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1 Full Research Paper

2

3 **The multilevel antibiotic-induced perturbations to biological systems: early-life exposure**  
4 **induces long-lasting damages to muscle structure and mitochondrial metabolism in flies**

5

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21

22 **AUTHOR CONTRIBUTIONS**

23 Conceptualization: H.Y., D.R., A.A.M.; Data analysis: A.A.M., H.Y., D.R.; Funding  
24 acquisition: H.Y., D.R.; Writing - original draft: D.R., A.A.M., H.Y.; Writing - review &  
25 editing: D.R., A.A.M.

26

27 **CONFLICT OF INTEREST**

28 The authors declare no conflict of interest.

29

30 **Abstract**

31 Antibiotics have been increasingly used over the past decades for human medicine, food-  
32 animal agriculture, aquaculture, and plant production. A significant part of the active  
33 molecules of antibiotics can be released into the environment, in turn affecting ecosystem  
34 functioning and biogeochemical processes. At lower organizational scales, these substances  
35 affect bacterial symbionts of insects, with negative consequences on growth and development  
36 of juveniles, and population dynamics. Yet, the multiple alterations of cellular physiology and  
37 metabolic processes have remained insufficiently explored in insects. We evaluated the  
38 effects of five antibiotics with different mode of action, i.e. ampicillin, cefradine,  
39 chloramphenicol, cycloheximide, and tetracycline, on the survival and ultrastructural  
40 organization of the flight muscles of newly emerged blow flies *Chrysomya albiceps*. Then, we  
41 examined the effects of different concentrations of antibiotics on mitochondrial protein  
42 content, efficiency of oxidative phosphorylation, and activity of transaminases (Glutamate  
43 oxaloacetate transaminase and glutamate pyruvate transaminase) and described the cellular  
44 metabolic perturbations of flies treated with antibiotics. All antibiotics affected the survival of  
45 the insects and decreased the total mitochondrial protein content in a dose-dependent manner.  
46 Ultrastructural organization of flight muscles in treated flies differs dramatically compared to  
47 the control groups and severe pathological damages/structures disorganization of  
48 mitochondria appeared. The activities of mitochondrial transaminases significantly increased  
49 with increased antibiotic concentrations. The oxidation rate of pyruvate+proline from isolated  
50 mitochondria of the flight muscles of 1-day-old flies was significantly reduced at high doses  
51 of antibiotics. In parallel, the level of several metabolites, including TCA cycle intermediates,  
52 was reduced in antibiotics-treated flies. Overall, antibiotics provoked a system-wide alteration  
53 of the structure and physiology of flight muscles of the blow fly *Ch. albiceps*, and may have  
54 fitness consequences at the organism level. Environmental antibiotic pollution is likely to

55 have unwanted cascading ecological effects of insect population dynamics and community  
56 structure.

57

58 **KEYWORDS**

59 Antibiotics; eukaryote; insect flight muscles; mitochondrial dysfunction; metabolomics;  
60 energy metabolism

61

## 62 INTRODUCTION

63 The use of pharmaceuticals has been consistently developed over the past decades (Aminov,  
64 2017), and antibiotics have revolutioned human medicine. Antibiotics are also highly  
65 employed for food-animal agriculture, aquaculture and plant production (Witte, 2000; Le et  
66 al., 2005; Cabello, 2006), for disease prevention, or for promoting growth and feed efficiency  
67 of livestock animals (Gaskins et al., 2002). However, the use of antibiotics is not without  
68 posing significant problems (Finley et al., 2013, Larsson 2014; Rico et al., 2014), and  
69 alternatives to these growth promoters are increasingly discussed (Seal et al., 2013). Besides  
70 antibiotic resistance (Neu, 1992; Laxminarayan et al., 2013), the adverse effects of antibiotics  
71 on environmental ecosystems is a rapidly emerging concern. Andersson & Hughes (2014)  
72 suggested that up to 80% of the active substances of antibiotics can be released into the  
73 environment. Several antibiotics are not degraded within the gut of livestock animals, and the  
74 several pathways that permit the wide-scale dissemination of these drugs (Sarmah et al., 2006;  
75 Chang et al., 2015) rapidly expand the antibiotic load in the environment (Perry et al., 2014;  
76 Amos et al., 2015). The effects of antibiotics on ecosystems and biogeochemical processes  
77 have thus become a major research topic (Roose-Amsaleg & Laverman, 2016).

78

79 Antibiotics are present in most ecosystems (Daghrir & Drogui, 2013), often as mixtures.  
80 Several antibiotics (tetracyclines) are widely used as crop protectants (McManus et al., 2002;  
81 Chang et al., 2015), but their exact ecological impacts remain insufficiently explored.  
82 Antibiotics have different modes of actions to kill or limit growth of bacteria: they can  
83 inhibit/regulate metabolism of nucleic acids (Floss & Yu, 2005), protein biosynthesis (Walsh,  
84 2000; Siibak et al., 2011; Wilson, 2014), activities of certain enzymes (Kohanski et al., 2010),  
85 or induce toxic metabolic perturbations (Belenky et al., 2015). Antibiotics are most efficient  
86 when cells of the microorganisms are multiplying (Stewart & Costerton, 2001), and can

87 particularly affect bacterial symbionts of organisms, with negative consequences on growth  
88 and development of juveniles (Kafil et al., 2013).

89

90 Insects are likely to be exposed to environmentally released antibiotics, and more  
91 particularly tetracyclines. Tetracyclines are highly persistent into the environment, and can  
92 reach very high amounts into winter manure, up to 43 and 183 mg kg<sup>-1</sup> for tetracycline and  
93 oxytetracycline, respectively (Hu et al. 2010), and up to 200 µg kg<sup>-1</sup> for tetracycline in the  
94 first layers of the soil (Hamscher et al. 2002). Surprisingly, most of the studies have focused  
95 on the fitness consequences of adding of these drugs to the food of laboratory cultured insects  
96 (Büyükgüzel & Kalender, 2008; Ridley et al., 2013; Xu et al., 2016). At low concentrations of  
97 ciprofloxacin (3.33 to 13.33 µg/mL), the development of the fly *Chrysomia putoria* was not  
98 altered (Ferraz et al., 2014), while addition of streptomycin to the food at concentrations of  
99 0.03 to 0.15% increased the growth of the larvae of the butterfly *Spodoptera litura*, without  
100 affecting its survival (Thakur et al., 2016). Dickel et al. (2016) reported that long-term  
101 exposure to prophylactic treatments (fumagillin) negatively affects the number of eggs laid by  
102 females of the wood tiger moth *Parasemia plantaginis*. Interestingly, metabolic perturbations  
103 provoked by antibiotics to insects, and their end-target consequences, have remained  
104 unexplored.

105

106 Bacteria and mitochondria share many similarities in terms of ribosomal machinery  
107 (Zhang et al., 2005), and there are growing evidence that antibiotics can affect mitochondrial  
108 protein synthesis and mitochondrial functioning in a wide range of non-bacterial taxa  
109 (Kalghatgi et al., 2013; Wang et al., 2015). For instance, doxycycline (tetracycline family) has  
110 been demonstrated to affect proteostasis in both invertebrate and vertebrate cells (Houtkooper  
111 et al., 2013). Ballard & Melvin (2007) demonstrated that tetracycline reduces ATP production

112 in *Drosophila simulans* and increases mtDNA density in insects free of *Wolbachia*. In  
113 parallel, several works have demonstrated that rapid metabolic changes occur after antibiotic  
114 treatments in bacteria (Belenky et al., 2015; Hoerr et al., 2016; Zampieri et al., 2017), but  
115 there are many unknowns regarding the mechanisms of action of antibiotics, and their  
116 impacts, at the intra-organismal level in insects. Hence, the use of extracellular footprints and  
117 physiological intracellular fingerprints should improve our understanding of the effects of  
118 antibiotics on insects.

119

120 Juveniles of dipterans are poorly mobile insects which often thrive in microhabitats near  
121 the surroundings of human settlements, including agricultural lands and farms. The  
122 probability of exposure of fly larvae and pupae to antibiotics is thus very high, and should  
123 greatly affect the biochemistry, physiology and morpho-anatomy of these developing  
124 individuals, with potential long-lasting effects. At the larval stage, wings and flight muscles  
125 are absent from the thorax (Paululat et al., 1999; Roy & VijayRaghavan, 1999), and muscle  
126 cells appear and progressively arrange and differentiate at the pupal stage (Roy et al., 1997).  
127 Also, in a number of fly species, the maturation of flight muscle mitochondria only occurs  
128 after the emergence of adults (Marden, 2000), and this process is associated with a high  
129 biosynthetic activity of mitochondrial proteins from flight muscles (Lennie & Birt, 1967;  
130 Tribe & Ashhurst, 1972). Because several antibiotics are inhibitor of protein biosynthesis, the  
131 exposure of juvenile flies to these drugs should affect the mitochondrial efficiency and  
132 mitochondrial DNA density, as shown in *Drosophila* flies (Ballard & Melvin, 2007).

