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3.5-GHz radiofrequency electromagnetic radiation promotes the development of *Drosophila melanogaster*☆

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ABSTRACT

With the rapidly increasing popularity of 5G mobile technology, the effect of radiofrequency radiation on human health has caused public concern. This study explores the effects of a simulated 3.5 GHz radiofrequency electromagnetic radiation (RF-EMF) environment on the development and microbiome of flies under intensities of 0.1 W/m^2, 1 W/m^2 and 10 W/m^2. We found that the pupation percentages in the first 3 days and eclosion rate in the first 2 days were increased under exposure to RF-EMF, and the mean development time was shortened. In a study on third-instar larvae, the expression levels of the heat shock protein genes *hsp22*, *hsp26* and *hsp70* and humoral immune system genes *AttC*, *TotC* and *TotA* were all significantly increased. In the oxidative stress system, *DuoX* gene expression was decreased, *sod2* and *cat* gene expression levels were increased, and SOD and CAT enzyme activity also showed a significant increase. According to the 16S rDNA results, the diversity and species abundance of the microbial community decreased significantly, and according to the functional prediction analysis, the genera *Acetobacter* and *Lactobacillus* were significantly increased. In conclusion, 3.5 GHz RF-EMF may enhance thermal stress, oxidative stress and humoral immunity, cause changes in the microbial community, and regulate the insulin/TOR and ecdysteroid signalling pathways to promote fly development.

1. Introduction

With the development of technology, mobile phone devices such as smartphones have occupied an important position in our social lives (Organization, 2011). In present society, mobile phones are used not only for communication but also online for financial transactions, social media, news dissemination and other convenient tools. As a result, the requirements for mobile communication performance are also increasing, as is the distribution density of signal transmission base stations, not only in urban areas but also in rural areas, where there are many base stations. The signal sources emitted by these base stations are considered to be important sources of radiofrequency radiation pollution in the environment (Balmori, 2015), and the resulting health effects are generating increasing concern.

Over the past decade, a large number of epidemiological and laboratory studies have demonstrated a correlation between health and RF-EMF exposure. Surveys show that people living near mobile phone base stations in France are exposed to higher doses of exposure when they are stay in the open air than when they are stay in their house (De Giudici et al., 2021), and that RF-EMF exposure is strongly associated with insomnia (Martin et al., 2021) (Rafique et al., 2020). Electromagnetic radiation may also cause health problems such as cancer (Carlberg et al., 2020) (Gorski et al., 2021) (Peleg et al., 2018), abnormal reproductive development (Erkut et al., 2016) (Altun et al., 2018), cardiovascular disease (Bandara and Weller, 2017), neurological disease (Gokcek-Sarac et al., 2021) (Balaguru et al., 2012), endocrine system disease (Meo et al., 2015), cognitive impairment (Guxens et al., 2016) (Verbeek et al., 2021), and sleep disorders (Eggert et al., 2020) (Danker-Hopfe et al., 2016). In 2011, the International Agency for Research on Cancer classified radiofrequency electromagnetic radiation (RF-EMF) as a possible carcinogen, but the evidence is weak and far from conclusive.

In recent years, the impact of mobile radiation on reproduction and

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development has been a topic of public concern and controversy. Regarding the impact on reproduction, studies have proposed that short-term radiofrequency radiation has no effect on the reproductive ability of wild invertebrates (Vijver et al., 2014). However, some studies have proposed different perspectives, and a study of the effects of radiofrequency radiation on queen bees found that mobile phone radiation can significantly reduce the hatching rate but does not reduce the successful mating rate (Odemer and Odemer, 2019). Some studies suggest that radiofrequency radiation exposure of the bee colony can also cause interference, so that bee population size, bee reserves, the number of foraging worker bees returning to hives, and the ability of the queen bee to lay eggs decrease significantly (Dalio,). Radiofrequency radiation of 900 MHz also affected female and male fruit flies and reduced their reproductive capacity (Panagopoulos et al., 2004). Therefore, electromagnetic radiation is considered to be a driver of the decline in insect populations (Balmori, 2021). Studies have suggested that nonthermal radiation from mobile phones may increase the number of offspring by increasing the expression levels of hsp70 and activating signal transduction pathways (Weisbrot et al., 2003), with differing views and unclear studies on the mechanism underlying the effect on reproductive capacity. There is little research on the growth and development of fruit flies exposed to radiofrequency radiation, and the specific mechanism is not very clear. It was reported that 100-MHz-radiofrequency radiation affected fertilized zebrafish embryos at 24–72 h, especially in the 48 h stage, by increasing oxidative stress gene transcription and changing cholesterol metabolism so that the growth rate is reduced (Piccinetti et al., 2018). Dasgupta et al. also proposed that exposure of zebrafish to 3.5 GHz, 8.27 W/kg for 6–48 h had no significant effect on mortality, morphology or photomotor response (Dasgupta et al., 2020). At the same time, many studies have suggested that bacteria in symbiosis with fruit flies are often involved in the growth and metabolic processes of the host. Compared with that of conventional fruit flies, the larval growth rate of sterile fruit flies decreased, and the size of the adults did not differ, indicating that the microbiome promoted the growth rate of the larvae (Ridley et al., 2012). Symbiotic Serratia promote the synthesis of fatty acids in pea aphid hosts and promote host growth and development (Zhou et al., 2021); thus, intestinal microorganisms are now also considered to be “host” factors affecting energy intake. Studies have suggested that plant Lactobacillus can control hormone signals (Storelli et al., 2011) by acting upstream of the host nutrition perception system on TOR. Another symbiotic bacterium, *Bacillus acetate*, has been found to regulate insulin/insulin-like growth factor signals (IISs) in fruit flies, thus regulating the balance mechanism within the host body and regulating developmental rate, body size and energy metabolism (Shin et al., 2011). Therefore, microbial immunity has a profound influence on many aspects of host physiology and is regarded as a factor affecting the growth of the host. The influence of RF electromagnetic radiation on microbial immunity is still unclear, and whether RF radiation can indirectly affect the development of organisms by affecting the microbiomes of the organisms remains unknown.

