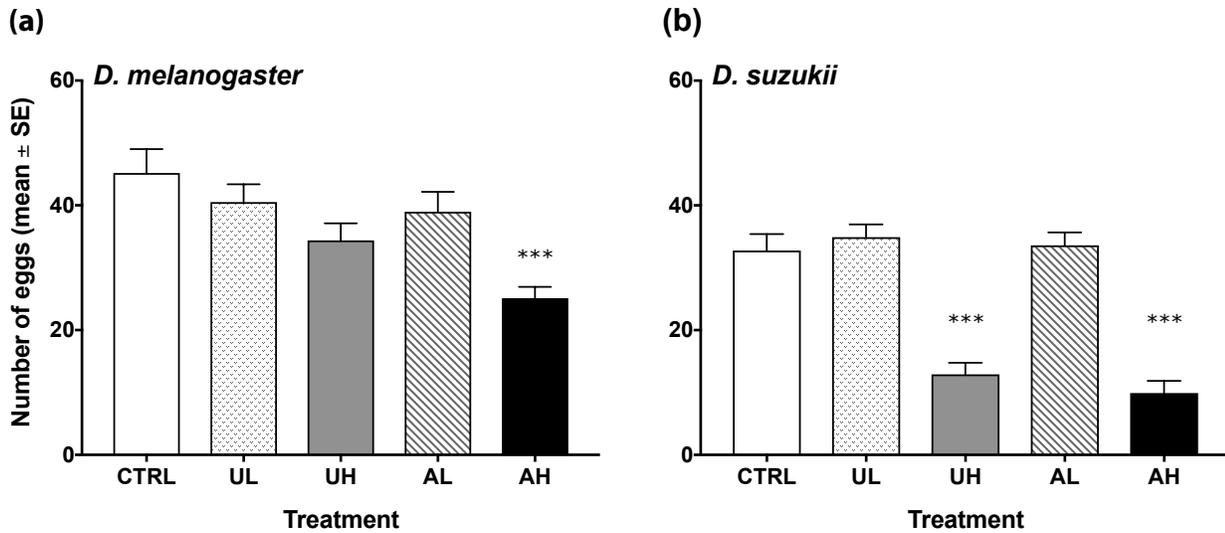
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555 **Figure 1.** Eggs laid during a 24-hour period by females exposed to standard food and standard  
556 food supplemented with urea and ammonia. CTRL: standard food; UL: standard food with 25 mM  
557 of urea; UH: standard food with 250 mM of urea; AL: standard food with 25 mM of ammonium  
558 chloride; AH: standard food medium with 250 mM of ammonium chloride. Mean ± standard error  
559 (SE) are shown, \*\*\*= $p < 0.001$ .

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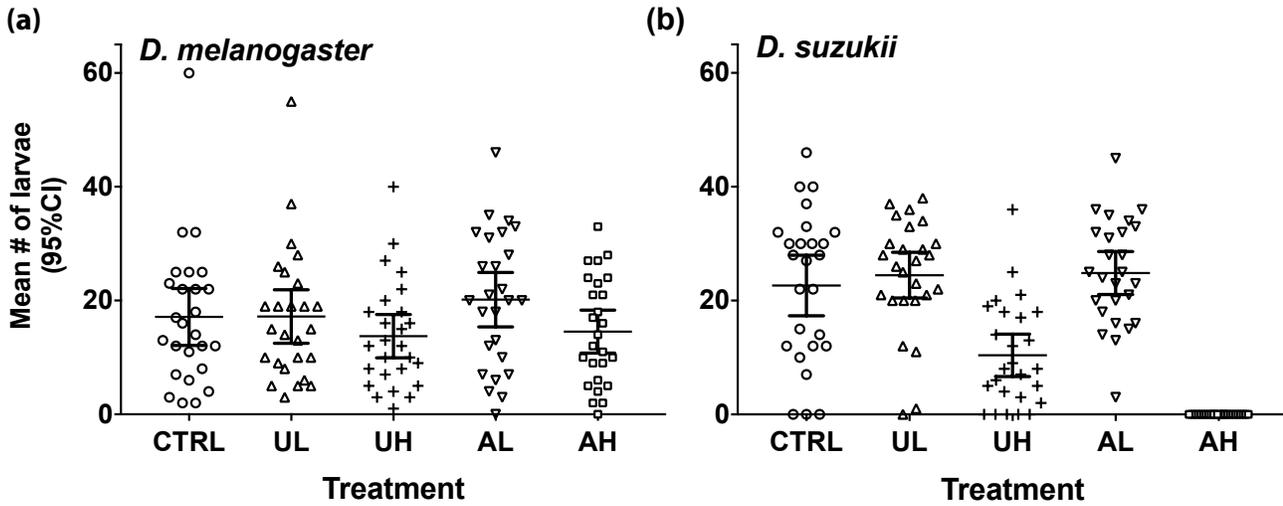
