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Developmental exposure of antibiotics shortens life span and induces teratogenicity in *Drosophila melanogaster*

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ABSTRACT

Antibiotics are the major therapeutic arsenal against bacterial infections. Yet, beneath this medical triumph lies an under investigated challenge of the potential teratological and toxicological impacts associated with the use of antibiotics. In the present study, we have explored the teratogenic potential of five commonly used antibiotics (streptomycin, metronidazole, tigecycline, doxycycline and norfloxacin) on *Drosophila melanogaster* Oregon-R strain. Except norfloxacin, all other tested antibiotics significantly delayed the onset of pupariation. Consistently, metronidazole, doxycycline and tigecycline resulted in statistically significant drops in egg-to-adult viability and adversely affected egg-to-pupa transition. In comparison, embryonic exposure of streptomycin impeded pupa-to-fly transition. All tested antibiotics induced morphological defects in antenna, wings, proboscis, eye, head, thorax, haltere and abdomen. Interestingly, developmental exposure of antibiotics resulted in statistically significant decrease in the lifespan of both male and female flies. This suggests an increased incidence of teratogenic faults at the systemic level, which are otherwise not manifested morphologically, due to the exposure of tested antibiotics during development. Taken together, our data show that developmental exposure of antibiotics may induce varying degrees of teratogenicity in *D. melanogaster*. Given the genomic homology and conservation of major molecular pathways that underpin development in humans and *D. melanogaster*, the findings do hold translational potential.

1. Introduction

Globally, infectious diseases account for over 50 % of human diseases, of these a significant portion is contributed by bacterial infections [1]. Since antibiotics are the most employed intervention for the treatment of bacterial infections, therefore, it is not surprising that usage of antibiotics has increased by 46 % from 2000 to 2018 [2]. Albeit antibiotics in different forms and shape have proven their efficacy in controlling bacterial infection, but nearly all these therapeutic molecules hold clinical shortcomings [3,4]. For example, antibiotics like penicillin, cephalosporins, carbapenems, clindamycin, erythromycin and norfloxacin are associated with the dysbiosis of normal flora in humans [5]. In addition, nitrofurantoin. azithromycin, penicillin V, cephalexin and linezolid antibiotics are linked with disturbance of normal physiology to even cellular or systemic toxicity [6]. Alarmingly, few antibiotics like chloramphenicol and metronidazole are included in the list of probably carcinogenic to humans (group 2 A) and possibly carcinogenic to humans (group 2B), respectively by International Agency for Research on Cancer (IARC) [7]. By the same token, teratogenic potential of several antibiotics, for example, metronidazole, ornidazole and tetracyclines have also been reported in animal and human studies [8–10].

Teratogenicity refers to the potential of an agent to induce developmental defects in the fetus during pregnancy. In humans, the most sensitive period for teratogenesis is usually the first trimester of pregnancy, when the major organs and systems of the fetus are formed, however, this is also the phase where diagnosis of pregnancy is conventionally uncertain [11–13]. Since, antibiotics constitute a dominant majority, around 80 %, of all medications prescribed during pregnancy, this ambiguity may result in the prescription and/or consumption of antibiotics with teratogenic potentials by pregnant females [14]. Antibiotics such as tigecycline, chloramphenicol, streptomycin, doxycycline, ciprofloxacin, norfloxacin, tobramycin, gentamicin, and

Abbreviations: BAM,, Banana Agar Medium; CM, Corn Meal; MIC, Minimum Inhibitory Concentrations; SEM, Scanning Electron Microscopy.

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sulfonamide-trimethoprim combinations have shown their teratogenic potential in different animal model studies [10,14,15]. For example, streptomycin, an aminoglycoside antibiotic, is prescribed to treat brucellosis, endocarditis, plague, tuberculosis, and tularemia disease, is linked to the increase ototoxicity [16]. Doxycycline, a bacteriostatic antibiotic, generally advised for skin, dental, respiratory tract, sexually transmitted and urinary tract infections have also been associated with liver necrosis, dental, skeletal malformations, and rare fetal hepatotoxicity [10,17]. The fluoroquinolone, norfloxacin, is used for the treatment of gonorrhea, prostate and urinary tract infections but can cause visceral abnormalities, cardiac hyperplasia, pulmonary hypoplasia, kidney hypoplasia or atrophy, hypoplasia, absence of thymus gland, and skeletal malformations in developing fetus [18]. Chloramphenicol, although effective against cholera, conjunctivitis, meningitis, plague, and typhoid fever, carries the risk of bone marrow suppression and gray baby syndrome [10]. Usage of sulfonamides are associated with incidence of anencephaly, hypoplastic left heart syndrome, coarctation of aorta, choanal atresia, transverse limb deficiency and diaphragmatic hernia [10].

Despite teratogenic effects of many antibiotics are known, there are several diverging hypotheses and controversial findings in this regard [19–21]. Moreover, no long term follow up studies have been conducted focusing on the wellbeing of newborns who may have been exposed to antibiotics during their embryonic development. Consistently, antibiotics have not been studied for their potential link to failed pregnancies. This all warrants a controlled investigation to assess the teratogenic potential of the antibiotics using animal models. The teratological effects of antibiotics have been investigated in albino rats, zebra fish, rabbits, and to a limited scale in *Drosophila melanogaster* [8,9,22–26]. Due to the shorter life cycle, simple genetics, high fecundity, and fast reproduction rate, *D. melanogaster* holds a peculiar advantage over other animal models [27,28]. In addition, *D. melanogaster* shares 60 % genomic similarity with humans and around 75 % of human disease genes have orthologues present in fruit fly, representing its translational importance [29]. Accounting this, herein we have assessed the teratogenic potential of five commonly used antibiotics using *D. melanogaster* as model organism. Our findings showed that exposure of antibiotics during development may render both short-term and long-term teratogenic effects on the progeny.