This study selected 3.5 GHz, the main 5G frequency band in China, and studied the development of fruit flies and the effect mechanisms of exposure to 3.5 GHz continuous wave radio frequency radiation (RF-EMF). By measuring the pupation percentages in the first 3 days and eclosion rate in the first 2 days of the pupation and feather stages, the results showed that 3.5 GHz radiofrequency radiation may promote the development of fruit flies. We further explored the mechanism by which RF radiation promotes the development of fruit flies through changes in thermal stress, oxidative stress, immunity and the structure of the microbiome in the body. Hopefully, this study can provide a reference for environmental health issues for the public living in a radiofrequency radiation environment for a long time.

2. Materials and methods

2.1. Experimental animal and experimental conditions

This study used wild-type black *Drosophila melanogaster* (Canton S strain) as the experimental subject, purchased from the Core Facility of Drosophila Resource and Technology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. After culture with yeast-cornmeal standard medium, the basic culture conditions were a temperature of 25 ± 0.5°C and a relative humidity (RH) of 60 ± 5%. The fruit flies selected for the experiment were all third-instar larvae.

2.2. Radiofrequency electromagnetic exposure device

The RF electromagnetic exposure simulation device mainly enlarges the radiation by a radio frequency vector signal generator (PSGE8267D, US Agilent) to a fixed amplifier (AV38701E, Qingdao Zhongdao Instrument Co., Ltd.), which then emits stable, continuous RF radiation (sine wave) through the transmitting antenna (AV89901, Qingdao Zhongdai Instrument Co., Ltd.). The frequency in our study is set to 3.5 GHz, and the electromagnetic radiation analyser (PMM8053A, PMM) measures the electromagnetic radiation power density at different sites, determines the position with intensities of 0.1 W/m^2, 1 W/m^2, and 10 W/m^2, and calculates the corresponding SAR values of 0.0026 W/kg, 0.026 W/kg, and 0.26 W/kg (Zhang et al., 2021) by detecting the conductivity of fruit fly tissue.

2.3. Long-term RF-EMF treatment

Newly hatched unmatched female flies and male flies were placed in the same culture tube, and each group of 10 parallel tubes was placed in a 3.5 GHz RF-EMF environment with an intensity of 0.1 W/m^2, 1 W/m^2 or 10 W/m^2. The control group was placed in a shielding box to ensure that the control group and treatment group were exposed to a consistent environment temperature. After three days of exposure, the parental fruit flies were poured out and placed in the original RF-EMF environment to observe the number of pupae and feathers each day. The pupation percentage in the first 3 days is the number of corresponding flies in the first three days/the total number in the pupa stage, the eclosion rate in the first 2 days is the number in the feather stage in the first 2 days/the total number in the feather stage, and the total eclosion rate is the total number in the feathre stage/the total pupa number. The above three treatments were repeated three times.

2.4. ROS detection

Third-instar larvae were collected from each experimental group (25 each), and living tissue was centrifuged at 12,000 g for 10 min at low temperature. A total of 10 μl of liquid was added to PBS solution containing 1 μl of DCFH-DA, and the fluorescence intensity was detected by fluorescent enzyme markers at 488 ± 10 and 525 ± 20 nm, after incubation at 37°C to avoid photo-bleaching for 40 min. This protocol is consistent with that used in previous research reports (Wang et al., 2020). The experiment was repeated three times.

2.5. Detection of antioxidant activity

Third-instar larvae from each experimental group were collected, and the supernatant was removed after crushing. A protein quantification (TP) assay box (Kemas bright blue method) and assay kits (Nanjing Jiancheng) for total antioxidant capacity (T-AOC), superoxide dismutase (SOD) (WST-1), glutathione peroxide (GSH-PX) and hydrogen peroxide enzyme (CAT) (ammonium niobium nitrite method) were used to determine the T-AOC, SOD, GSH-PX and CAT contents in fruit flies. The data were analysed using standard methods. The experiment was

repeated three times.

2.6. RT-PCR assay

After collecting the third-instar larvae from each experimental group, 10 larvae were collected in 1.5 ml tubes, and three tubes were collected each time. Samples were ground after being frozen in liquid nitrogen, and total RNA was extracted using the TRIzol method (Invitrogen). Then, the RNA was reverse transcribed into cDNA using a reverse transcription kit with Prime ScriptTM RT Master Mix (TaKaRa). Using SYBR Premix Ex Taq II Kits (TaKaRa) and a Light Cycler 480 II Fluorescence Quantitative PCR Instrument (Roche), the relative expression levels of heat stress-related genes (*hsp22, hsp26, hsp70*), humoral immune system-related genes (*AttC, TotC, TotA*), oxidative stress-related genes (*DuoX, sod1, sod2, cat*) and key insulin/TOR and ecdysteroid signalling pathway genes (*InR, Eip74B, Sad*, *Dilp2, Dilp3*) were detected. In this study, rp49 was selected as the internal gene. Amplification was performed with the two-step method, a TM value of 55°C, and 40 cycles, and the results were calculated by the $2^{-\triangle\triangle Ct}$ value, which was repeated three times in the experiment. The primer sequence is shown in Supplementary Table 1 below.