133

134 In this work, we investigated the effects of different antibiotics inhibiting (i) cell wall  
135 synthesis (ampicillin and cefradine) and (ii) protein synthesis (chloramphenicol,  
136 cycloheximide, and tetracycline) (Table S1) on the survival and ultrastructural organization of



137 the flight muscles of newly emerged blow fly, *Chrysomya albiceps*. Having these phenotypic  
138 data, we further examined the effects of these drugs on the physiology and metabolism of the  
139 individuals. Considering the prominent importance of mitochondria for flight muscles, we  
140 examined the effects of different concentrations of antibiotics on mitochondrial protein  
141 content, efficiency of oxidative phosphorylation, and activity of transaminases. Among  
142 transaminases that are localized in mitochondria, glutamate oxaloacetate transaminase (GOT)  
143 and glutamate pyruvate transaminase (GPT) are the most active in insects (Gäde, 1985).  
144 These enzymes are crucial for amino acid metabolism, but the effects of antibiotics on their  
145 activity have received little attention. Finally, we were interested in describing the cellular  
146 metabolic perturbations of flies treated with antibiotics, and run metabolomics assays to  
147 obtain a system-wide picture of biochemical networks altered by antibiotics.

148

149 We expected that moderate to high concentrations of protein synthesis inhibitors would be  
150 detrimental to the survival of newly emerged adult *Ch. albiceps*, whose mitochondriogenesis  
151 and biosynthetic processes should be affected. Chloramphenicol and tetracycline bind on  
152 large and small subunits of the mitochondrial ribosomes, and can inhibit mitochondrial  
153 protein synthesis and biogenesis in mammals (Brodersen et al., 2000; Houtkooper et al.,  
154 2013), while cycloheximide provokes a complete inhibition of cytoplasmic protein synthesis  
155 of eukaryotic cells. We assumed that mitochondrial structures and protein amounts would be  
156 altered in muscle tissues of flies injected with these three drugs. Ampicillin and cefradine are  
157 cell wall synthesis inhibitors, and these bactericidal antibiotics can strongly reduce the activity  
158 of the electron transfer chain of mitochondria (Kalghatgi et al., 2013). At low to moderate  
159 concentrations, mitochondrial metabolism of adult *Ch. albiceps* treated with ampicillin,  
160 cefradine, chloramphenicol, cycloheximide and tetracycline should be reduced, rendering  
161 cells' energy deficient (reduced oxidative phosphorylation). Finally, we hypothesized that

162 metabolic alterations would occur after the flies would be injected by the different antibiotics,  
163 and that the effects of the metabolic profiles would mirror the mode of actions of these  
164 different drugs.

165

## 166 **MATERIALS AND METHODS**

167

### 168 **Insect rearing**

169 Adults of the hairy maggot blow fly (GenBank common name: [taxid:81419](#)) *Chrysomya*  
170 *albiceps* (Wiedemann, 1819) (Diptera: Calliphoridae) were field-collected from Giza, Egypt  
171 (30°01'34.4"N 31°12'21.6"E). Colonies were established at the insectary of the Faculty of  
172 Science (Cairo University, Giza, Egypt) as described in [Queiroz \(1996\)](#). Adult flies were  
173 placed into wooden cages (50X50X80 cm, LxLxH) with sides made of fine mesh wire-gauze;  
174 cages containing flies were maintained under controlled conditions (27±1 °C, 60-70%  
175 Relative Humidity (R.H.), 14:10 h light:dark), and reared for five generations before being  
176 used for the experiments. Newly emerged adults were supplied with sucrose and water. Two  
177 to three days after emergence, adults were offered fresh meat (about 250 mg) as food and  
178 oviposition media. Females oviposit batches of up to 250-300 eggs, which were periodically  
179 transferred to larval rearing dishes (38 cm diameter, 7 cm height) containing a rearing  
180 medium (moistened sawdust and fresh meat blocks). These dishes were covered with muslin  
181 cloth fastened with rubber bands. Larval populations of *Ch. albiceps* are cannibals; hence, 2<sup>nd</sup>  
182 instars were regularly sampled from the semi-dried meat, and transferred to new rearing  
183 dishes. Pupation took place in the sawdust, or within dried meat chunks, and the pupae were  
184 collected and transferred to adult cages for emergence.

185

186 **Experimental treatments: injection of antibiotics and monitoring of the survival of the**  
187 **flies**

188 The effects of antibiotics with bacteriostatic or bactericidal activity (Table S1) on the biology  
189 and physiology of the blow flies were assessed by injection of different concentrations of:  
190 ampicillin (A9518 Sigma-Aldrich), cefradine (CAT # C0690000 Sigma-Aldrich),  
191 chloramphenicol (CAT # C3787 Sigma-Aldrich), cycloheximide (CAT # C7698 Sigma-  
192 Aldrich), and tetracycline (CAT # T7660 Sigma-Aldrich). To standardize the amount of drug  
193 given to each insect, antibiotics were injected into the adults. Before injections, newly  
194 emerged adults were allowed to harden for 1-2 hour, and they were then briefly cooled on ice  
195 prior to injection. A 10- $\mu$ L Hamilton microsyringe was used for injections. Different  
196 concentrations of antimicrobial solutions were freshly prepared into distilled water and  
197 protected from light by wrapping tubes in foil. A volume of 2  $\mu$ L of antimicrobial drugs was  
198 injected to each fly, meaning that we had different independent groups of flies that received  
199 distinct antibiotics at different concentrations (ampicillin: 100, 200, 300, 400 and 500  $\mu$ g/fly;  
200 cefradine: 100, 200, 300, 400, 500, 600 and 700  $\mu$ g/fly; chloramphenicol: 100, 150, 200, 250  
201 and 300  $\mu$ g/fly; cycloheximide: 2.5, 5, 10, 20, 30 and 40  $\mu$ g/fly; tetracycline: 10, 20, 30 and  
202 40  $\mu$ g/fly). The concentrations of the antibiotics were determined by running a preliminary  
203 test that measured the mortality dose-response of the flies (each experimental group started  
204 with an initial number of 50 flies). Two groups of control flies were also prepared: flies  
205 pricked with the syringe (no injection, control), and flies that were injected 2  $\mu$ L of saline  
206 solution (control 'saline'). All injections were made in the flight muscles, by entering the  
207 syringe beneath the wings, between the thorax and the abdomen.

208

209 For this part of the experiment, flies were individually injected one of the above-  
210 mentioned antimicrobial concentration. Three independent survival assays (N = 50 flies per

211 survival assay) were done for each experimental condition, including control flies. After  
212 injection, flies were maintained into rearing cages on sugar and water diet at  $27\pm 1$  °C, 60-70%  
213 R.H, and 14:10 h (light: dark) photoperiod. Flies from each experimental condition were  
214 examined daily for mortality for up to four days. The results from this experiment were used  
215 for selecting the antibiotic concentrations used for the second part of the study, i.e. for the  
216 characterization of extracellular footprints and physiological intracellular fingerprints of  
217 antimicrobial and antifungal treatments.

218

### 219 **Extracellular footprints of the effects of antibiotic treatments on flight muscles**

220 For this part of the experiment, antibiotic quantities generating 50% mortality after one day  
221 ( $LD_{50}$  values obtained from the survival experiment) were injected to the flies (cefradine: 500  
222  $\mu\text{g}/\text{fly}$ ; chloramphenicol: 200  $\mu\text{g}/\text{fly}$ ; cycloheximide: 20  $\mu\text{g}/\text{fly}$ ; Tetracycline: 30  $\mu\text{g}/\text{fly}$ ),  
223 except for ampicillin (300  $\mu\text{g}/\text{fly}$ ) for which lower quantities were injected to have flies  
224 surviving up to the fourth day post injection.

225 Heads, legs and abdomens of flies collected 1 and 4 days after injection of antibiotics  
226 were carefully removed after the flies were immobilized at 4 °C. The mid line of the dorsal  
227 surface of the thorax was cut with a sharp razor, so that the flight muscles were exposed on  
228 each side of the insect. A droplet of 2.5% glutaraldehyde fixative was put onto the flight  
229 muscles, which were then left for *in situ* fixation for 30 min. Small strips of about 1 mm of  
230 muscles were collected and immersed again in glutaraldehyde solution at 4 °C for 2 hours.  
231 Then, muscles were washed in several baths of 0.05 M cacodylate buffer (pH 7.3) and let  
232 overnight at 4 °C. Postfixation was carried out in 1% osmium tetroxide for 45 min at room  
233 temperature. Muscles were then washed by cacodylate buffer, and dehydrated in graded  
234 ethanol concentrations, from 60 to 100%. A final dehydration step was carried by washes with  
235 toluene or propylene oxide. The tissues were then left overnight in 1:1 toluene and embedding

236 medium (Epon) for infiltration. After this step, muscles were transferred to closed plastic  
237 capsules containing fresh epon. Epon capsules were polymerized at 60 °C for 48 hours.  
238 Ultrathin sections were cut with glass knives by an LKB 2088-Ultramicrotome V (Cambridge  
239 Instruments Ltd., Cambridge, UK). Sections were mounted on uncoated copper grids.  
240 Mounted sections were stained with uranyl acetate, followed by lead citrate and ultra  
241 automatic stainer (Leica-Reichert). The sections were examined with a Jeol 120 kV high  
242 resolution TEM (JEOL Ltd. Tokyo, Japan), and structural elements were identified based on  
243 [Smith \(1984\)](#).