2. Materials and methods

2.1. Drosophila stock

Wild type Oregon-R strain of *Drosophila melanogaster* was maintained in Banana Agar Medium (BAM) containing 50 % (w/v) banana, 1.667 % (w/v) agar technical, 0.3 % (v/v) propionic acid and 0.05 % (w/v) instant yeast. The flies were kept at 25 ± 3 °C under a 10/12-hour day-night cycle with 40–60 % relative humidity.

2.2. Antibiotics tested

Streptomycin sulfate (BIOSYNTH Carbosynth: 3810–74–0), metronidazole (BIOSYNTH Carbosynth: 443–48–1), doxycycline monohydrate (SIGMA-ALDRICH: 17086–28–1), norfloxacin (AmBeed: 70458–96–7) and tigecycline (AmBeed: 220620–09–7) were assessed in the present study. The streptomycin sulfate and metronidazole were solubilized in milli-Q water, whereas norfloxacin and doxycycline monohydrate were dissolved in 99 % and 33 % of glacial acetic acid, respectively. DMSO was used to dissolve tigecycline. All media were standardized with the corresponding amount of solvent in the control set as well.

2.3. Experimental setup

2.3.1. Experimental treatment

Standard Corn Meal (CM) medium containing 10.09 % (w/v) corn meal, 3.04 % (w/v) sucrose, 6.07 % (w/v) dextrose, and 0.5 % (w/v) technical agar, 0.125 % methyl 4-hydroxybenzoate (nipagin) and 0.05 % yeast were used during the experiment. Three different concentrations of antibiotics were selected and incorporated in the CM medium according to the Minimum Inhibitory Concentrations (MIC), lowest drug concentrations reported to inhibit the growth of bacterial pathogens [30], as test vials (Table 1). Corresponding controls containing appropriate volume of the dissolving solvent (DMSO and glacial acetic acid) were also used.

2.3.2. Egg collection

For egg collection, 10 males and 10 female flies were placed in a glass vial containing 8 mL of 2.5 % agar medium, with a drop of yeast suspension in distilled water (1:2). The vials were incubated at 25 ± 3° C temperature, 40–60 % relative humidity in 10/12-hour day-night cycle for two days. After two days, flies were removed and 1 mL of 29 % sucrose solution was dispensed in a vial and stirred gently. The contents were then filtered through a 70 μm cell strainer (FALCON). Under stereomicroscope (Leica Zoom 2000), eggs were sieved out from the suspension. In each of the control and test vials, 40 eggs of *D. melanogaster* were placed using a fine-tip brush. A minimum of 13 replicates (developmental exposure) and a maximum of 25 replicates (survival curve) were run during the investigation for each control and test concentration of each antibiotic.

2.4. Developmental teratogenicity

In order to check teratogenic effect of antibiotics on the development of *D. melanogaster*, a total of 500 eggs (40eggs/vial) were placed in control and experimental sets and incubated at 25 ± 3 °C, 40–60 % relative humidity and a 10/12-hour day-night ratio. The vials were daily observed for the onset of pupariation and eclosion. Subsequently, egg-to-adult viability, partial and complete failure and stillbirth fraction were determined at the termination of experiment (day 20) as follows.

2.4.1. Egg-to-adult viability

The egg-to-adult viability was deduced as a percentage between the total number of flies hatched (x) per 40 eggs placed in each vial as following.

$$\text{Egg-to-adult viability} = (x/40)*100$$

2.4.2. Complete failure

At day 20 (termination day), the media from each vial is extracted and carefully examined for the number of puparium (z). Operationally, complete failure refers to the number of eggs that failed to transfer into pupa and deduced as following:

$$\text{Complete failure} = (40\text{-}z/40)*100$$

Table 1
Concentrations of antibiotics (μg/mL) used in the present investigation.

Antibiotics	Half MIC (μg/mL)	Standard MIC (μg/mL)	Double MIC (μg/mL)
Streptomycin Sulfate	512	1024	2048
Metronidazole	512	1024	2048
Tigecycline	16	32	64
Doxycycline Monohydrate	4	8	16
Norfloxacin	32	64	128

2.4.3. Partial failure

Partial failure refers to the number of flies that failed to emerge from pupa and deduced as following.

Partial failure = $(z–x/40)*100$

2.4.4. Fraction of stillbirth

The fraction of stillbirth was deduced by dividing the number of stillbirths (flies found dead immediately after eclosion) with the total number of hatched flies.

2.5. Morphological teratogenicity

The teratogenic effect of antibiotics on the morphological and/or anatomical structures of the flies were assessed by both stereomicroscopy and ultramicroscopy.

2.5.1. Stereomicroscopy

Adult flies were examined for morphological defects under a stereomicroscope (Olympus SZX 12) at 7 X – 95 X magnifications. Morphological traits such as eye shape and texture, mouth parts, head shape, bristles (scutum, scutellum and abdomen), legs, tergites, haltere shape, wing shape and wing venation were observed at different magnifications. Defected flies were photographed at 50 X – 95 X as deemed appropriate.