2.7. Analysis of microbial community of flies

The same number of long-term-exposed third-instar larvae was collected in centrifuge tubes (50 larvae per tube, 6 repetitions per group). The larval surface was washed with 70% alcohol, washed three times with PBS, frozen in liquid nitrogen and stored in a refrigerator for later use. The bacterial DNA of third-instar larvae was extracted by a FastDNA® Spin Kit for Soil (MP Biomedicals, US) bacterial DNA extraction kit following the sequencing company protocols. The amplification primers 338F (ACTCCACGGGAGGGAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) for the 16S rRNA gene in the V3–V4 highly variable region were selected. Then, 16S amplification sequencing was carried out by Shanghai Meiji Biopharmaceutical Technology Co., Ltd., on the MiSeq PE300 platform.

For microbiome data analysis, FASTP was used to further filter raw data containing adapters or low-quality reads (https://github.com/OpenGene/fastp, version 0.20.0). Splicing was performed using FLASH (http://www.cbcb.umd.edu/software/flash, version 1.2.7) with a minimum overlap length of 10 bp, and the maximum mismatch allowed for the overlap area of the stitching sequence was 0.2. Using the UCHIME algorithm to search for “Clean” in the reference database, execution based on the combination of reference checks (http://drive5.com/uparse/, version 7.1) was performed to remove all nested labels, and finally, the resulting valid label was used for further analysis. The comparison threshold was set to 70% for each sequence using RDP Classifier (http://rdp.cme.msu.edu/, version 2.2) to classify the species according to the Silva 16S rRNA database (v138).

2.8. Statistical analysis

The experimental data were analysed by using IBM SPSS 20 Statistics and ORIGIN 2021 software. The averages of each set were analysed using one-way ANOVA, and the LSD method was used to test for significance. $P < 0.05$ represents a statistically significant difference (”*” $0.01 < P < 0.05$; "**"$P < 0.01$).

3. Results

3.1. Effect of long-term RF-EMF on the development of flies

After long-term RF-EMF exposure of female and male flies during mating, the number of offspring, the pupation percentages in the first 3 days, the total eclosion rate, the eclosion rate in the first 2 days and the mean development time were analysed. It was found that under long-term 3.5-GHz RF radiation conditions, the number of pupae and the pupation percentages in the first 3 days showed significant increases (Fig. 1A and B), the overall eclosion rate was not significantly changed (Fig. 1C), and the eclosion rate in the first 2 days was significantly increased ($P < 0.05$) (Fig. 1D). In addition, the mean developmental time was reduced by 10.70%, 11.98% and 13.79% in the E0.1 group, E1 group, and E10 group, respectively (Fig. 1E). Long-term radiofrequency radiation promotes the development of fruit flies, and the effect is significant under 10 W/m^2 conditions.

3.2. Effect of long-term RF-EMF on heat stress responses and humoral immunity in third-instar larvae

Thermal stress is one of the main ways in which radiofrequency radiation affects organisms, so we explored the relative expression of the key heat stress genes *hsp22*, *hsp26* and *hsp70* and key humoral immune system genes *AttC*, *TotC* and *TotA* under exposure to long-term RF radiation. The results showed that the *hsp22*, *hsp26* and *hsp70* genes showed significant increases in E0.1, E1 and E10 ($p < 0.01$) compared to the control group (Fig. 2A). In the humoral immune system, the *AttC* gene showed significant increases in E0.1, E1 and E10 ($p < 0.01$) compared to the control group, and the Turandot family genes *TotC* and *TotA*, which encode humoral immune factors, showed significant increases in E0.1, E1 and E10 ($p < 0.05$) (Fig. 2B). Long-term radiofrequency radiation enhances the thermal stress response and humoral immune system in third-instar fruit fly larvae.

3.3. Effect of long-term RF-EMF on oxidative stress in third-instar larvae

Oxidative damage is considered to be another important way that radio frequency radiation can cause harm to the body. This study explored the relative expression level of the *DuoX* gene, which induces ROS, the *sod1* and *sod2* genes encoding SOD and the *cat* gene encoding CAT and explored ROS levels, T-AOC, CAT activity, SOD activity and GSH-PX activity. The results showed that the *DuoX* gene was significantly decreased in the E1 and E10 groups ($p < 0.01$), and the *sod1* gene was significantly increased in the E0.1 group and E1 group and significantly decreased in the E10 group ($p < 0.0$ 1). The *sod2* gene showed significant increases in the E0.1 and E10 groups ($p < 0.01$), while the *cat* gene showed significant increases in the E0.1 and E10 groups ($p < 0.05$) (Fig. 3A). Compared to the control group, there were no significant differences in ROS levels in E0.1, E1 and E10 (Fig. 3B) and no significant changes in the T-AOC average (Fig. 3C). CAT activity significantly increased in E0.1, E1 and E10 ($p < 0.01$) (Fig. 3D), SOD activity showed a significant increase in E0.1, E1 and E10 ($p < 0.05$) (Fig. 3E), and GSH-PX vitality showed a significant decrease only in E1 and E10 ($p < 0.01$) (Fig. 3F). Therefore, long-term radiofrequency radiation may enhance enzyme activity by regulating the relative expression of key genes, removing oxidative free radicals, and enhancing the antioxidant stress system in third-instar larvae.