244

#### 245 **Physiological intracellular fingerprints of the effects of antibiotics**

246 Here, antibiotic quantities generating around 50% mortality after one day (according to LD<sub>50</sub>  
247 values obtained from the survival experiment), and one lower and one higher dose, were  
248 injected to the flies for each drug (cefradine: 200, 400 or 600 µg/fly; chloramphenicol: 100,  
249 200 or 250 µg/fly; cycloheximide: 10, 20 or 30 µg/fly; tetracycline: 20, 30 or 40 µg/fly),  
250 except for ampicillin for which we used an intermediate dose lower than LD<sub>50</sub> (100, 300 or  
251 500 µg/fly). Flies were collected for physiological analyses 24 hours after the injections.

252

#### 253 *Isolation of mitochondria*

254 Twenty-four hours after the injection of antimicrobial solutions or distilled water, adult blow  
255 flies were collected from cages and immobilized by transferring them to -20 °C for 5 min.  
256 Flies were subsequently kept on ice, and heads, wings, legs and abdomens were quickly  
257 removed. Thoraces were conserved for isolation of flight muscle mitochondria as described  
258 by [Tribe \(1967\)](#). Thoraces from 25-50 flies were homogenized in a Dounce homogenizer  
259 containing pre-cooled isolation medium [(0.32 M sucrose, 0.01 M Tris buffer, pH 7.4 and  
260 0.01 M EDTA (ethylenediaminetetraacetic acid disodium salt)]. Homogenates were filtered

261 through four layers of muslin, previously soaked in ice-cold sucrose medium, and filtrates  
262 were centrifuged at 750 g for 10 min at 4 °C. The pellet was discarded, and the supernatant  
263 that contained mitochondria was centrifuged at 12,000 g for 20 min at 4 °C. The supernatant  
264 was discarded, and the mitochondrial pellet was resuspended gently in isolation solution using  
265 a pre-cooled fire-polished glass rod, and centrifuged again at 12,000 g for 20 min at 4 °C. The  
266 samples were then used for analyses of the protein content of the mitochondria.

267

268 *Protein content of the mitochondria and sodium dodecyl sulphate polyacrylamide gel*  
269 *electrophoresis (SDS-PAGE) separation*

270 The mitochondrial pellet was suspended into 5 mL of ice-cold 10% TCA. After 10 min at 4  
271 °C, the samples were centrifuged at 12,000 g for 10 min, and the pellet was washed twice  
272 with ice-cold 10% TCA. The last mitochondrial TCA insoluble protein pellet was dissolved  
273 into 0.5 mL of NaOH (1 N). Aliquots of 100 µL were used for measuring protein amounts  
274 with the Folin-phenol method of [Lowry et al. \(1951\)](#). Bovine serum albumin (BSA; Sigma-  
275 Aldrich, fraction V) was used as the standard for the protein calibration curve. Absorbance of  
276 the samples was read at 750 nm.

277

278 SDS-PAGE gels were performed according to the method of [Laemmli \(1970\)](#) in a  
279 Tris-glycine discontinuous gel system under denaturing conditions. Electrophoresis was  
280 performed on either 10 or 12% separating gel, overlaid with 5% stacking gel, at a constant  
281 current of 30 mA for 4 hours. The following reference proteins were used as molecular weight  
282 markers (Sigma-Aldrich), and ran concomitantly in SDS-PAGE:  $\alpha$ -lactalbumin (14,200 Da),  
283 trypsin inhibitor (21,500 Da), bovine carbonic anhydrase (31,000 Da), egg albumin (42,699  
284 daltons), bovine serum albumin (66,200 Da) and phosphorylase b (97,400 Da). Gel-Pro

285 Analyzer 3.1 software (Media Cybernetics, Washington - MD 20850, USA) was used for  
286 estimating the relative molecular weight (Mwt) and relative mobility (Rf) values.

287

288 *Measurement of the effects of antibiotics on energy production of the mitochondria*

289 Oxygen consumption of mitochondria was determined with the Warburg manometric  
290 technique at 25 °C (Tribe 1967), and expressed in terms of oxygen consumed per hour per  
291 milligram dry mass of mitochondrial flight muscles ( $\mu\text{L O}_2/\text{mg/h}$  or  $\text{QO}_2$ ). We focused on the  
292 mitochondria of the flies injected with antibiotics at the  $\text{LD}_{50}$ . For each replicate, a volume of  
293 100  $\mu\text{L}$  of the mitochondrial extract was homogenized into a standard reaction mixture  
294 containing 130 mM KCl, 10 mM Tris HCl (pH 7.4), 30 mM potassium phosphate, 30 mM  
295 glucose, 1 mg BSA/mL, and 10  $\mu\text{L}$  50 mM ADP. For this assay, pyruvate (10 mM) and  
296 proline (4 mM) were the supplied substrates. Then, a volume of 100  $\mu\text{L}$  of sarcosomal  
297 suspension (containing 0.2-0.4 mg protein) was added. The center of the flasks was filled with  
298 200  $\mu\text{L}$  of 20% KOH, and an acid washed filter paper wick was added. An incubation period  
299 of 25 min at 25 °C was conducted, with air as the gas phase. The reaction was stopped by the  
300 addition of 1 mL of ice-cold 25% TCA, and the flasks were placed immediately on ice in a  
301 refrigerator at 0 °C to prevent degradation of ATP. The contents of the flasks were  
302 centrifuged (12,000 g for 5 min at 4 °C). Then, inorganic phosphate of the supernatant was  
303 determined by mixing 100  $\mu\text{L}$  from each sample with 3 mL of copper acetate buffer, 0.5 mL  
304 of ammonium molybdate and 0.5 mL of reducing agent. After 10 min at room temperature,  
305 absorbance of the samples was read at 880 nm. Corrections for endogenous oxidation and  
306 phosphorylation were not done, since these amounts are small when mitochondria are isolated  
307 from intact tissues.

308

309 *Measurement of the glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate*  
310 *transaminase (GPT)*  
311 Enzymatic assays were carried out as described in [Reitman & Frankel \(1957\)](#), with minor  
312 modifications. Oxaloacetate and pyruvate formed by the transaminase enzymes GOT and  
313 GPT, respectively, react with dinitrophenylhydrazine (DNPH) to form hydrazone. The  
314 absorbance of hydrazone produced in the alkaline medium was measured at 546 nm. For each  
315 test, 100  $\mu$ L of the mitochondrial suspension was pipetted and transferred to 500  $\mu$ L of  
316 phosphate buffer (GOT substrate: phosphate buffer (pH 7.2) 100 mM/L, L-aspartate 80  
317 mM/L,  $\alpha$ -ketoglutarate 4 mM/L; GPT: phosphate buffer (pH 7.2) 100 mM/L, D.L-alanine 80  
318 mM/L,  $\alpha$ -ketoglutarate 4 mM/L). After incubation for 30 min at 37 °C, a volume of 500  $\mu$ L of  
319 the color reagent (DNPH) was added. The samples were mixed for 20 min at room  
320 temperature before adding 5 mL of 0.4 M NaOH. The enzyme activity was expressed in  
321 standard international units (U) per g of mitochondrial protein. One U is defined as the  
322 amount of enzyme that catalyzes the conversion of 1  $\mu$ mole of substrate per min at 25 °C.

323

#### 324 **Metabolomic fingerprinting of flight muscles treated with antibiotics**

325 In the last part of the work, we focused on antibiotics with bacteriostatic activity  
326 (chloramphenicol, cycloheximide, and tetracycline). We decided to collect the flies two days  
327 after the injections to ensure that we passed the peak of mortality of day 1. Based on the  
328 results of the survival assays, we selected one mild and one moderate antibiotic concentration  
329 for the metabolomic assays: 100 or 200  $\mu$ g of chloramphenicol per fly (Chloramph C2 and  
330 C1, respectively), 2.5 or 10  $\mu$ g of cycloheximide per fly (Cyclohex C2 and C1, respectively),  
331 10 or 30  $\mu$ g of tetracycline per fly (Tetracl C2 and C1, respectively). Untreated flies were  
332 injected saline solution (2  $\mu$ L, hereafter refer to as ‘control saline’), and a second group of  
333 flies was just pricked with the syringe (no injection, hereafter refer to as ‘control’). After



334 injections (or prick), all flies were returned to optimal growth conditions, and were collected  
335 for subsequent analyses when they were aged of 2 days. Each replicate consisted of flight  
336 muscles from at least nine flies. Four to six independent replicates were run for each  
337 experimental condition. The replicates were vacuum-dried and stored at -20 °C prior to  
338 analyses.