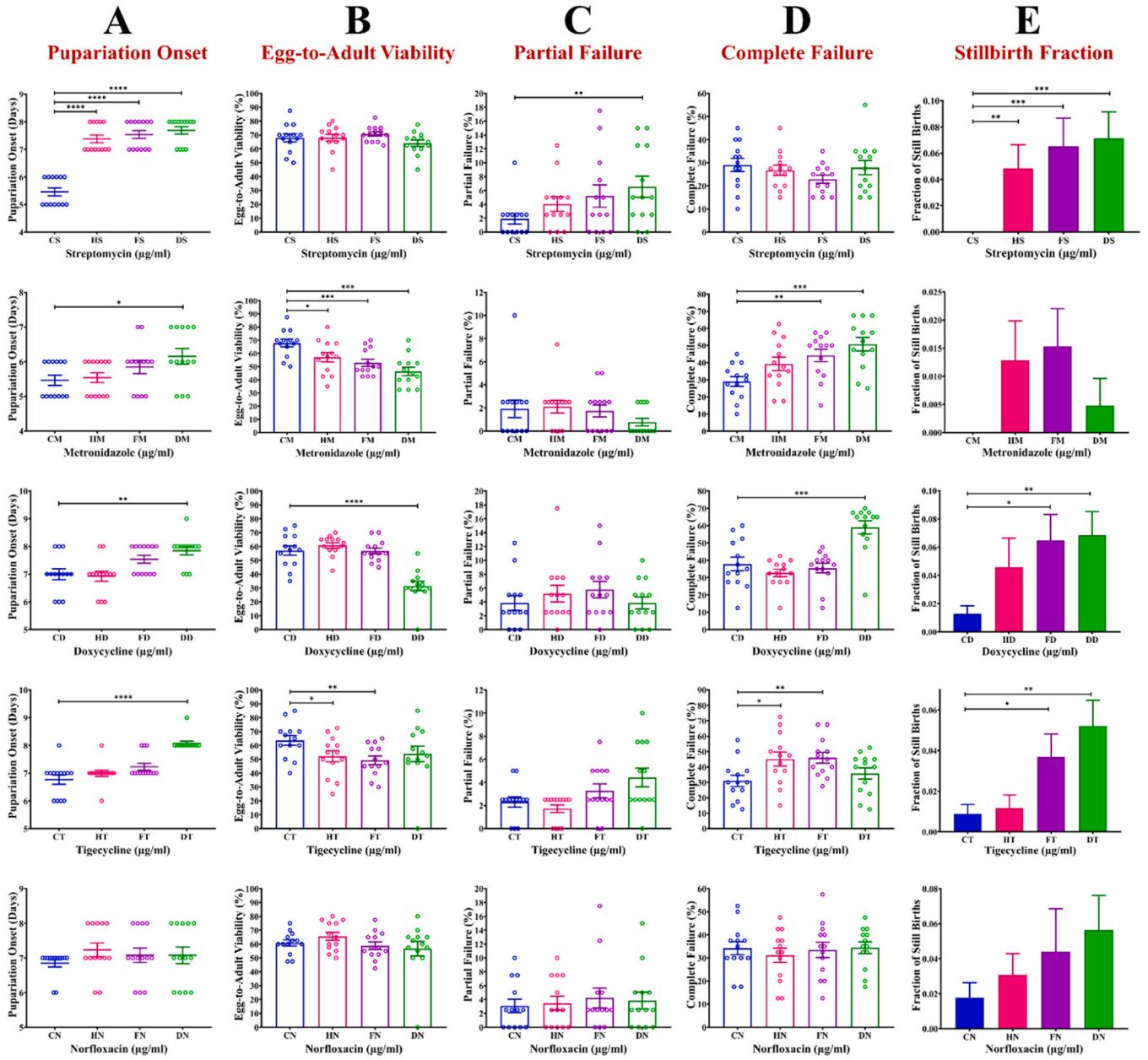

Fig. 1. Graphs showing effect of developmental exposure of antibiotics on (A) pupariation onset, (B) egg-to-adult viability, (C) partial failure, (D) complete failure and (E) stillbirth fractions in *D. melanogaster*. Where C: control, H: half of Minimum Inhibitory Concentration (MIC), F: standard MIC, D: 2X MIC, S: streptomycin sulfate, M: metronidazole, D: doxycycline monohydrate, T: tigecycline and N: norfloxacin as indicated in Table 2. The large and small horizontal lines or height of the bars and error bars represents mean and standard error of mean, respectively. Whereas "*" represents the degree of statistical significance. <0.0001) was found to delay pupariation span in *D. melanogaster* compared to corresponding control groups (Fig. 1A).

2.5.2. Scanning electron microscopy (SEM)

Flies with discernable morphological defects were examined under scanning electron microscope (JSM-IT100). The flies were initially subjected to dehydration as defined by Tare et al. [31], to keep the morphology of flies intact. The dehydrated flies were coated with thin platinum layer using JEOL sample coater (JEC-3000FC) for better conductivity. The coated flies were then examined and photographed at different magnifications.

2.6. Kaplan-Meier survival curve

Virgin flies (100 males and 100 females per control and experimental set) eclosed from control and test vials were transferred to standard cornmeal medium (without antibiotics) and incubated at 25 ± 3° C temperature, 40–60 % relative humidity in 10/12-hour day-night cycle. In each vial 10–12 flies were placed and after every 10 days, the files were shifted to the fresh cornmeal medium (without antibiotics). Mortality of files were monitored daily till all the flies died.

2.7. Statistical analysis

All data were statistically analyzed using GraphPad Prism v.8.0.1. To evaluate the distribution of the data, both Sapiro Wilk and Kolmogorov Smirnov test was used as deemed appropriate. Statistical significance was determined using t-test and Mann-Whitney test where applicable. Survival estimation was done using a Kaplan-Meier survival curve. A p-value of less than 0.05 was considered statistically significant in all cases.

3. Results

3.1. Antibiotics disturbed pupariation onset span

Except norfloxacin, developmental exposure of four out of five tested antibiotics disturbed the span of pupariation onset in Oregon R-strain of *D. melanogaster* (Fig. 1A, Table 2). In this regard, all three tested concentrations of streptomycin showed significantly delay (p= <0.0001) in the pupariation onset in *D. melanogaster* compared to control. In comparison, exposure to only highest concentration of metronidazole (p= 0.0323), doxycycline (p= 0.0062) and tigecycline (p=

3.2. Antibiotics dropped egg-to-adult viability

Among five tested antibiotics, three antibiotics showed a significant decline of egg-to-adult viability in *D. melanogaster* during development (Fig. 1B, Table 2). Metronidazole displayed a concentration dependent decline in egg-to-adult viability in *D. melanogaster* at all three concentrations (HM: p= 0.0316, FM: p= 0.0007, and DM: p= 0.0001) compared to the control. Whereas exposure to both half (p= 0.0160) and standard MIC (p= 0.0012) of tigecycline resulted in a significant decrease in egg-to-adult viability in *D. melanogaster*. Additionally, a significant reduction (p= <0.0001) in egg-to-adult viability of *D. melanogaster* was found only at exposure of highest concentration of doxycycline compared to control. Conversely, streptomycin and norfloxacin did not exhibit any significant effects on egg-to-adult viability of *D. melanogaster* (Fig. 1B).