3.4. Effect of long-term RF-EMF on the microbial community in third-instar larvae

The variation in microbial community structure in third-instar larvae after long-term exposure to RF-EMF was analysed by 16S rDNA sequencing, and 1 domain, 1 kingdom, 23 phylums, 41classes, 94 orders, 139 families, 204 genuses, 252 species, and 291 OTUs were detected. In comparison to the control group of fruit flies, principal coordinate analysis (PCoA) showed that the three biological repetitions in the four groups could be well grouped into one group (Fig. 4A), and the results of the α diversity analysis showed that radiofrequency electromagnetic radiation had a significant effect on the microbiome of fruit flies (Table 1). The species abundance of bacteria and the Venn diagram of the core OTUs of the fruit flies exposed to different radiofrequency radiation intensities show a significant difference in microorganisms after

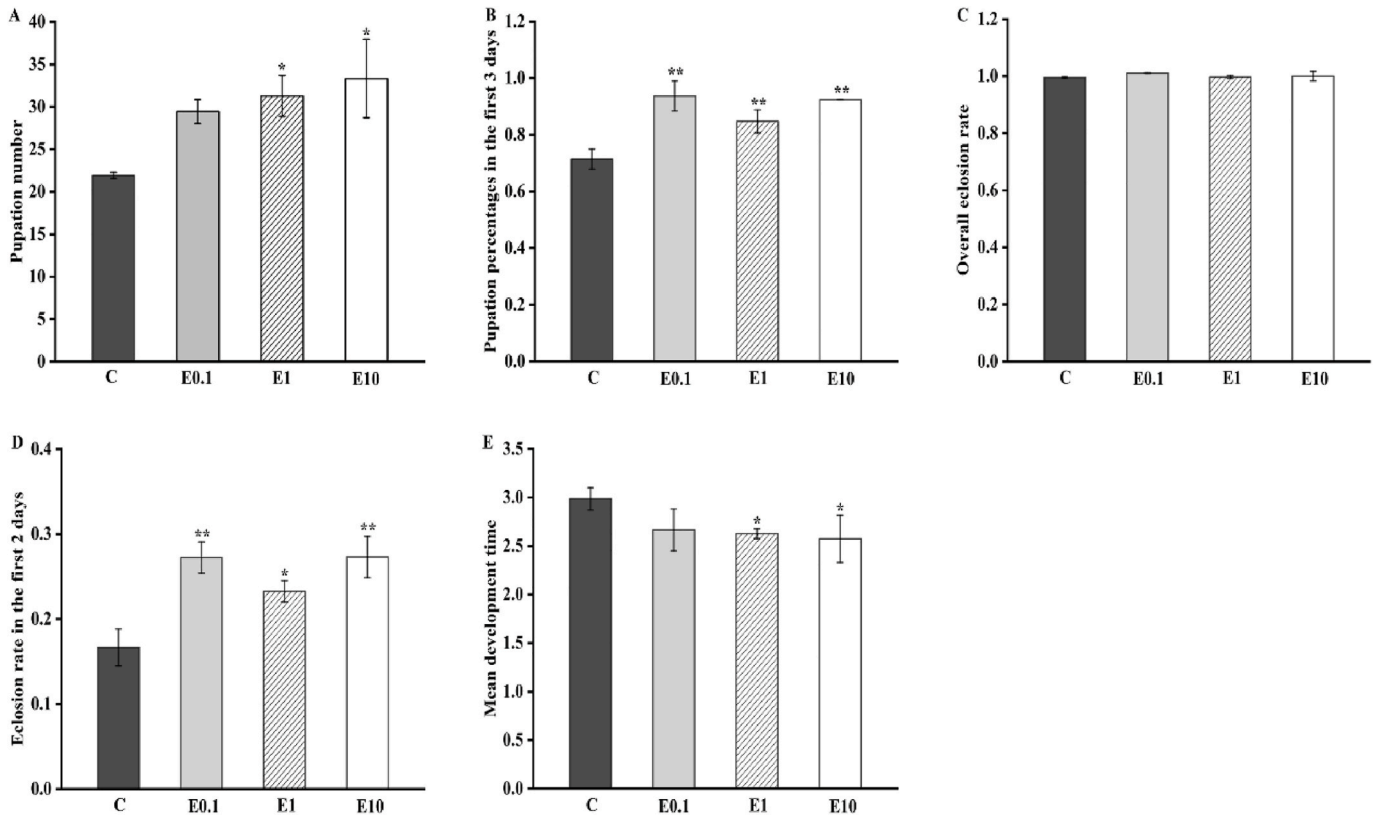


Fig. 1. Effects of long-term exposure to 3.5 GHz (0.1 W/m^2, 1 W/m^2 and 10 W/m^2) RF-EMF on flies. (A) The pupa number in the C, E0.1, E1 and E10 groups of F1 flies. (B) Pupation percentages in the first 3 days. (C) The overall eclosion rate. (D) Eclosion rate in the first 2 days. (E) Mean development time. Values represent mean ± SEM. (*, 0.01 < p < 0.05; **, p < 0.01).