339

340 Gas chromatography coupled with mass spectrometry (GC-MS) was used detect the 62  
341 metabolites of our database, encompassing amino acids, organic acids, polyamines, polyols,  
342 and sugars. We used the method described in [Khodayari et al. \(2013\)](#). Each sample was  
343 homogenized in 900 µL of a solution of ice-cold methanol: chloroform (2:1, v:v). Then, a  
344 volume of 600 µL of ultra-pure water was added. Samples were homogenized and centrifuged  
345 for 10 min at 4,000 g (4 °C). Ninety µL of the upper phase containing metabolites was  
346 transferred to new glass vials and samples were vacuum-dried (Speed Vac Concentrator,  
347 MiVac, Genevac Ltd., Ipswich, England) at 34 °C. Then, we used the derivatization process  
348 and GC-MS settings described by [Khodayari et al. \(2013\)](#). Randomized sample sequences  
349 were established for sample injection, chromatograms were analyzed using *XCalibur* v2.0.7.  
350 Standard samples, consisting of 62 pure reference compounds at 1, 2, 5, 10, 20, 50, 100, 200,  
351 500, 750, 1000, and 1500 µM were run and metabolite levels were quantified using the  
352 quadratic calibration curves for each reference compound.

353

#### 354 **Statistical analysis**

355 The Kaplan-Meier estimates and Mantel-Cox two-sided log-rank tests were used to calculate  
356 the mean cumulative mortality of the flies after antibiotic treatments ( $\pm$  the standard error of  
357 mean, SEM), and draw the effects of doses of antibiotics on survival probabilities. For  
358 estimating LC<sub>50</sub> and comparing the different antibiotic doses, probit analysis was applied to fit

359 multiple regression using SAS 9.2 (SAS Institute Inc., NC, USA). LC<sub>50</sub> were obtained by  
360 drawing a cumulative 96 hours mortality curve (dependent variable).

361

362 Biochemical data were analyzed with independent *t*-tests to assess for differences among  
363 the treated groups and controls. All experiments were performed in four replicates, unless  
364 otherwise indicated. The results are presented as the mean ± SEM. Data were analyzed with  
365 IBM SPSS Statistics V22.0 (IBM Corp. Armonk, NY, USA).

366

367 For metabolomics, the concentration of the metabolites was log-transformed, and data  
368 were mean-centered. The effects of antibiotics on the metabolic signatures of the flight  
369 muscles were investigated using Principal Component Analysis (PCA). A heatmap was  
370 constructed using Ward's method as the clustering method, and the Pearson correlation as the  
371 distance measure. The dendrogram was constructed according to Ward's method, and  
372 Euclidean correlation as the distance measure. Variations in the quantities of individual  
373 metabolites were then examined among the experimental conditions using an ANOVA with a  
374 Tukey post-hoc procedure. Analyses of metabolomic data were conducted with  
375 MetaboAnalyst 3.0 (<http://www.metaboanalyst.ca>; Xia & Wishart, 2016).

376

## 377 **RESULTS**

378

### 379 **Effect of different concentrations of antibiotics on the survival of the flies**

380 All injected antibiotics affected the survival of the flies (Fig. 1, Table 1), while little mortality  
381 was observed in flies injected with the saline solution, or in flies that were just pricked. Log-  
382 rank tests demonstrated that increased antibiotic doses significantly increased mortality of the  
383 flies ( $p < 0.001$ , Chi-square value ( $\chi^2$ ) were: ampicillin  $\chi^2 = 67.98$ ,  $df=4$ ; cefradine  $\chi^2 = 93.15$ ,

384  $df=5$ ; chloramphenicol  $\chi^2 = 101.35$ ,  $df=4$ ; cycloheximide  $\chi^2 = 168.85$ ,  $df=5$ ; tetracycline  $\chi^2 =$   
385  $107.56$ ,  $df=3$  (Fig. 1).  $LD_{50}$  values for each antibiotic are presented in Table 1. At the lowest  
386 concentration, the injected antibiotics had small effects on the survival of *Ch. albiceps*. The  
387 number of dead individuals was the highest 1-day after injection for cefradine,  
388 chloramphenicol and tetracycline, whatever the concentration of the drug. As a result,  $LD_{50}$   
389 values remained stable for these three drugs when calculated 1 or 4 days after the injection  
390 (Table 1). Conversely, mortality progressed constantly over the four days post-injection for  
391 the flies that were treated with ampicillin or cycloheximide, and  $LD_{50}$  values greatly varied.  
392 For instance,  $LD_{50}$  was 21.76 (19.63-23.89)  $\mu\text{g}/\text{fly}$  for cycloheximide when calculated at day  
393 1, and dropped to 7.09 (6.47-7.71)  $\mu\text{g}/\text{fly}$  when  $LD_{50}$  was calculated at day 4.

394

#### 395 **Extracellular footprints of the effects of antimicrobial treatments on flight muscles**

396 Electron micrographs of flight muscles of flies treated with antibiotics showed a degenerative  
397 alteration of the fine structure of flight muscles and mitochondria; alterations were observed 1  
398 (Fig. S1) and 4 (Fig. S2) days after the injection of the antibiotics. Except for flies treated  
399 with cefradine, mitochondria were swollen, cristae were distorted, and glycogen granules  
400 were dense (Fig. S2). In 4-day-old flies, some organelles appeared, without any internal  
401 compartment; moreover, mitochondrial and flight muscles interspaces were relatively large.  
402 Fewer effects were observed with cefradine (Fig. S2). Ultrastructural sections of ampicillin-  
403 treated flies showed few irregularities in mitochondrial shapes but were overall very similar to  
404 controls (data not shown).

405

#### 406 **Physiological intracellular fingerprints of the effects of antibiotics**

407

408 *Effects of antimicrobial on the protein content of the mitochondria of the cells from flight*  
409 *muscles*

410 A consistent pattern was observed across the experimental treatments, with the amount of  
411 proteins from flight muscles being decreased when the concentration of the drugs was  
412 increased (Fig. 2). At the highest antibiotic concentration, a larger decrease of the protein  
413 amount was observed for chloramphenicol and cycloheximide (reduction of about 29%),  
414 while the lowest decrease was observed for cefradine and tetracycline (reduction of about  
415 14%).

416 Mitochondrial proteins of the flight muscles were characterized by SDS-PAGE, which  
417 allowed the separation of 41 bands (Fig. S3, Table S2). The 10% SDS-PAGE separated 27  
418 protein subunits, ranging from 11,000 to 120,500 daltons, while the 12.5% SDS-PAGE  
419 separated 24 protein bands, ranging from 28,500 to 97,000 daltons. Proteins with molecular  
420 weights of 50, 42.5, 38 and 22 kilodaltons for the 10% gel, and 34.5, 33 and 17.5 kDa for the  
421 12.5% gel were the most concentrated protein subunits. The highest changes were observed  
422 for flies injected with cycloheximide and chloramphenicol, whose gels were characterized by  
423 a lower number of bands of fainter colors, as compared with control flies (Fig. S3).

424

425 *Oxidative phosphorylation of flight muscle mitochondria*

426 The oxygen consumption (oxidation rate of pyruvate and proline) was  $102 \pm 0.2 \mu\text{L O}_2/\text{h}/\text{mg}$   
427 mitochondrial proteins in 1-day-old control flies (Fig. 3a). It was two times lower in flies that  
428 were injected with antibiotics, with values ranging from  $39.5 \pm 0.9 \mu\text{L O}_2/\text{h}/\text{mg}$  for  
429 chloramphenicol at  $200 \mu\text{g}/\text{fly}$  to  $55.7 \pm 1.5 \mu\text{L O}_2/\text{h}/\text{mg}$  for cycloheximide at  $20 \mu\text{g}/\text{fly}$ .

430 The considerable inhibition of the rate of oxygen uptake following the treatment with high  
431 doses of antibiotics altered P/O ratio, which oscillated around 2 across in the experimental  
432 treatments (Fig. 3b).

433

434 *Effects of antimicrobial on the transaminase activity*

435 Flight muscle mitochondria from untreated newly emerged flies were characterized by similar  
436 transaminase (GOT & GPT) activities, of about 14 U/g mitochondrial protein (Fig. 4). Except  
437 for the GOT activity of the flies injected with 100 µg/fly ampicillin, all antibiotic treatments  
438 significantly affected the activity of GOT and GPT. In three groups, all from the cefradine  
439 treatment, activity of GPT was reduced significantly as compared with control flies.  
440 Conversely, activity of GPT and GOT was significantly increased in all of the other  
441 experimental treatments. Activity of mitochondrial GPT was increased, relative to control, in  
442 flies injected with moderate and high doses of ampicillin (2.4- and 2.7-fold, respectively),  
443 chloramphenicol (3.6- and 3.4-fold, respectively), cycloheximide (1.6- and 3.0-fold,  
444 respectively), and tetracycline (1.23- and 1.20-fold, respectively). Mitochondrial GOT activity  
445 was increased significantly in all flies that were treated with antibiotics as compared with  
446 control flies (Fig. 4).

447

448 *Metabolomics*

449 Forty-nine metabolites were quantified from our samples, with alanine, glycerol and lactic  
450 acid being the most abundant metabolites whatever the experimental condition. The first and  
451 second principal components (PC1, PC2) of the PCA accounted for 71.4% and 9.3% of the  
452 total inertia (Fig. 5a). The metabolic phenotypes of the samples injected with two different  
453 doses of antibiotics remained very similar, i.e. largely overlapped. PC1 strongly separated the  
454 flies treated with cycloheximide from the other experimental groups. Concentrations of  
455 ethanolamine, pipercolic acid, putrescine, glutamic acid and ribose (ranked by contribution to  
456 the axis) were positively correlated to PC1, and were more abundant in flies that were injected  
457 cycloheximide (Fig. 5b). Lactic acid, trehalose, fructose-6-P, glucose, fructose, glucose-6-P,

458 spermine, sorbitol and alanine were positively correlated to PC2, and were in lower amounts  
459 in flies treated with cycloheximide.