3.3. Antibiotics increased developmental failure

Out of all tested antibiotics, developmental exposure to only the highest concentration of streptomycin showed a statistically significant increase (p= 0.0086) in the partial failure (pupa- to-fly transition) in *D. melanogaster* (Fig. 1C, Table 2). In comparison, exposure of three out of five tested antibiotics during development resulted in an increased incidence of complete failure at different concentrations in *D. melanogaster* (Fig. 1D, Table 2). For example, a statistically significant increase in complete failure (egg-to-pupa transition) in *D. melanogaster* was observed at both medium (p= 0.0032) and highest (p= 0.0005) of metronidazole compared to control. Similarly, tigecycline exposure also resulted in a significant increase in complete failure at half (p= 0.0161) and standard (p= 0.0041) MIC in *D. melanogaster* and doxycycline showed complete developmental arrest during the egg-to-pupa transition (p= 0.0006) in *D. melanogaster* only at the exposure of the highest concentration (Fig. 1D). In addition, developmental exposure of streptomycin, tigecycline, and doxycycline exhibited a dose-dependent teratogenic impact in *D. melanogaster*, manifested as a statistically

Table 2
Effect of antibiotics on the developmental parameters of *D. melanogaster*.

Antibiotics (µg/mL)	Pupariation Onset	Egg-to-Adult Viability (%)	Partial Failure (%)	Complete Failure (%)	Stillbirth Fraction
Streptomycin Sulfate					
Control (0.0)	5.5 ± 0.52	67.88 ± 10.25	1.92 ± 2.73	29.04 ± 10.18	0.00 ± 0.00
Half MIC (512)	7.4 ± 0.51	68.08 ± 9.08	4.04 ± 3.75	26.73 ± 7.99	0.05 ± 0.06
Standard MIC (1024)	7.5 ± 0.52	70.77 ± 5.53	5.19 ± 5.82	22.88 ± 6.36	0.06 ± 0.08
Double MIC (2048)	7.7 ± 0.48	64.04 ± 8.19	6.54 ± 5.45	27.88 ± 11.27	0.07 ± 0.07
Metronidazole					
Control (0.0)	5.5 ± 0.52	67.88 ± 10.25	1.92 ± 2.73	29.04 ± 10.18	0.00 ± 0.00
Half MIC (512)	5.5 ± 0.52	57.12 ± 12.7	2.11 ± 2.00	39.23 ± 14.34	0.01 ± 0.02
Standard MIC (1024)	5.8 ± 0.69	52.88 ± 9.89	1.73 ± 1.88	44.23 ± 13.01	0.01 ± 0.02
Double MIC (2048)	6.1 ± 0.80	46.35 ± 11.57	0.77 ± 1.20	50.77 ± 14.30	0.01 ± 0.02
Tigecycline					
Control (0.0)	6.8 ± 0.59	63.65 ± 12.89	2.31 ± 1.60	30.96 ± 12.97	0.01 ± 0.02
Half MIC (16)	7.0 ± 0.41	52.12 ± 14.39	1.73 ± 1.20	45.19 ± 16.60	0.01 ± 0.02
Standard MIC (32)	7.2 ± 0.44	49.23 ± 11.29	3.27 ± 2.14	45.96 ± 12.52	0.04 ± 0.04
Double MIC (64)	8.1 ± 0.28	53.85 ± 20.15	4.42 ± 2.91	35.77 ± 13.28	0.05 ± 0.04
Doxycycline Monohydrate					
Control (0.0)	7.0 ± 0.71	57.12 ± 12.11	3.85 ± 3.77	37.88 ± 13.99	0.01 ± 0.02
Half MIC (4)	6.9 ± 0.64	60.77 ± 7.25	5.19 ± 4.38	32.69 ± 7.87	0.05 ± 0.07
Standard MIC (8)	7.5 ± 0.52	56.73 ± 7.66	5.77 ± 4.25	35.58 ± 10.06	0.06 ± 0.07
Double MIC (16)	7.8 ± 0.55	31.54 ± 12.1	3.85 ± 3.16	59.04 ± 13.52	0.07 ± 0.06
Norfloxacin					
Control (0.0)	6.8 ± 0.37	60.77 ± 8.06	3.08 ± 3.41	34.23 ± 10.33	0.02 ± 0.03
Half MIC (32)	7.2 ± 0.72	65.58 ± 10.52	3.46 ± 3.61	31.15 ± 11.26	0.03 ± 0.04
Standard MIC (64)	7.1 ± 0.76	58.85 ± 9.72	4.23 ± 5.14	33.46 ± 12.14	0.04 ± 0.09
Double MIC (128)	7.1 ± 0.86	56.73 ± 18.89	3.85 ± 4.40	34.42 ± 8.97	0.06 ± 0.08

Mean ± Standard deviation
MIC: Minimum Inhibitory Concentration

significant increase in stillbirth fraction (Fig. 1E, Table 2).

3.4. Antibiotics induced morphological aberrations

Exposure to all five antibiotics during development resulted in a significant increase in the proportion (1.3–1.76 x) of morphologically defective flies compared to their corresponding controls (Fig. 2A). However, the frequency of errors of different body parts of *D. melanogaster* varied in magnitude among the tested antibiotics where defects in antennae, wings, and halteres are most observed, whereas legs were found to be least affected (Fig. 2B).