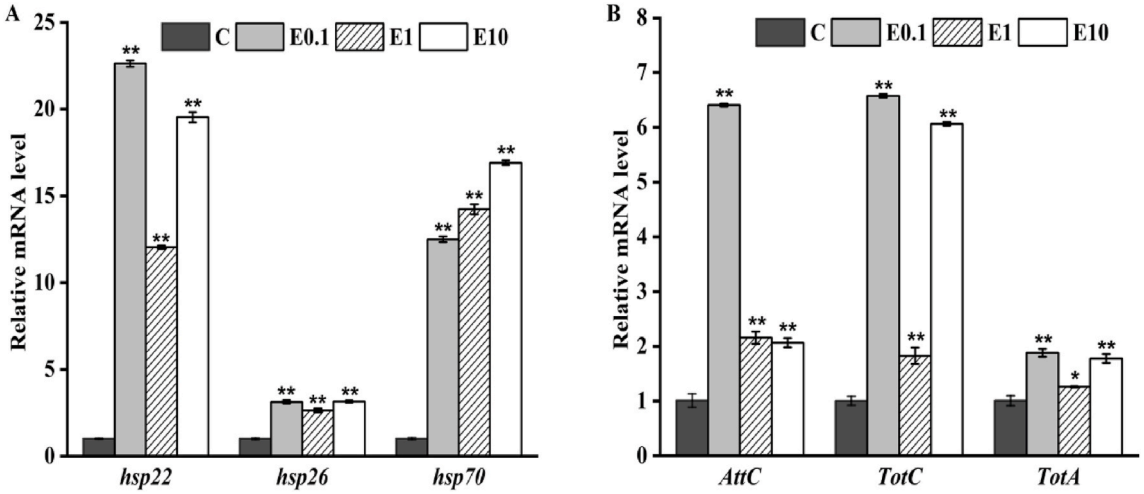


Fig. 2. Effects of long-term exposure to 3.5 GHz (0.1 W/m^2, 1 W/m^2 and 10 W/m^2) RF-EMF on heat responses and the immune system in L3 larvae. (A) Changes of *hsp22, hsp26* and *hsp70* gene expression in F1 L3 larvae. (B) Changes of *AttC, TotC* and *TotA* gene expression in F1 L3 larvae. Values represent mean ± SEM. (*, 0.01 < p < 0.05; **, p < 0.01).

radiofrequency electromagnetic radiation exposure and significant differences in the total number of species (Fig. 4B). For the relative abundance of species at each classification level, compared with the control group, at the phylum level, the effects of RF electromagnetic radiation in the E0.1 and E1 groups were to promote Proteobacteria and inhibit Firmicutes (Fig. 4C). At the class level, the effects of RF electromagnetic radiation were to promote Alphaproteobacteria and inhibit Gammaproteobacteria (Fig. 4D). At the family level, the effects of RF electromagnetic radiation were to promote Anaplasmataceae and inhibit Pseudomonadaceae, Alcaligenaceae and Burkholderiaceae (Fig. 4E). At the genus level, the effects of RF electromagnetic radiation were to promote Wolbachia, Pseudomonas and Achromobacter (Fig. 4F). Species difference analysis revealed microorganisms with significant differences between the RF-EMF and control groups under different exposure intensities and showed that the abundance of Pseudomonas, Ralstonia, Burkholderia-Caballeronia-Paraburkholderia and Dyella significantly decreased under different exposure intensities (Supplementary Fig. 1).

