Full Research Paper

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

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26

27 **CONFLICT OF INTEREST**

28 The authors declare no conflict of interest.

29

Abstract

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

INTRODUCTION

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

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

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

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

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

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

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

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

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

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

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

MATERIALS AND METHODS

Insect rearing

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

Experimental treatments: injection of antibiotics and monitoring of the survival of the flies

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

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

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

Extracellular footprints of the effects of antibiotic treatments on flight muscles

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

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

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

Physiological intracellular fingerprints of the effects of antibiotics

Here, antibiotic quantities generating around 50% mortality after one day (according to LD₅₀ values obtained from the survival experiment), and one lower and one higher dose, were injected to the flies for each drug (cefradine: 200, 400 or 600 µg/fly; chloramphenicol: 100, 200 or 250 µg/fly; cycloheximide: 10, 20 or 30 µg/fly; tetracycline: 20, 30 or 40 µg/fly), except for ampicillin for which we used an intermediate dose lower than LD₅₀ (100, 300 or 500 µg/fly). Flies were collected for physiological analyses 24 hours after the injections.

Isolation of mitochondria

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

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

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

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

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

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

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

Oxygen consumption of mitochondria was determined with the Warburg manometric technique at 25 °C (Tribe 1967), and expressed in terms of oxygen consumed per hour per milligram dry mass of mitochondrial flight muscles ($\mu\text{L O}_2/\text{mg/h}$ or QO_2). We focused on the mitochondria of the flies injected with antibiotics at the LD_{50} . For each replicate, a volume of 100 μL of the mitochondrial extract was homogenized into a standard reaction mixture containing 130 mM KCl, 10 mM Tris HCl (pH 7.4), 30 mM potassium phosphate, 30 mM glucose, 1 mg BSA/mL, and 10 μL 50 mM ADP. For this assay, pyruvate (10 mM) and proline (4 mM) were the supplied substrates. Then, a volume of 100 μL of sarcosomal suspension (containing 0.2-0.4 mg protein) was added. The center of the flasks was filled with 200 μL of 20% KOH, and an acid washed filter paper wick was added. An incubation period of 25 min at 25 °C was conducted, with air as the gas phase. The reaction was stopped by the addition of 1 mL of ice-cold 25% TCA, and the flasks were placed immediately on ice in a refrigerator at 0 °C to prevent degradation of ATP. The contents of the flasks were centrifuged (12,000 g for 5 min at 4 °C). Then, inorganic phosphate of the supernatant was determined by mixing 100 μL from each sample with 3 mL of copper acetate buffer, 0.5 mL of ammonium molybdate and 0.5 mL of reducing agent. After 10 min at room temperature, absorbance of the samples was read at 880 nm. Corrections for endogenous oxidation and phosphorylation were not done, since these amounts are small when mitochondria are isolated from intact tissues.

Measurement of the glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT)

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

Metabolomic fingerprinting of flight muscles treated with antibiotics

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

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

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

Statistical analysis

The Kaplan-Meier estimates and Mantel-Cox two-sided log-rank tests were used to calculate the mean cumulative mortality of the flies after antibiotic treatments ($\pm$ the standard error of mean, SEM), and draw the effects of doses of antibiotics on survival probabilities. For estimating LC₅₀ and comparing the different antibiotic doses, probit analysis was applied to fit

multiple regression using SAS 9.2 (SAS Institute Inc., NC, USA). LC₅₀ were obtained by drawing a cumulative 96 hours mortality curve (dependent variable).