Stereomicroscopic examination revealed a variety of morphological aberrations in flies developed under different concentrations of antibiotics (Fig. 3). Streptomycin, metronidazole and doxycycline exposure during embryonic development in flies induced antennal and proboscis blackening, eye depression and spotting, undeveloped/anencephalic head with ectopic structures, wing darkening and crumpling with spotting, abnormal thoracic curvature with depression, trident-shaped thoracic pigmentation, pigmented halteres, tergite malformations, and severe abdominal depression with tumor-like growths (Fig. 3A-C). Metronidazole exposure specifically induced thoracic malformations, including spots and a thoracic cleft-like depression (Fig. 3B). Doxycycline exposure resulted in malformations such as missing eye, proboscis outgrowth, tumor-like growth on cephalic furrow, defective thoracic closure and missing wing (Fig. 3C). In comparison, tigecycline exposure also induced various structural abnormalities, including reduced eye size, undeveloped head structures, abdominal darkening and tumor-like growth, wing crumpling, blistering and spotting, tergite malformations, and thoracic deformation with tumor-like growth, defective thoracic closure and depression (Fig. 3D).

Ultramicroscopic examination of representative defected flies further resolved antibiotic induced structural malformations in head, thorax, haltere and wings (Fig. 4). Compared to morphologically normal fly (Fig. 4A), partial head formation (Fig. 4B), reduced eye size/ reduced eye facets (Fig. 4C), and absence of one eye (Fig. 4D), abnormal thoracic curvature including defective thoracic closure and cleft like depression (Fig. 4E-I), blistered and vestigial wings (Fig. 4F,G), lack of a wing development (Fig. 4I) were observed.

3.5. Antibiotics exposure reduced lifespan

Kaplan-Meier survival curves showed a significant decrease in lifespan in both male and female flies following developmental exposure to all tested antibiotic (Fig. 5). Male and female flies developed in the presence of all concentrations of streptomycin, doxycycline and tigecycline showed significant reduction in the lifespan compared to their respective controls (Fig. 5A,B). The only small exception is the insignificant difference in survival of male flies developed in the presence of half MIC of tigecycline compared to control (Fig. 5A). A significant decreased in survival was observed in male progeny flies exposed to only 2X MIC of norfloxacin during development (Fig. 5A) whereas the female flies showed decreased in life span at all concentrations of norfloxacin (Fig. 5B). Similarly, male flies developed at half and 2X MIC of metronidazole showed significant decreased in lifespan whereas female flies showed decreased in lifespan due to developmental exposure of metronidazole at all concentrations (Fig. 5A,B).

4. Discussion

Drosophila melanogaster has been increasingly employed as a model organism to screen the biological and toxicological effects of different bioactive molecules including drugs and/or antibiotics [32–35]. However, limited studies are conducted in relation to the exploration of teratogenic effect of antibiotics using *D. melanogaster* as model organism [26,36,37]. In this investigation, we assessed the teratogenic potential and long-term effect in terms of longevity of five commonly used antibiotics in Oregon-R strain of *D. melanogaster*.

Pupariation span is considered one of the most sensitive traits of *D. melanogaster* that gets affected in the presence of variety of physical and/or chemical stresses [38–40]. Except norfloxacin, all assessed antibiotics delayed the pupariation in *D. melanogaster* (Fig. 1A). Previously, exposure of gemifloxacin, streptomycin and minocycline has also been shown to cause delay in pupariation in w^{1118} and wild type strains of *D. melanogaster* [32,36,41]. Likewise, noticeable delay in pupal development has also been noticed in other insect species like *Galleria mellonella*, *Calliphora vomitoria*, *Lucilia sericata* and *Protophormia terraenovae* when exposed to penicillin, streptomycin, fluconazole, ceftriaxone and levofloxacin during development [42–45]. It is important to mention that streptomycin, tigecycline and doxycycline are protein synthesis inhibitors and included in the category "D" by Food and Drug Administration (FDA) due to existence of strong evidence regarding their detrimental effect on human developing fetuses [14,46]. Consistent to this, compared to DNA replication inhibitors (metronidazole and norfloxacin), protein synthesis inhibitors (streptomycin, doxycycline and tigecycline) showed a more pronounced effect on the pupariation span of *D. melanogaster* (Fig. 1A). Moreover, pupariation in *D. melanogaster*

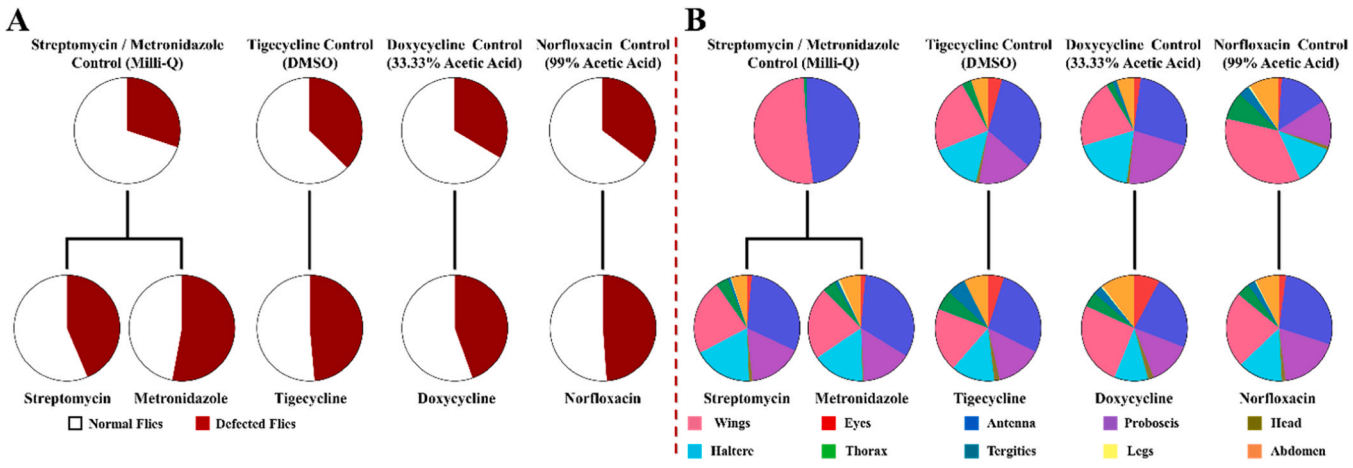

Fig. 2. Pie chart graphs represent (A) the percentage of normal and defective flies developed under exposure to different antibiotics and their respective controls, as labelled. Pie charts showing (B) distribution of different anatomical defects in flies developed under exposure of different antibiotics and their respective controls, as labelled. The color key on the bottom right panel indicates corresponding anatomical regions in pie charts. For details kindly see supplementary file 1 (Table S1 and S2). Solvents used for dissolving the antibiotics are also mentioned.