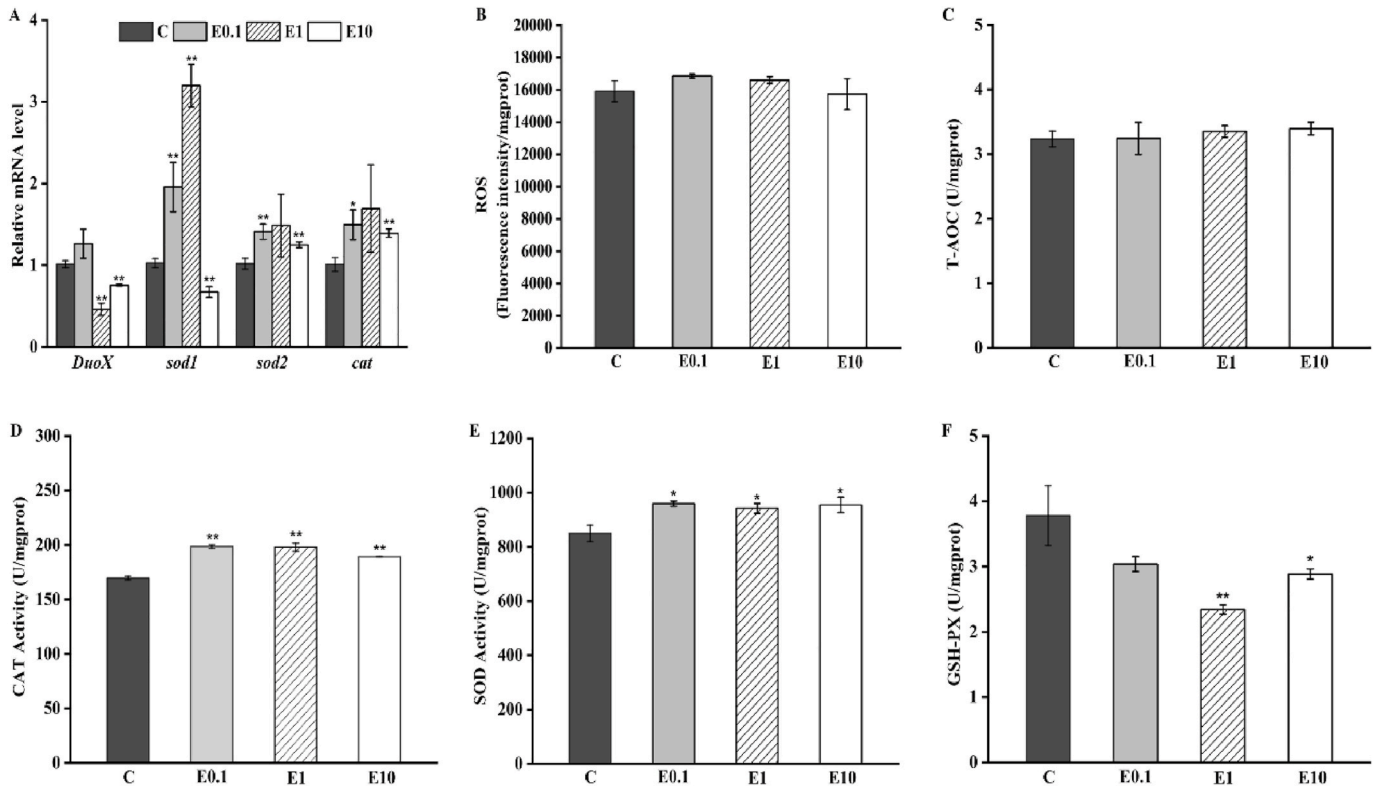


Fig. 3. Effects of long-term exposure to 3.5 GHz (0.1 W/m^2, 1 W/m^2 and 10 W/m^2) RF-EMF on oxidative stress in L3 larvae. (A) Long-term exposure to 3.5 GHz (0.1 W/m^2, 1 W/m^2 and 10 W/m^2) RF-EMF affected the expression levels of the *DuoX, sod1, sod2* and *cat* genes in L3 larvae. (B) The change in ROS levels in F1 L3 larvae. (C) The change in the total antioxidant capacity (T-AOC). (D) The change in CAT. (E) The change in SOD enzyme activity. (F) The change in GSH-PX enzyme activity. Values represent mean ± SEM. (*, $0.01 < p < 0.05$; **, $p < 0.01$).

The results of functional phenotype prediction showed a significant increase in the Acetobacter and Lactobacillus genera in the gram-positive and gram-negative groups (Fig. 5ABC). The functional prediction results also showed that the COG functional composition of all samples was similar to that of the species. All bacteria in fruit flies had increased cellular processes, replication, repair, transcription and translation functional abundance, while RNA synthesis, energy production and conversion, lipid transport and metabolism, carbohydrate transport and metabolism, signalling and defence mechanisms were associated with reduced functional abundance (Fig. 5D). Long-term RF-EMF affects the species abundance and diversity of the microbial community in fruit flies and affects the structure and function of the flora, with obvious differences between *Bacillus acetate* and *Lactobacillus*.

3.5. Effect of long-term RF-EMF on insulin/TOR and the ecdysteroid signalling pathways in third-instar larvae

Changes in the microbiome affect fruit fly development by regulating the insulin/TOR and ecdysteroid signalling pathways, and this study analysed the relative expression levels of the key genes *InR*, *Eip74B*, *Sad*, *Dilp2* and *DILP3*. The results showed that in third-instar larvae, the InR gene decreased significantly under radiofrequency radiation conditions compared to the control ($p < 0.01$) (Fig. 6A), the *Eip74B* gene showed a significant increase in the E10 group ($p < 0.01$) (Fig. 6B), and the *Sad* gene showed significant increases in the E1 and E10 groups ($p < 0.01$) (Fig. 6C), and the *Dilp2* and *Dilp3* gene showed significant increases (Fig. 6DE). In summary, 3.5 GHz radiofrequency radiation can affect the insulin/TOR and moulting hormone signalling pathways in third-instar larvae.

4. Discussion

With the continuous development of global 5G, the impact of 5G RF-EMF on health has attracted great attention, but there has been little research and its mechanism is unclear. This study selected a main Chinese frequency band of 3.5 GHz to simulate its radiofrequency radiation environment and to investigate the development of fruit flies under 3.5 GHz RF-EMF.

Regarding the effects of RF-EMF on fruit flies' development, a recent study showed that mobile phone radiation will reduce the hatching rate of fruit flies. Electromagnetic radiation may be an emerging factor (Balmori, 2021). However, there are also studies suggesting that high-frequency RF-EMF can delay the development of fruit flies (Atli and Unlu, 2006). In our study, it was found that long-term RF radiation exposure significantly increased the developmental rate of the first three days of fruit fly offspring. The feathering rate in the first two days also significantly increased, and the average development time was reduced, indicating that long-term 3.5 GHz RF-EMF promote the development of fruit flies.