460

461 The second axis of the PCA (PC2) separated control flies (pricked flies or flies injected  
462 with the saline solution) from flies treated with tetracycline or chloramphenicol. Lactic acid,  
463 glycerol-3-P, saccharose and gluconolactone were measured in higher amounts in pricked and  
464 saline-injected flies, whose metabolic phenotypes did not significantly differ. Glucose,  
465 fructose-6-P, free phosphate, phenylalanine, spermine, glycerol, glucose-6-P and trehalose  
466 were quantified in higher concentrations in flies that were injected with chloramphenicol.

467

468 Hierarchical clustering of the samples and heatmap (Fig. S4 and S5) were drawn to  
469 facilitate pattern recognition, i.e. visualize similarities / differences among replicates without  
470 *a priori* on the experimental conditions. The hierarchical cluster analysis confirmed the  
471 separation of the experimental groups observed in the PCA, with samples from a given  
472 experimental condition clustering together for all experimental treatments (Fig. S4). A subset  
473 of amino acids, polyamines and organic acids were decreased significantly in flies treated  
474 with chloramphenicol and tetracycline, with 21 metabolites having decreased amounts in  
475 these two conditions as compared with controls (Fig. S5). The concentrations of most  
476 metabolites from the flies injected with cycloheximide were altered as compared with  
477 controls, while only few metabolites (i.e. most of those positively correlated to PC1 in the  
478 PCA, putrescine, fumaric acid, ethanolamine, pipercolic acid, aspartic acid, and glutamic acid)  
479 being accumulated (Table S6).

480

## 481 **DISCUSSION**

482

483 **Effect of antibiotics on the survival of the flies**

484 While the lowest doses of ampicillin, cefradine, chloramphenicol, cycloheximide and  
485 tetracycline induced little mortality to the flies, survival was very quickly impaired as soon as  
486 the quantity of drugs was increased. For cefradine, chloramphenicol, and tetracycline, most of  
487 the mortality of the flies was recorded one day after injection, and survival remained  
488 relatively stable afterwards. Contrariwise, mortality progressed over the course of the  
489 experiment when the flies were injected ampicillin or cycloheximide, as depicted by the  
490 decrease of LD<sub>50</sub> from day 1 to 4; yet, the highest mortalities were observed after a day at the  
491 highest doses for these two antibiotics. Our findings suggest that cefradine, chloramphenicol,  
492 and tetracycline may have diffused and reached their targets more quickly within the flies  
493 after the injection than the two other antibiotics. Moreover, the time-dependent sequence of  
494 antibiotic-associated effects that inhibit various vital cellular functions, such as transcription,  
495 translation, or DNA supercoiling, or alter the microbiome of the flies, must be drug-specific.  
496 At the highest antibiotic doses, the little to null activity of the ribosomes into the cells of the  
497 bacteria and flies likely explains the death of our insects.

498

499 The toxic effects of chloramphenicol were particularly striking, with survival of the flies  
500 dropping very quickly when the injected amount was only slightly increased (1.5 or 2 times as  
501 compared with the lowest dose), and no survival was measured at 300 µg  
502 chloramphenicol/fly. These results are consistent with the findings of [Ashour et al. \(1978,](#)  
503 [1980a\)](#) who reported low mortality in newly emerged flies of *Calliphora erythrocephala*  
504 injected with 100 µg chloramphenicol/fly, whereas all flies died when doses of this drug were  
505 higher than 400 µg/fly. There are now growing suspicions that chloramphenicol could induce  
506 aplastic anemia to human, and that it could be carcinogenic for vertebrates ([Baynes et al.,](#)  
507 [2016](#)), including ectothermic vertebrates ([El-Mofty et al., 2000](#)). However, the main

508 physiological causes that provoked the death of our blow flies at moderate to high  
509 concentrations of chloramphenicol remain uncertain.

510

511 Tetracycline, which is often used for clearing insects infected by *Wolbachia*, had very low  
512 effects on the survival of the flies when injected at low amounts. However, a 3- or 4-fold  
513 increase of the injected dose greatly reduced survival, which reached 0% after 4 days at the  
514 highest concentration of tetracycline. A similar finding was found with cycloheximide.  
515 Working with cycloheximide and other protein DNA synthesis inhibitors, [Campbel & Birt](#)  
516 [\(1975\)](#) reported in the sheep blow fly *Lucilia cuprina* that doses of 1 µg of cycloheximide  
517 were quickly detoxified by the individuals, whereas longer durations were required for higher  
518 concentrations, like for instance puromycin (mitochondrial protein inhibitor) at 5 µg. At  
519 moderate to high antibiotic concentrations, the detoxifying capacities of the blow flies may  
520 have been exceeded, resulting in the death of the individuals.

521

522 Our present survival data were used to calibrate the doses of antibiotics that were further  
523 injected to the insects to investigate the physiological effects of drugs on developing/maturing  
524 flies. We speculated that the different target / mode of action of each drug, and the differential  
525 rates of detoxification of each of these molecules by the insect, would explain the different  
526 survival kinetics we measured.

527

### 528 **Effects of antibiotics on mitochondrial structure and oxidative rate**

529 Most of the mitochondria of 1-day-old flies were not fully differentiated, i.e. cristae were not  
530 densely packed, which is consistent with the findings of [Gregory et al. \(1968\)](#) on the blow fly  
531 *Lucilia cuprina*. The cytoplasmic and mitochondrial protein inhibitors we used induced  
532 marked changes to the mitochondrial ultrastructure of the flies, with chloramphenicol,



533 cycloheximide and tetracycline having the most severe effects, by provoking swelling,  
534 disorganization of cristae, vacuolation and whorl shape formation. Consistently, the  
535 mitochondrial protein inhibitors chloramphenicol and ethidium bromide caused irregular  
536 alignment of cristae in *C. erythrocephala* (Ashour et al., 1980b); higher doses inflicted  
537 considerable swelling to whole mitochondria, most likely because cristae maintenance and  
538 maturation post emergence require considerable amounts of ATP production from  
539 mitochondria. Similar changes, i.e. swollen, short and fragmented mitochondria, have also  
540 been reported from mammalian cells (Kalghatgi et al., 2013), demonstrating the genericity of  
541 these antibiotic-induced changes across taxa.

542

543 Whorl-like shapes of the mitochondria were particularly significant for the blow fly *Ch.*  
544 *albiceps* treated with cycloheximide. Bulos et al. (1972), and Webb & Tribe (1974) suggested  
545 that whorls are associated with the absence of cytochrome oxidase band, and possibly of other  
546 key enzymes. In the absence of a repair mechanism, configuration of the membranes could  
547 change towards an organization observed in myelin sheath i.e. concentric lipoprotein bilayers  
548 which seem more stable configuration in these circumstances.

549

550 Three out of the five injected antibiotics used in this study are inhibitors of protein  
551 synthesis. Consistently, the amount of proteins of mitochondria was decreased for up to ca.  
552 25% in flies treated with chloramphenicol, cycloheximide and tetracycline. These results  
553 confirmed that the drugs reached their targets and affected the physiology of the flies. Sebald  
554 et al. (1969), in *Locusta migratoria*, and Bartelink & DeKort (1973), in the Colorado beetle,  
555 found that the mitochondrial protein synthesized in the presence of cycloheximide was about  
556 15% lower than in controls. Ashour et al. (1980a) found that the highest rate of protein  
557 synthesis occurred 3 to 4 days after emergence in *C. erythrocephala*, and the highest values of

558 flight muscle mitochondrial proteins were recorded in 6-10 days old flies. Consistent with this  
559 observation, [Ashour et al. \(1980a\)](#) found that flies displayed the greatest susceptibility to drug  
560 treatment when the protein synthesis was the most intense (in 3 to 4 days old flies), while  
561 older flies (6-10 days old flies) displayed the greatest resistance to drug treatment. As we  
562 worked with newly emerged flies, we may have minimized the effects of drugs on protein  
563 synthesis, which could have been greater some few days later.

564

565 An injection of chloramphenicol (100 or 200  $\mu\text{g}/\text{fly}$ ) was expected to limit protein  
566 synthesis (50S ribosomal subunits) by inhibiting the activity of the peptidyl transferase and  
567 cytochrome oxidase enzymes ([Houtkooper et al., 2013](#)). The earlier investigations conducted  
568 on this drug reported a 92% inhibition of the protein synthesis in *Saccharomyces cerevisiae*  
569 ([Schweyen & Kaudewitz, 1970](#)); in the Colorado beetle, doses of chloramphenicol of 50, 100  
570 and 150  $\mu\text{g}$  induced an inhibition of the mitochondrial protein ranging from 40 to 70%  
571 ([Bartelink & DeKort, 1973](#)). In the present work, the amount of mitochondrial proteins of the  
572 flight muscles were progressively reduced when the dose of chloramphenicol was increased,  
573 and the highest dose of this antibiotic resulted in a 30% reduction of the protein content. The  
574 electrophoretical differentiation of the proteins, as compared with control flies, revealed that  
575 chloramphenicol, but also cycloheximide, rapidly decreased mitochondrial protein synthesis,  
576 and had the highest effect on protein subunits differentiation. Cycloheximide alters ribosomes  
577 when translation is ongoing ([de Loubresse et al., 2014](#)). As the turnover of the synthesis of  
578 protein subunits from blow fly flight muscle mitochondria is relatively low over time ([Jeffreys  
579 & Craig, 1976a; Jeffreys & Craig, 1976b; Ashour and Tribe, 1981](#)), the observed changes are  
580 maintained over several days.