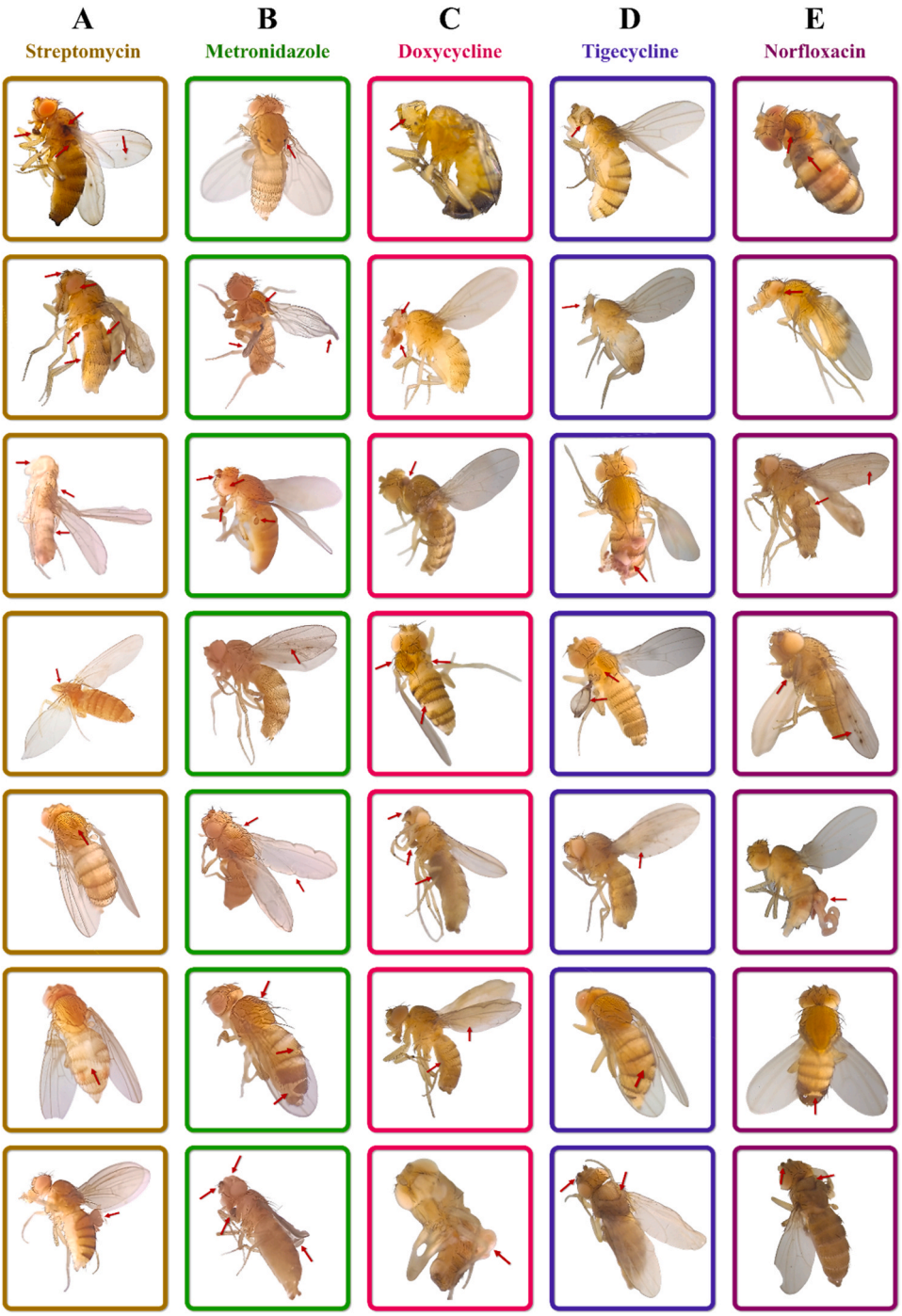

Fig. 3. Stereomicrographs representing different morphological aberrations in representative flies developed under different concentrations of (A) streptomycin, (B) metronidazole, (C) doxycycline, (D) tigecycline and (E) norfloxacin. The defective regions are indicated by red arrows.

and other insects are underpinned by ecdysone pathway [47–49]. Sequence homology studies have shown the orthologous relationship between proteins of ecdysone pathway and proteins engaged in human cancer. For example dBrms1 and Eip75B proteins of *D. melanogaster*, involved in ecdysone pathway, are orthologous to the human BRMS1 and NR1D2 proteins, known for their association with brain and skin cancers [50,51]. Since most teratogenic agents are carcinogenic as well [52–55], therefore, it is conceivable that observed delay in pupariation due to antibiotics exposure may be due to disturbance in ecdysone pathway and represent teratogenic potential of antibiotics. Collectively, the data suggest that the pupariation span of *D. melanogaster* may be a valuable marker to assess the potential teratological impact of compounds in preclinical studies.