Long-term exposure to RF-EMF may induces an environmental stress response, and the most common physiological response is an increase in the expression of heat shock proteins (HSPs) (Colinet et al., 2010). It has been reported that RF-EMF exposure significantly increases the gene expression levels of the heat shock protein (Hsp) and thermal shock transcription factor (Hsf) families (Ohtani et al., 2016). Previous studies have also found that RF-EMF significantly increases the relative expression levels of *hsp22*, *hsp26* and *hsp70* genes in fruit flies (Zhang et al., 2021). In our study, we also found that the *hsp22*, *hsp26* and *hsp70* genes in larvae significantly increased, enhancing the thermal stress response. And the same time, the changes of *hsp70* gene were dose-dependent. HSP70 is one of the most easily induced cell protection proteins under stress conditions and has been found in many animal

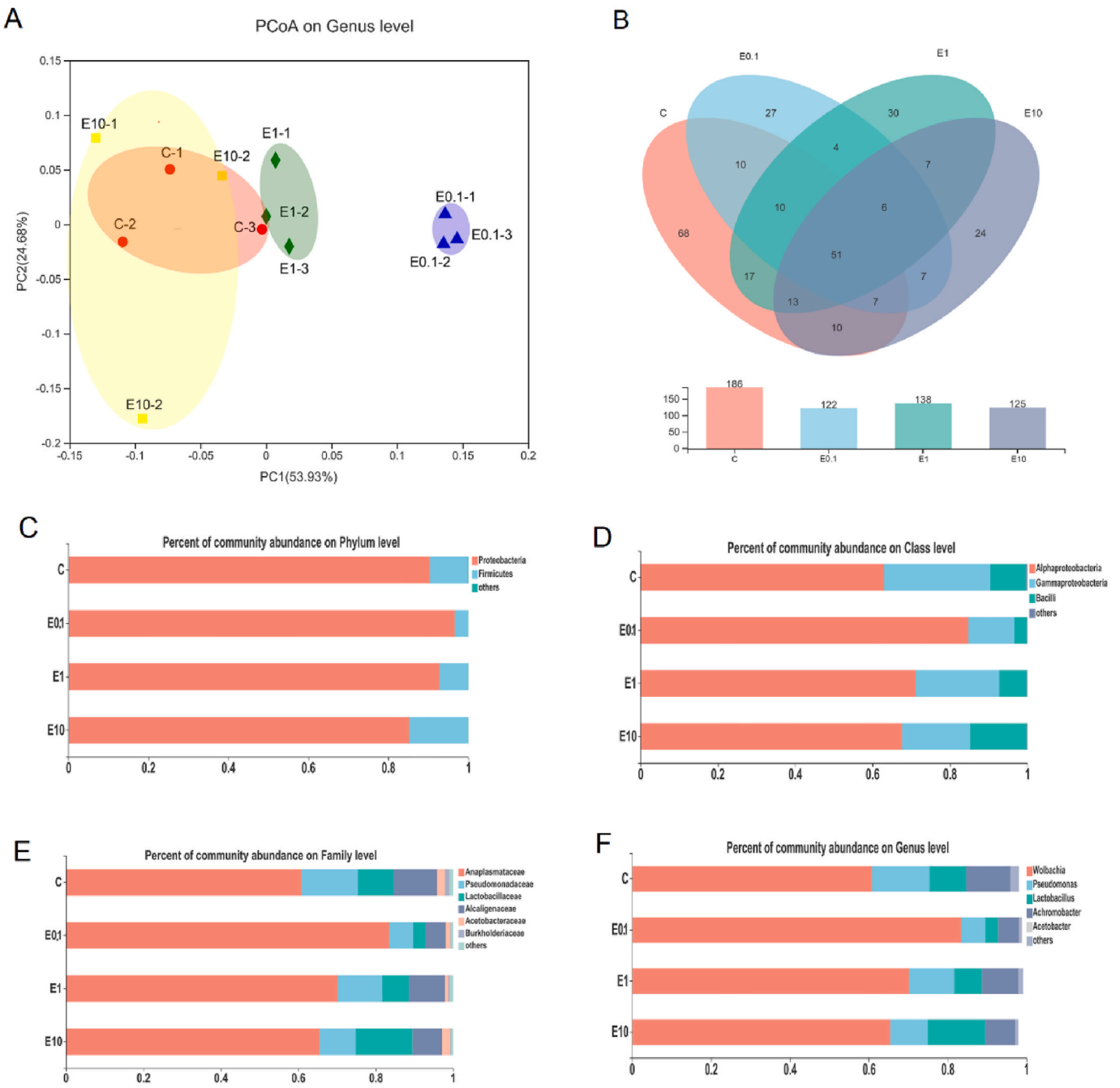


Fig. 4. Microbiome analysis of 16S rDNA sequencing data from control Drosophila (C) and exposed Drosophila (E). (A) Principal coordinate analysis of samples from four groups, n = 3. (B) Prediction of species composition level similarity with a Venn diagram. (C) Microbiome analysis performed at the phylum level. (D) Microbiome analysis performed at the class level. (E) Microbiome analysis performed at the family level. (F) Microbiome analysis performed at the genus level.

Table 1
α diversity indices statistics.