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

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

RESULTS

Effect of different concentrations of antibiotics on the survival of the flies

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

df=5; chloramphenicol $\chi^2 = 101.35$, df=4; cycloheximide $\chi^2 = 168.85$, df=5; tetracycline $\chi^2 = 107.56$, df=3 (Fig. 1). LD₅₀ values for each antibiotic are presented in Table 1. At the lowest concentration, the injected antibiotics had small effects on the survival of *Ch. albiceps*. The number of dead individuals was the highest 1-day after injection for cefradine, chloramphenicol and tetracycline, whatever the concentration of the drug. As a result, LD₅₀ values remained stable for these three drugs when calculated 1 or 4 days after the injection (Table 1). Conversely, mortality progressed constantly over the four days post-injection for the flies that were treated with ampicillin or cycloheximide, and LD₅₀ values greatly varied. For instance, LD₅₀ was 21.76 (19.63-23.89) µg /fly for cycloheximide when calculated at day 1, and dropped to 7.09 (6.47-7.71) µg/fly when LD₅₀ was calculated at day 4.

Extracellular footprints of the effects of antimicrobial treatments on flight muscles

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

Physiological intracellular fingerprints of the effects of antibiotics

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

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

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

Oxidative phosphorylation of flight muscle mitochondria

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

The considerable inhibition of the rate of oxygen uptake following the treatment with high doses of antibiotics altered P/O ratio, which oscillated around 2 across in the experimental 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, pipecolic 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,

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

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

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

DISCUSSION

Effect of antibiotics on the survival of the flies

While the lowest doses of ampicillin, cefradine, chloramphenicol, cycloheximide and tetracycline induced little mortality to the flies, survival was very quickly impaired as soon as the quantity of drugs was increased. For cefradine, chloramphenicol, and tetracycline, most of the mortality of the flies was recorded one day after injection, and survival remained relatively stable afterwards. Contrariwise, mortality progressed over the course of the experiment when the flies were injected ampicillin or cycloheximide, as depicted by the decrease of LD₅₀ from day 1 to 4; yet, the highest mortalities were observed after a day at the highest doses for these two antibiotics. Our findings suggest that cefradine, chloramphenicol, and tetracycline may have diffused and reached their targets more quickly within the flies after the injection than the two other antibiotics. Moreover, the time-dependent sequence of antibiotic-associated effects that inhibit various vital cellular functions, such as transcription, translation, or DNA supercoiling, or alter the microbiome of the flies, must be drug-specific. At the highest antibiotic doses, the little to null activity of the ribosomes into the cells of the bacteria and flies likely explains the death of our insects.

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

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

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

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

Effects of antibiotics on mitochondrial structure and oxidative rate

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

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

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

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

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

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

In mammalian cells, antibiotics have been demonstrated to induce mitochondria dysfunction (Houtkooper et al., 2013; Kalghatgi et al., 2013; Moullan et al., 2015). Damages and swelling of mitochondria may lead to reduction to suppression of ATP production, because antibiotics alter the inner membrane of mitochondria, in turn affecting the coupling of oxygen consumption to ADP phosphorylation (Brand & Nicholls, 2011). In this part of the study, all antibiotics were injected at doses that cause *ca.* 50% mortality after one day (except for ampicillin), and we reported a considerable decrease of the oxidative rate of surviving flies as compared with controls. There are few data on the mode of action of antibiotics in insects; in mammals, antibiotics have been proven to alter the electron transfer chain, thus decreasing the mitochondrial membrane potential and ATP levels (Kalghatgi et al., 2013). The leakage of electrons from the electron transfer chain also contributes to increase the production of reactive oxygen species. For instance, treatments of mammalian cells with ampicillin increased the amount of superoxide ions, which potentially induces increased DNA and protein damages (Kalghatgi et al., 2013). While electron leaks are expected to affect mitochondrial coupling efficiency, the P/O ratios were similar among the different experimental conditions. This result suggests that the electrochemical gradient was not affected by the drugs, and revealed that ATP production *versus* O₂ consumption was reduced in antibiotic-treated flies.