Except norfloxacin, exposure of all assessed antibiotics during development impeded transition in D. melanogaster. For example, failure of pupa-to-fly transition, refers to as partial failure was observed in case of streptomycin (Fig. 1C). Likewise, failure in the transition of egg-to-pupa, refer to as complete failure, was observed in case of metronidazole, doxycycline and tigecycline exposure (Fig. 1D). This represents that teratogenic impact of streptomycin may vary in relation to stage of *D. melanogaster* development compared to metronidazole, doxycycline and tigecycline. Interestingly, teratogens also vary in their impacts in relation to human gestation period. For example, certain antibiotics like clarithromycin, trimethoprim, valproic acid and streptomycin pose risks

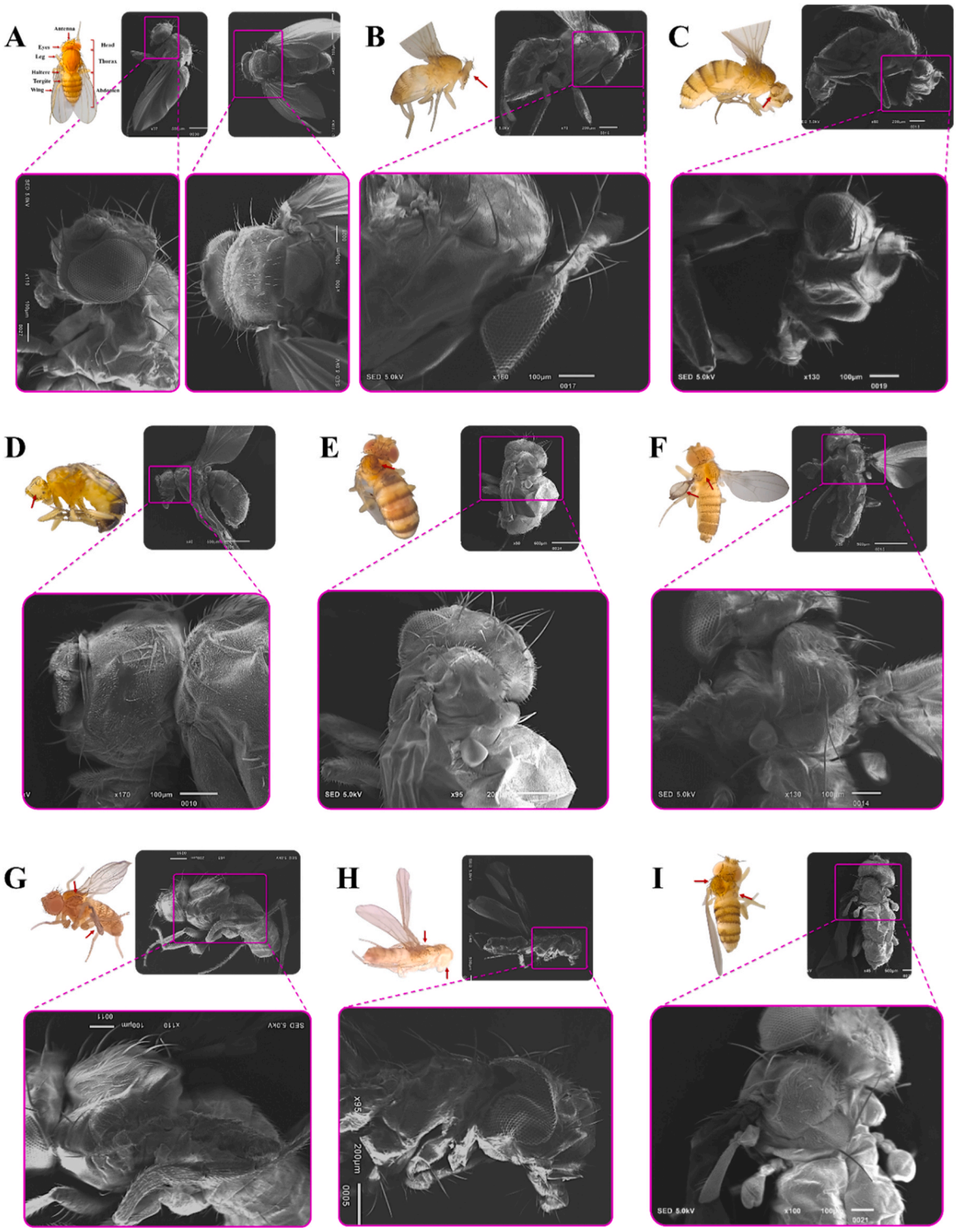

Fig. 4. Scanning electron micrographs of (A) control fly and defected flies exhibiting (B) incomplete head formation, (C) small eye, (D) missing eye, (E) abnormal thoracic closure with missing wing, (F) defective thoracic closure with blistered wing, (G) thoracic cleft like depression with vestigial wing, (H) abnormal thoracic curvature and depressed eye and (I) defective thoracic closure with one wing absent and indentation in haltere.

to the developing fetus when administered during early pregnancy [15, 56–58] however, other antibiotics (oxytetracycline, sulfonamides, amoxicillin, aspirin and nonsteroidal anti-inflammatory drugs) may carry heightened risks during the later stages of pregnancy [59–62]. It is also important to note that egg-to-pupa transition engaged molecular pathways like Fibroblast Growth Factor (FGF), wingless (Wnt), hedgehog (Hh), Transforming Growth Factor-beta/Activin (TGFβ), Bone Morphogenetic Protein (BMP) and notch signaling [63–67] whereas metamorphosis (pupa-to-fly) entails ecdysone, insulin signaling, Target of Rapamycin Complex 1 (TORC1) and JAK/STAT pathways [47, 68–71]. In humans, development of organs and systems mostly occurs in the first trimester [11], and the stage is analogous with egg-to-pupa transition in *D. melanogaster* due to similarities in underlying molecular events [72–76]. Therefore, developmental exposure of antibiotics like metronidazole, doxycycline and tigecycline that has shown increased incidence of failure of egg-to-pupa transition may likely