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**Figure 2.** Estimated number of alive larvae 24 hours after hatching in standard food and in standard food supplemented with urea or ammonia. CTRL: standard food; UL: standard food with 25 mM of urea; UH: standard food with 250 mM of urea; AL: standard food with 25 mM of ammonium chloride; AH: standard food medium with 250 mM of ammonium chloride. Horizontal bars indicate mean and 95% confidence interval.

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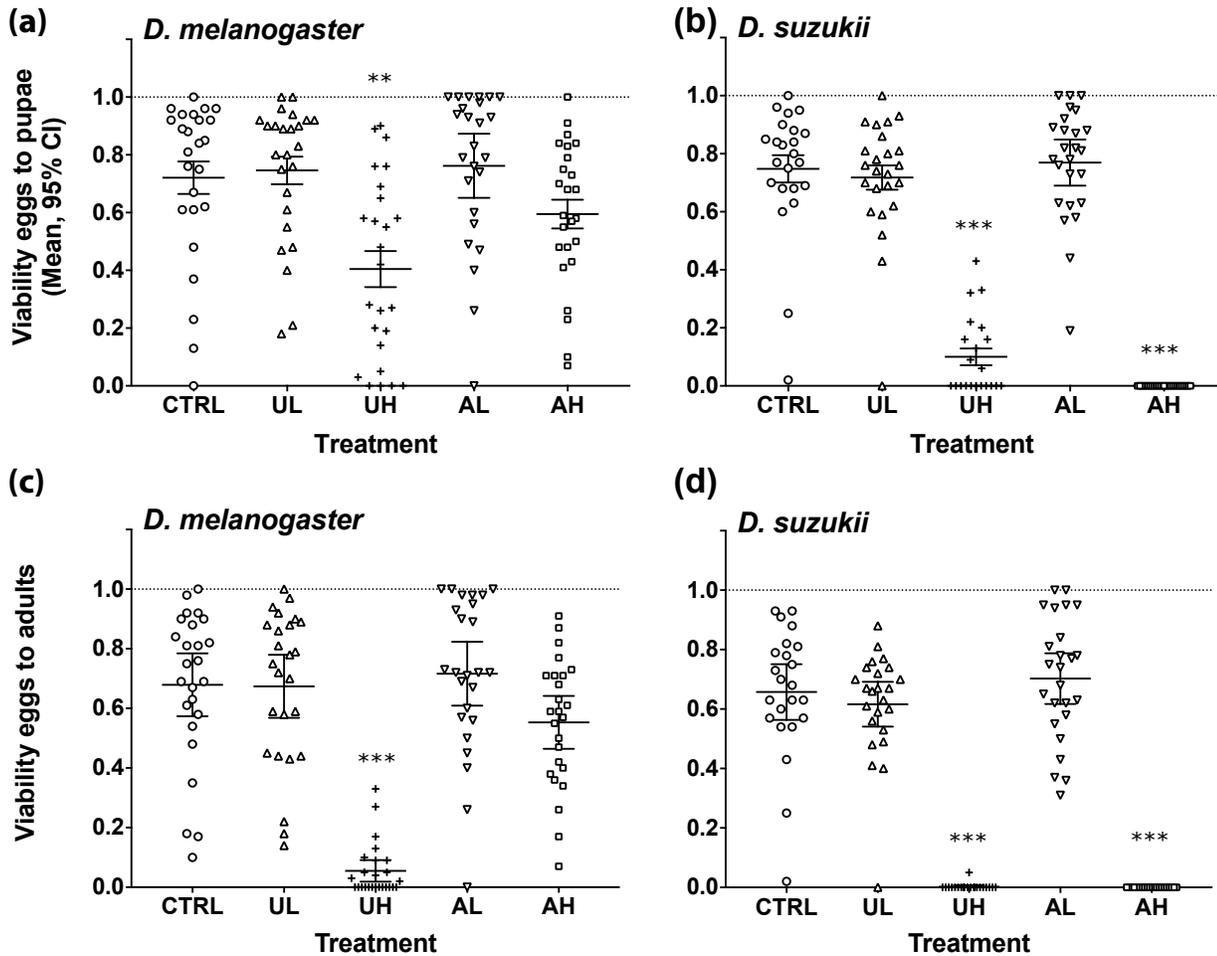
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**Figure 3.** Viability eggs-to-pupae (a, b) and eggs-to-adults (c, d) in standard food and standard food supplemented with dietary urea and ammonia. CTRL: standard food; UL: standard food with 25 mM of urea; UH: standard food with 250 mM of urea; AL: standard food with 25 mM of ammonium chloride; AH: standard food medium with 250 mM of ammonium chloride. Horizontal bars indicate mean and 95% confidence interval. \*\*= $p < 0.01$ , \*\*\*= $p < 0.001$ .

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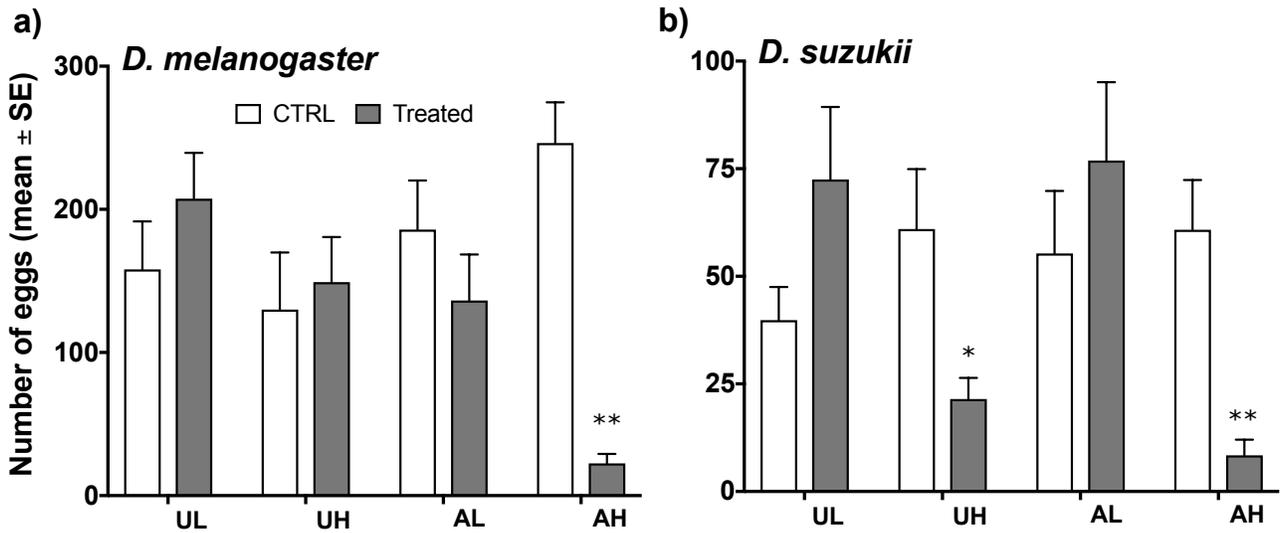
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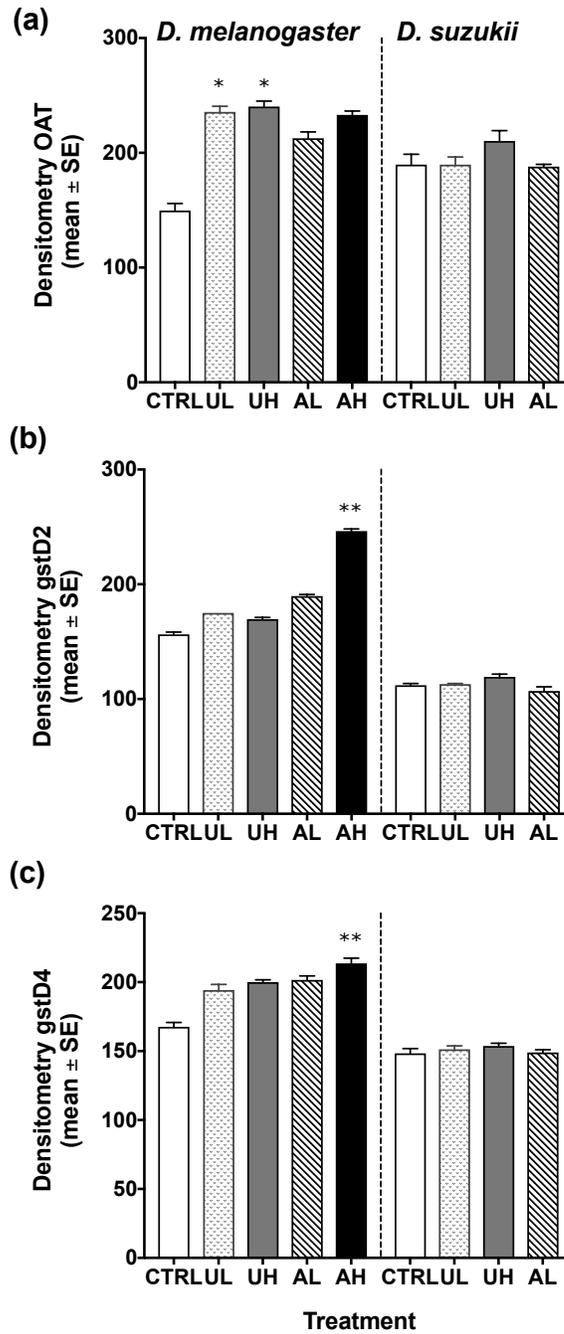
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**Figure 4.** Oviposition site preference for standard food supplemented with dietary urea and ammonia (Treated) against non-supplemented standard food (CTRL). UL: standard food with 25 mM of urea; UH: standard food with 250 mM of urea; AL: standard food with 25 mM of ammonium chloride; AH: standard food medium with 250 mM of ammonium chloride. Mean  $\pm$  standard error (SE) are shown.

\*= $p < 0.5$ , \*\*= $p < 0.01$ .

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660 **Figure 5.** Semi-quantitative RT-PCR analysis of genes coding for OAT (a), gstD2 (b), and gstD4 (c)  
661 in *D. melanogaster* and *D. suzukii*, in control specimens (CTRL), in the presence of low (UL) and  
662 high (UH) concentrations of urea and in the presence of low (AL) and high (AH) concentrations of  
663 ammonium chloride. Data are mean ± standard error (SE). \* = p < 0.05, \*\* = p < 0.01.