The putative mitochondrial dysfunctions post-antibiotic treatments can be explored by ratiometric analysis of O₂ consumption (QO₂) after addition of pyruvate and proline (state 2 of mitochondrial respiration), followed by the addition of ADP to reach state 3, and returning to basal oxygen mitochondrial consumption when all ADP had been phosphorylated (state 4). Respiratory control ratio of state 3/state 4 is a measure of the level of mitochondrial coupling and phosphorylation efficiency (Brand & Nicholls, 2011). In antibiotic-treated flies,

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

Physiological underpinnings of the effects of antibiotics

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

Consistent with survival patterns, metabolic phenotypes of flies treated with chloramphenicol and tetracycline were similar, but distinct from the one measured from flies treated with cycloheximide. Injection of cycloheximide, an antifungal antibiotics that inhibits protein synthesis and that is used in agriculture (McManus et al., 2002; Wang et al., 2014), had the most striking effect on the metabolic phenotype of the flies and provoked a decrease of the levels of most metabolites. In *C. erythrocephala*, Ashour et al. (1980a) found that cycloheximide can inhibit amino acid incorporation in the young flies, a finding consistent with the inhibition of RNA translocation induced by cycloheximide (Schneider-Poetsch et al., 2010). The depletion of the quantities of most metabolites in flies treated with cycloheximide suggests that intermediary metabolism was globally depressed. The increased levels of fumaric acid measured from flies treated with cycloheximide should also be highlighted. We speculate that the conversion of succinic acid to fumaric acid remained functional in these flies, generating FADH₂, but the transfer of the electrons, and their entry into the electron-transfer chain at the complex II, may have been blocked. This could have resulted in a greater production of fumaric acid and FADH₂ in regards to their demand, leading to the accumulation of fumaric acid.

Increased levels of putrescine were observed in chloramphenicol- and cycloheximide-treated flies. Putrescine often accumulates during anoxia in plant cells, and indirectly contributes to H₂O₂ production under oxygen shortage (Blokhina et al., 2003).

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

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

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

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

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

Conclusion

Low doses of antibiotics had limited effects on the survival of newly emerged flies of *Ch. albiceps*. Yet, the extracellular footprints and physiological intracellular fingerprints we used demonstrated that these drugs have multiple direct and cascading effects on the structure of the flight muscles and physiology of the flies. The time-dependent sequence of antibiotic-associated effects was drug-specific, with LD₅₀ that greatly varied for ampicillin and cycloheximide from day 1 to 4 post injection. Flies treated with chloramphenicol and tetracycline had altered oxidative phosphorylation and activity of the Krebs cycle, likely compensated by an increased activity of transaminase enzymes. The drop of oxidative phosphorylation correlated with damages of mitochondria. Cycloheximide had the most pronounced physiological effects on the phenotype of the flies, and quickly provoked their death, even at low doses. Overall, our findings suggest that antibiotic pollution could greatly affect insect population dynamics, which could be particularly problematic for beneficial insects and insect community structure. The effects on the trophic network should be explored in future studies.

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

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

FIGURE 3 Oxidation rate of pyruvate+proline (mean $\pm$ S.E.) from flight muscle mitochondria (a), and P/O ratio (mean $\pm$ S.E.) from with flight muscle mitochondria (b). 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 ± 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; ■ $p<0.05$, ● $p<0.01$, * $p<0.001$.

FIGURE 5 Scores and contribution plots of principal component analysis (PCA) of flight muscle metabolome. **a:** Sample projection (4-6 replicates per experimental condition) onto the first PCA plane (PC1 *versus* PC2) of the metabolic phenotypes of the fight muscles of the blow flies collected two days after the injection of 100 or 200 μg of chloramphenicol (Chloramph C2 and C1, respectively), 2.5 or 10 μg of cycloheximide per fly (Cyclohex C2 and C1, respectively), 10 or 30 μg of tetracycline per fly (Tetracl C2 and C1, respectively). Untreated flies were injected saline solution (Control_saline), and a second group of flies was just pricked with the syringe (control). **b:** Contribution plots of the metabolites to PC1 and PC2.