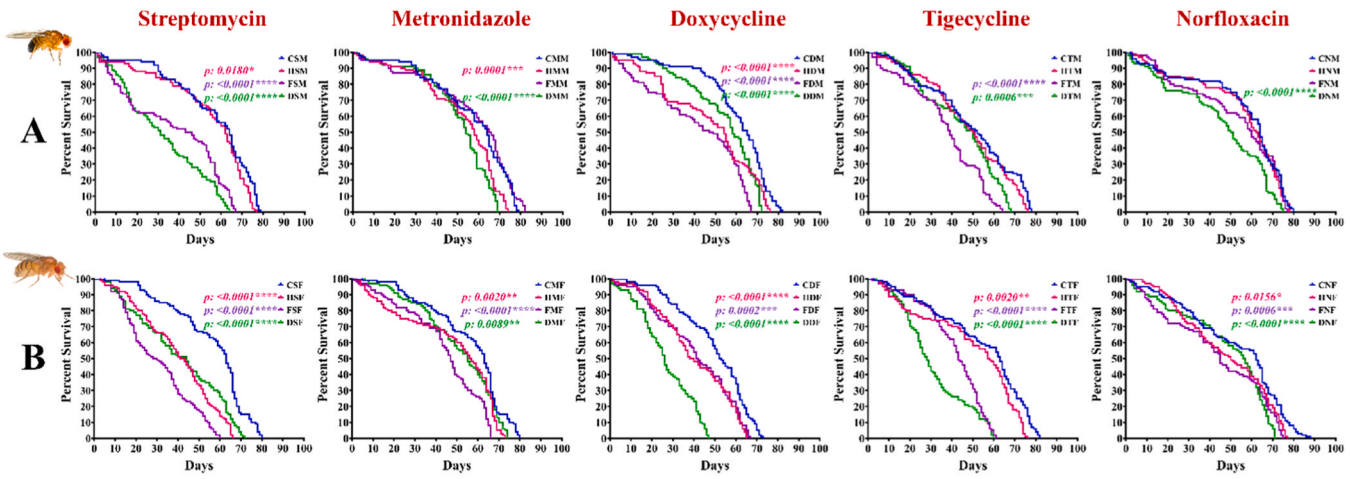

Fig. 5. Kaplan-Meier survival curve illustrates percent survival of (A) male and (B) female flies exposed to varying concentrations of antibiotics during development. Blue, pink, purple, and green line represent control, half, standard and 2X Minimum Inhibitory Concentrations (MIC), respectively, of each antibiotic, as labelled. The statistical significance of the difference in longevity was shown in inset.

impact the developing fetus in humans during first trimester whereas streptomycin may impact later stages of development in humans. Moreover, the first trimester is the most critical phase of development in humans with more than 60 % of pregnancies are failed in this phase [77]. In line with this observation, antibiotics (metronidazole, doxycycline and tigecycline) which adversely affect egg-to-pupa transition in *D. melanogaster* also leads to low egg-to-adult viability compared to streptomycin which affect mainly on metamorphosis but not egg-to-pupal transition and egg-to-adult viability despite showing high fraction of stillbirth (Fig. 1).

Stratification of teratogenic error showed wings are the most affected region in flies due to developmental exposure of antibiotics (Fig. 2B). There are nineteen imaginal discs in *D. melanogaster* responsible for the development of all major organs [78]. Out of these, wings are formed by the largest imaginal disc located at thoracic segment (T2) of 3rd instar larvae containing approximately 50,000 cells at L3 stage originating from merely 30 progenitor cells in few days [79,80]. Therefore, it is conceivable that wing imaginal discs are more prone to mutation and/or develop aberrations upon exposure of mutagens due to active cellular proliferation or genomic amplification [81].

Streptomycin, doxycycline and tigecycline are included in class "D", therefore already prohibited to be prescribed during pregnancy [14,46] and our data aligns well with the current positioning of these antibiotics. In comparison norfloxacin is included in class "C" where strong evidence in relation to teratogenicity is not available [82]. Our study shows that norfloxacin by and large did not show any effect on the development and fecundity of *D. melanogaster*. This may suggest that norfloxacin may be readjusted in the teratogenesis classification scheme of antibiotics to class B. Conversely, metronidazole is a class "B" antibiotic and considered safe to be used during pregnancy [82,83]. However, many studies in albino rats have shown that exposure of metronidazole during gestational period results in significant reduction in the number of implantation sites and concurrently increases the rate of embryonic resorption or dead embryos [8,84]. This is consistent with our findings where developmental exposure of metronidazole resulted in more profound loss in egg-to-adult viability and increase frequency of aborted development and teratological faults in *D. melanogaster* (Figs. 1B,D and 2–4).

Developmental exposure of all five tested antibiotics showed decreased in longevity of both male and female flies. Compared to other antibiotics, norfloxacin did not profoundly affect the longevity of flies, which is consistent to our observation that norfloxacin did not show any noticeable impact on the development of *D. melanogaster* (Figs. 1,5). Nevertheless, the decreased longevity of flies developed under the exposure of antibiotics demonstrates the existence of teratogenicity beyond anatomical level. In humans, prenatal and early life antibiotic exposure has been linked to increased risks of atopic and metabolic disorders later in life, potentially contributing to a decreased lifespan [85]. Alternatively, this could be due to the depletion of normal flora in the fly. Normal flora of *D. melanogaster* is constituted by mainly *Lactobacillus* and *Acetobacter* species of bacteria [86,87]. Dysbiosis in normal flora has also been shown to decrease the lifespan of flies [88–91]. However, this possibility could be weakened or even ruled out since only developing stages (larvae) were exposed to antibiotics and flies after eclosing were transfer to media without antibiotics during the survival curve analysis, where axenic conditions were not in place. Therefore, it is rather more plausible that decreased survivability of otherwise normal flies is because of the metabolic teratogenicity where structure or expression of enzymes crucial for normal physiology may have been affected.

5. Conclusion

In summary, the present study showed that except for norfloxacin, all other screened antibiotics showed teratogenic potential in *D. melanogaster* at varying levels. Moreover, long-term effect of developmental exposure of antibiotics have also been explored, showing reduced longevity in *D. melanogaster*. Though streptomycin, doxycycline and tigecycline are prohibited to be used during pregnancy, however, metronidazole is included in class "B" and commonly prescribed to the pregnant female. But our data corroborates studies conducted on mammalian models showing that metronidazole may be considered contraindicated during pregnancy. However, more studies in relation to functional resolution are warranted in this regard.

CRediT authorship contribution statement

Sanya Shabbir: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Mushtaq Hussain:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Nusrat Jabeen:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Abdullah Hadi:** Writing – review & editing, Methodology, Investigation.

Declaration of Competing Interest

The authors declare that there are no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2024.101784.

Data availability

Data will be made available on request.

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