581

582 In mammalian cells, antibiotics have been demonstrated to induce mitochondria  
583 dysfunction (Houtkooper et al., 2013; Kalghatgi et al., 2013; Moullan et al., 2015). Damages  
584 and swelling of mitochondria may lead to reduction to suppression of ATP production,  
585 because antibiotics alter the inner membrane of mitochondria, in turn affecting the coupling of  
586 oxygen consumption to ADP phosphorylation (Brand & Nicholls, 2011). In this part of the  
587 study, all antibiotics were injected at doses that cause *ca.* 50% mortality after one day (except  
588 for ampicillin), and we reported a considerable decrease of the oxidative rate of surviving flies  
589 as compared with controls. There are few data on the mode of action of antibiotics in insects;  
590 in mammals, antibiotics have been proven to alter the electron transfer chain, thus decreasing  
591 the mitochondrial membrane potential and ATP levels (Kalghatgi et al., 2013). The leakage of  
592 electrons from the electron transfer chain also contributes to increase the production of  
593 reactive oxygen species. For instance, treatments of mammalian cells with ampicillin  
594 increased the amount of superoxide ions, which potentially induces increased DNA and  
595 protein damages (Kalghatgi et al., 2013). While electron leaks are expected to affect  
596 mitochondrial coupling efficiency, the P/O ratios were similar among the different  
597 experimental conditions. This result suggests that the electrochemical gradient was not  
598 affected by the drugs, and revealed that ATP production *versus* O<sub>2</sub> consumption was reduced  
599 in antibiotic-treated flies.

600

601 The putative mitochondrial dysfunctions post-antibiotic treatments can be explored by  
602 ratiometric analysis of O<sub>2</sub> consumption (QO<sub>2</sub>) after addition of pyruvate and proline (state 2  
603 of mitochondrial respiration), followed by the addition of ADP to reach state 3, and returning  
604 to basal oxygen mitochondrial consumption when all ADP had been phosphorylated (state 4).  
605 Respiratory control ratio of state 3/state 4 is a measure of the level of mitochondrial coupling  
606 and phosphorylation efficiency (Brand & Nicholls, 2011). In antibiotic-treated flies,

607 mitochondrial functions were clearly impaired, as shown by the significant decrease of  
608 oxidation rate of pyruvate+proline. It is however not possible to decipher if the reduced  
609 oxidation of these two compounds resulted from a dysfunction of state 2 or state 3, or both.  
610 An alteration of the state 3, for instance, by alteration of the activity of the Krebs cycle  
611 enzyme  $\alpha$ -ketoglutarate dehydrogenase, could have lowered the demand for substrate for  
612 oxidative phosphorylation.

613

#### 614 **Physiological underpinnings of the effects of antibiotics**

615 We observed two distinct survival patterns, for flies injected with cefradine,  
616 chloramphenicol, or tetracycline on the one hand, and flies injected with ampicillin or  
617 cycloheximide on the other hand. In parallel, we demonstrated that all five antibiotics caused  
618 mitochondria dysfunctions. To date, metabolic changes resulting from antibiotic treatments  
619 have been mostly investigated in microorganisms ([Olszewska & Szewczyk, 2013](#)). For the  
620 metabolomics approach, we restricted our analyses to flies treated with two doses of  
621 chloramphenicol, cycloheximide and tetracycline, in addition to control and saline injected  
622 flies. The metabolic profiles of the two untreated groups (controls, saline-injected flies)  
623 overlapped and clustered in the same branch of the HCA. Metabolism of the organisms is often  
624 very sensitive to environmental stressors and/or injuries, by mediating adequate responses to  
625 maintain physiological homeostasis ([Colinet & Renault, 2012](#); [Hidalgo et al., 2013](#); [Colinet &](#)  
626 [Renault, 2018](#)). Importantly, the quasi superposition of the metabolic phenotypes of the two  
627 control groups ensures that the observed repatterning of the metabolism of antibiotic-treated  
628 flies depicts the sole effects of the drugs. Interestingly, the metabolic changes were not  
629 affected by drug dosage, and were specific to the antibiotics that were injected.

630

631 Consistent with survival patterns, metabolic phenotypes of flies treated with  
632 chloramphenicol and tetracycline were similar, but distinct from the one measured from flies  
633 treated with cycloheximide. Injection of cycloheximide, an antifungal antibiotics that inhibits  
634 protein synthesis and that is used in agriculture (McManus et al., 2002; Wang et al., 2014),  
635 had the most striking effect on the metabolic phenotype of the flies and provoked a decrease  
636 of the levels of most metabolites. In *C. erythrocephala*, Ashour et al. (1980a) found that  
637 cycloheximide can inhibit amino acid incorporation in the young flies, a finding consistent  
638 with the inhibition of RNA translocation induced by cycloheximide (Schneider-Poetsch et al.,  
639 2010). The depletion of the quantities of most metabolites in flies treated with cycloheximide  
640 suggests that intermediary metabolism was globally depressed. The increased levels of  
641 fumaric acid measured from flies treated with cycloheximide should also be highlighted. We  
642 speculate that the conversion of succinic acid to fumaric acid remained functional in these  
643 flies, generating FADH<sub>2</sub>, but the transfer of the electrons, and their entry into the electron-  
644 transfer chain at the complex II, may have been blocked. This could have resulted in a greater  
645 production of fumaric acid and FADH<sub>2</sub> in regards to their demand, leading to the  
646 accumulation of fumaric acid.

647

648 Increased levels of putrescine were observed in chloramphenicol- and cycloheximide-  
649 treated flies. Putrescine often accumulates during anoxia in plant cells, and indirectly  
650 contributes to H<sub>2</sub>O<sub>2</sub> production under oxygen shortage (Blokhina et al., 2003).

651

652 Commonalities in the metabolic profiles were observed between chloramphenicol and  
653 tetracycline, with amounts of glucose, fructose-6-P, free phosphate, glucose-6-P and trehalose  
654 being increased in flies that received one of these two drugs. Studies conducted on  
655 microorganisms reported that bactericidal drugs alter the oxidative phosphorylation and the

656 activity of the Krebs cycle, and resulted in increased amounts of metabolites from energetic  
657 metabolism (Belenky et al., 2015). Yet, in our study, the amounts of TCA cycle intermediates  
658 were overall decreased, and more particularly at the intermediate antibiotic dose, which is  
659 consistent with our measurements of lower oxidative rates in these conditions. The reduction  
660 of the activity of the TCA cycle can be compensated by an increased activity of the  
661 glutamate-oxaloacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT),  
662 which can provide alpha ketoglutarate (Huang et al., 2006). Our results are in accordance with  
663 this assumption, as the activity of the transaminases was significantly increased at 200 µg  
664 chloramphenicol/fly, likely accompanying the increased amounts of succinic, fumaric and  
665 malic acids, whereas levels of citric acid were reduced. In parallel, levels of alanine and  
666 aspartic acid remained high, suggesting the higher demand for these compounds that may be  
667 converted to glutamic acid instantaneously transformed to alpha ketoglutarate.

668

669 Transamination permits the biosynthesis of amino acids and the shift from sugar to protein  
670 metabolism according to the ecological situations encountered by organisms (Bursell, 1981;  
671 Cooper & Hanigan, 2018). The ratio GOT/GPT is often used as a biomarker of the health  
672 status of liver in human (Kim et al., 2008), and has been used to assess the level of  
673 xenobiotic-induced stress in insects (Içen et al., 2005; Zibae et al., 2011). In our study, the  
674 large increase of GPT activity in flies treated with chloramphenicol altered GOT/GPT ratio  
675 may have revealed cell lesion to the flight muscles tissues of the treated flies.

676

677 Lactic acid amounts were measured in high amounts from flight muscles of untreated  
678 flies. Lactic acid is a well-known product of anaerobic glycolysis but has also been  
679 experimentally demonstrated as a prominent oxidized substrate by muscles of rainbow trouts  
680 during glycogenesis (Kam and Milligan 2006). Levels of lactic acid were highly significantly

681 reduced in flies treated with chloramphenicol and cycloheximide, and we speculated that this  
682 reduction affects the energy (ATP) required for the glycogenesis in muscles (Kam &  
683 Milligan, 2006; Kane, 2014). Further studies should compare the amount of stored glycogen  
684 in treated and non-treated fly muscles to comfort this assumption. The activity of lactate  
685 dehydrogenase and lactate transporters (Kane, 2014) that may have been greatly altered by  
686 chloramphenicol and cycloheximide treatments could also be quantified.

687

## 688 **Conclusion**

689 Low doses of antibiotics had limited effects on the survival of newly emerged flies of *Ch.*  
690 *albiceps*. Yet, the extracellular footprints and physiological intracellular fingerprints we used  
691 demonstrated that these drugs have multiple direct and cascading effects on the structure of  
692 the flight muscles and physiology of the flies. The time-dependent sequence of antibiotic-  
693 associated effects was drug-specific, with LD<sub>50</sub> that greatly varied for ampicillin and  
694 cycloheximide from day 1 to 4 post injection. Flies treated with chloramphenicol and  
695 tetracycline had altered oxidative phosphorylation and activity of the Krebs cycle, likely  
696 compensated by an increased activity of transaminase enzymes. The drop of oxidative  
697 phosphorylation correlated with damages of mitochondria. Cycloheximide had the most  
698 pronounced physiological effects on the phenotype of the flies, and quickly provoked their  
699 death, even at low doses. Overall, our findings suggest that antibiotic pollution could greatly  
700 affect insect population dynamics, which could be particularly problematic for beneficial  
701 insects and insect community structure. The effects on the trophic network should be explored  
702 in future studies.

703

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709

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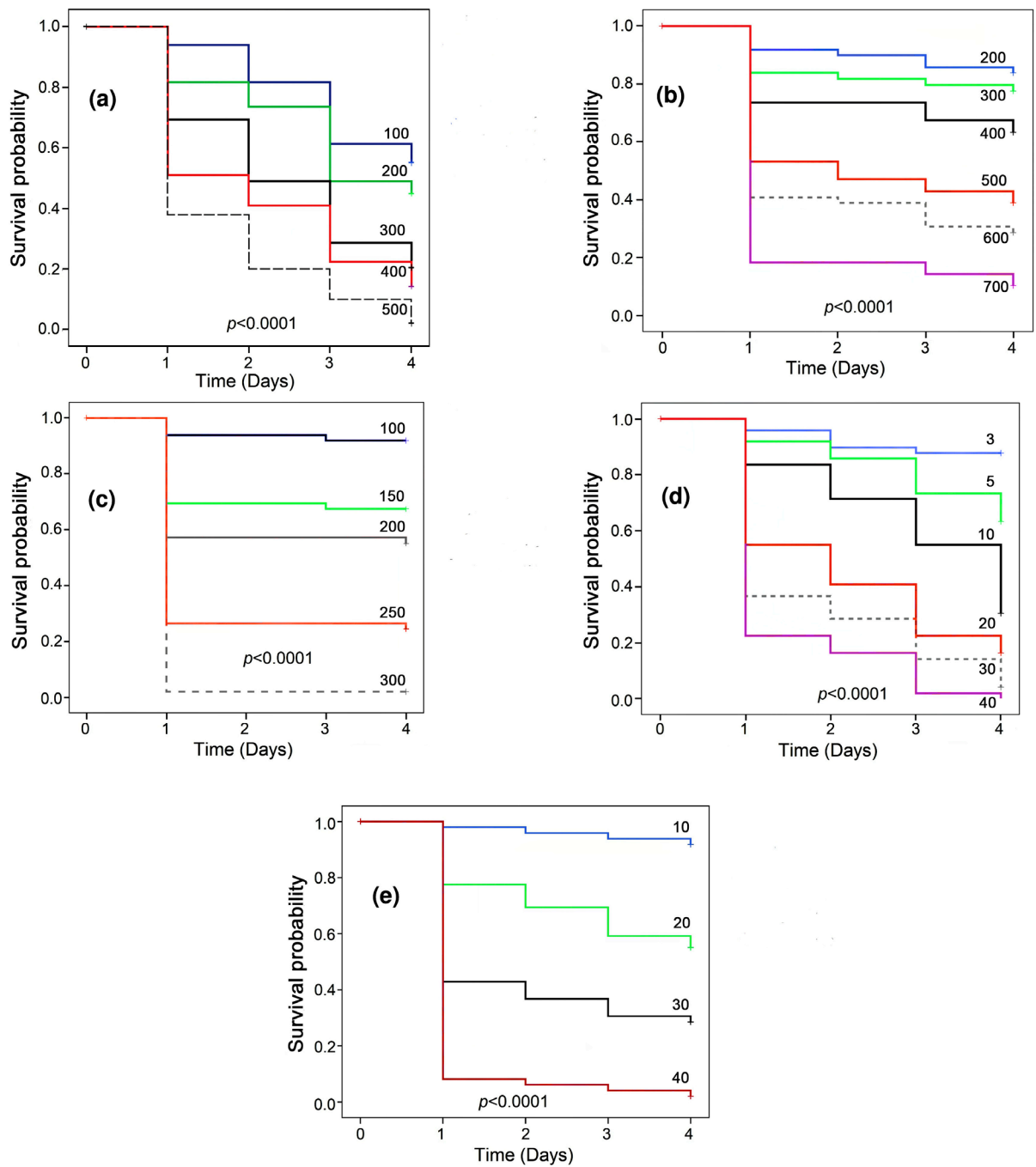
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957

958 **Figure legends**

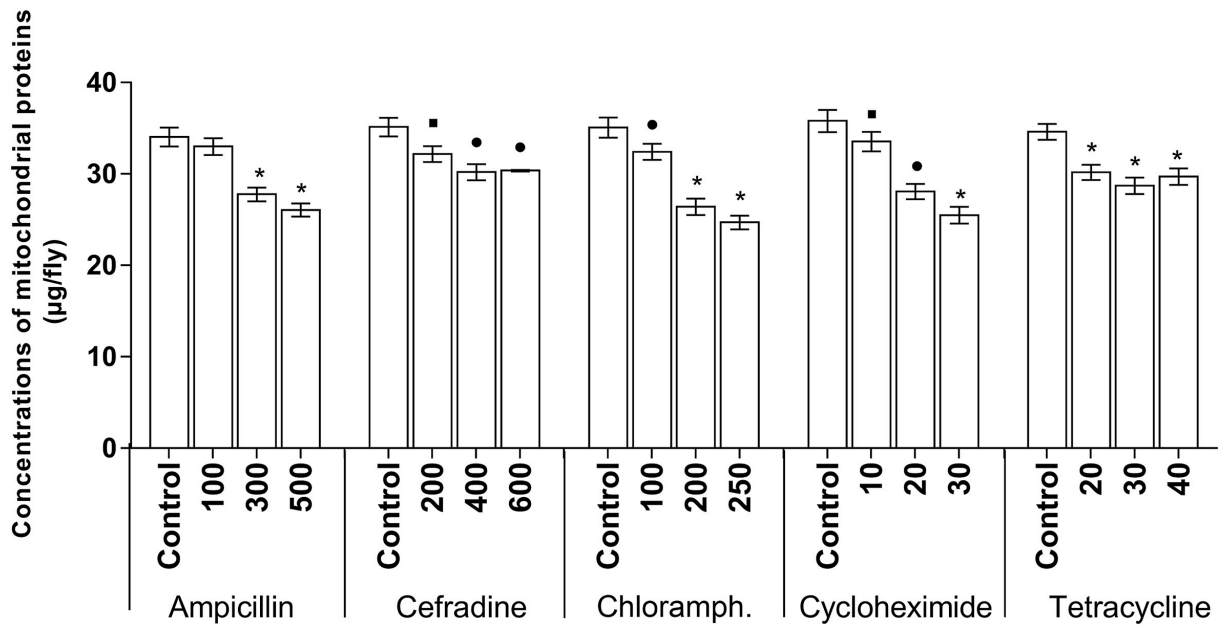


959

960 **FIGURE 1** Kaplan-Meier survival probabilities of the blow flies over four days after the  
 961 injection of different doses of (a) Ampicillin, (b) Cefradine, (c) Chloramphenicol, (d)  
 962 Cycloheximide, and (e) Tetracycline.

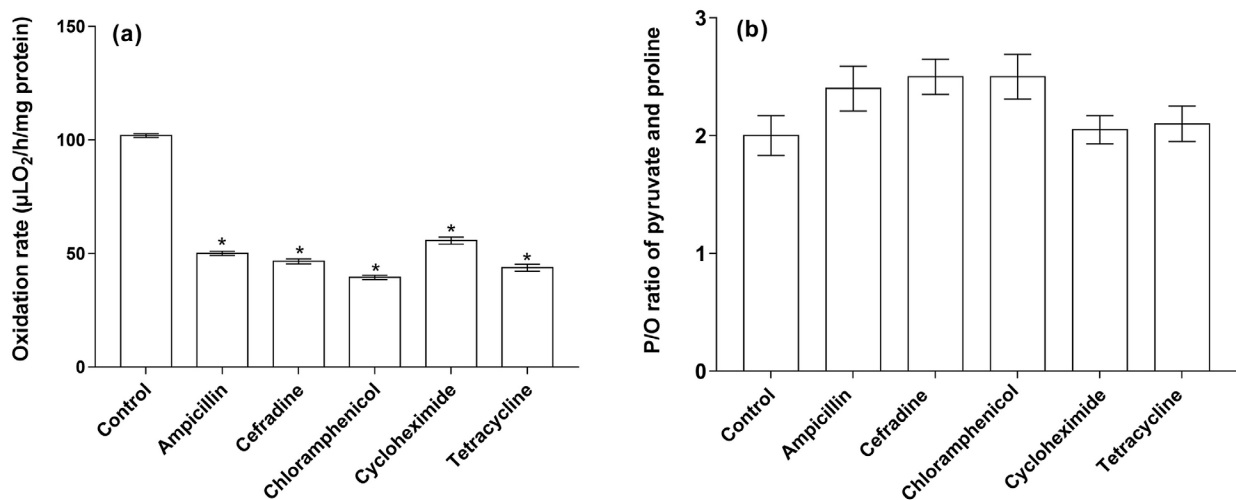
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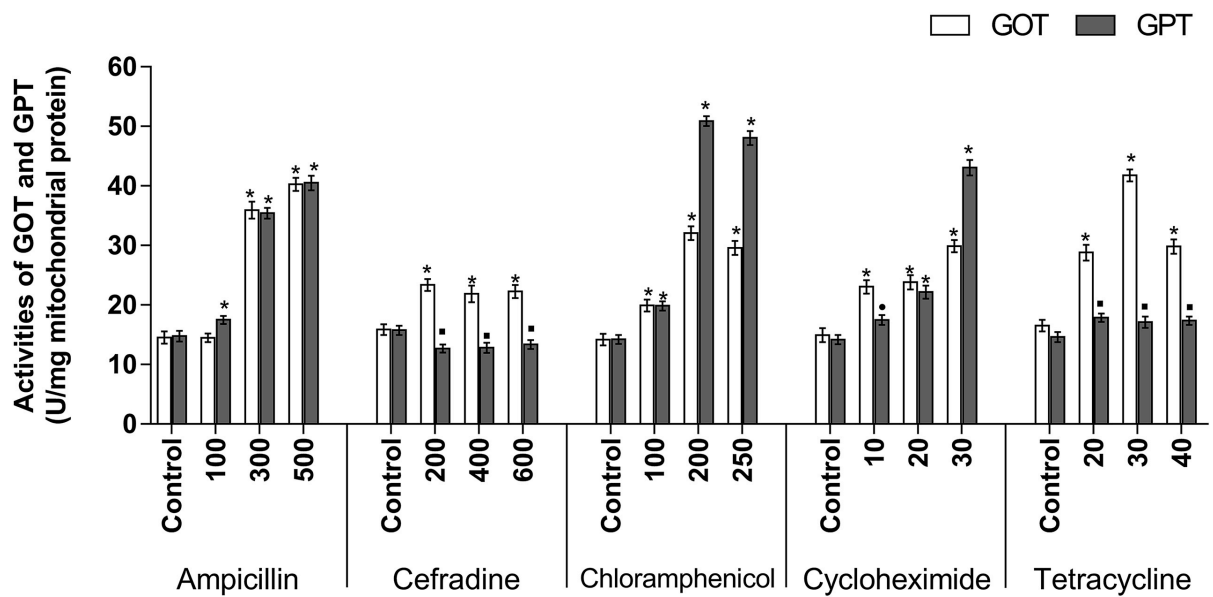
966 **FIGURE 2** Concentrations of mitochondrial proteins (mean  $\pm$  S.E.; N = 4 replicates per  
 967 experimental condition) from blow flies injected with different doses of Ampicillin,  
 968 Cefradine, Chloramphenicol, Cycloheximide, and Tetracycline. Dark squares, dark circles and  
 969 asterisks report differences among the injected doses of a given antibiotic; ■ $p$ <0.05, ● $p$ <0.01,  
 970 \* $p$ <0.001.



971

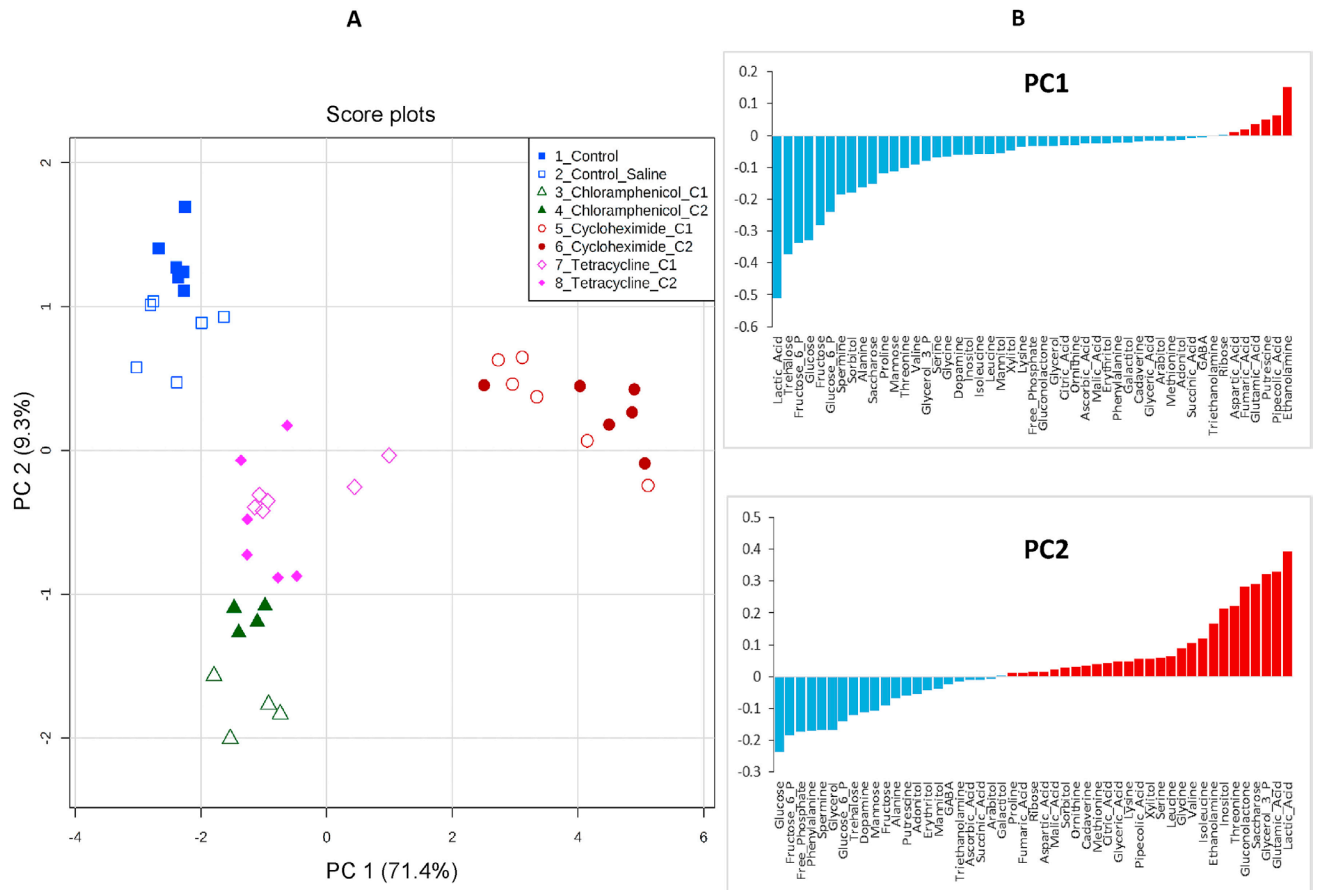
972 **FIGURE 3** Oxidation rate of pyruvate+proline (mean  $\pm$  S.E.) from flight muscle  
 973 mitochondria (a), and P/O ratio (mean  $\pm$  S.E.) from with flight muscle mitochondria (b).  
 974 Measurements were realized from 1-day-old flies, 24 hours after they were injected the saline

975 solution (control, or 300 µg/fly ampicillin, or 500 µg/fly cefradine, or 200 µg/fly  
 976 chloramphenicol, or 20 µg/fly cycloheximide, or 30 µg/fly tetracycline. \* $p < 0.001$ .



977

978 **FIGURE 4** Activity of glutamate-oxaloacetate transaminase (GOT) and glutamate-pyruvate  
 979 transaminase (GPT) (mean  $\pm$  S.E., N = 4 replicates per experimental condition) in the flight  
 980 muscle mitochondria of flies injected with saline solution (control) or different doses of  
 981 antibiotics. Dark squares, dark circles and asterisks report differences among the injected  
 982 doses of a given antibiotic;  $\blacksquare p < 0.05$ ,  $\bullet p < 0.01$ ,  $* p < 0.001$ .



983

984 **FIGURE 5** Scores and contribution plots of principal component analysis (PCA) of flight  
 985 muscle metabolome. **a:** Sample projection (4-6 replicates per experimental condition) onto the  
 986 first PCA plane (PC1 *versus* PC2) of the metabolic phenotypes of the flight muscles of the  
 987 blow flies collected two days after the injection of 100 or 200  $\mu\text{g}$  of chloramphenicol  
 988 (Chloramph C2 and C1, respectively), 2.5 or 10  $\mu\text{g}$  of cycloheximide per fly (Cyclohex C2  
 989 and C1, respectively), 10 or 30  $\mu\text{g}$  of tetracycline per fly (Tetracl C2 and C1, respectively).  
 990 Untreated flies were injected saline solution (Control\_saline), and a second group of flies was  
 991 just pricked with the syringe (control). **b:** Contribution plots of the metabolites to PC1 and  
 992 PC2.

993