Sample Name	shannon	P	simpson	P	ace	P	chao	P
C	1.31 ± 0.10		0.41 ± 0.040		116.01 ± 2.33		117.04 ± 3.37	
E0.1	0.72 ± 0.05	**	0.70 ± 0.02	**	122.89 ± 10.51	n. s.	90.68 ± 2.85	*
E1	1.052 ± 0.09	n. s.	0.53 ± 0.04	n. s.	87.11 ± 5.53	*	79.22 ± 4.30	**
E10	1.19 ± 0.09	n. s.	0.46 ± 0.05	n. s.	85.49 ± 10.00	*	79.68 ± 11.51	**

Note: * indicates that the difference between the exposure and control groups is significant, $0.01 < P < 0.05$; ** indicates that the difference between the exposure and control groups is significant, $P < 0.01$; n. s. means that the difference is not significant.

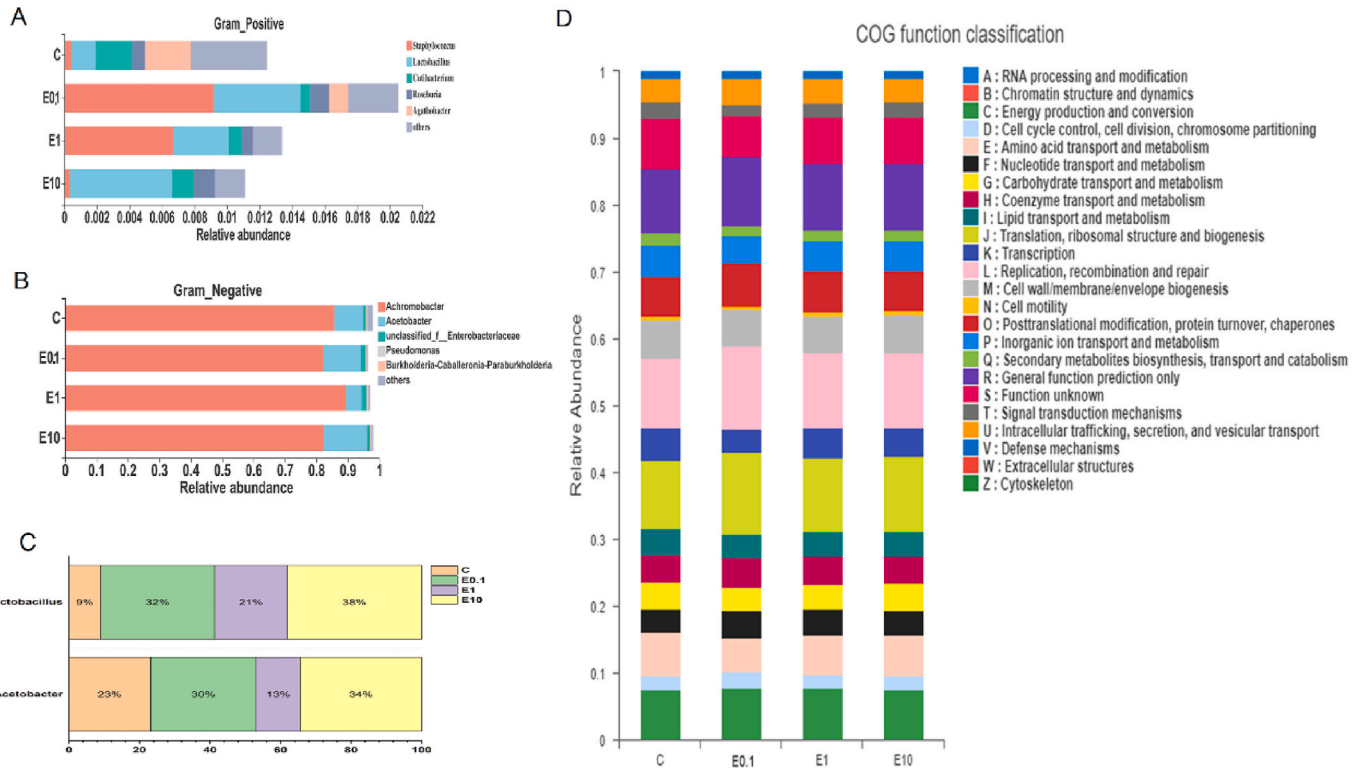


Fig. 5. Microbiome function analysis of 16S rDNA sequencing data from control Drosophila (C) and exposed Drosophila (E). (A) Gram-positive-level microbiome phenotypes predicted with Bugbase. (B) Gram-negative-level microbiome phenotypes predicted with Bugbase. (C) The change in *Acetobacter* and *Lactobacillus*. (D) COG function classification.

experiments to respond to RF-EMF (Lee et al., 2005) (Gaestel, 2010). HSP70 is a biomarker considered to be a systemic inflammatory response to radiofrequency radiation (Balakrishnan et al., 2014), and changes or imbalances in HSP70 that trigger stress in the body (Othman et al., 2021), cause the body's immune system to respond.

There are 8 Turandot family genes in humoral immunity of flies, all of which can be induced by various stress conditions, such as thermal stress, bacterial infection or ultraviolet radiation, and they can improve the stress resistance of flies (Ekengren and Hultmark, 2001), but the mechanism by which they enhance immunity is unclear. *TotA* enhances the high-temperature resistance of fruit flies (Mahapatra and Rand, 2012), and *TotC* encodes a protein expressed in the body in response to stress through the JAK-STAT pathway. In addition, *AttC* is one of the key genes involved in the humoral immune system. Our study revealed that long-term 3.5 GHz RF-EMF significantly increased the relative expression of the *AttC*, *TotC* and *TotA* genes.

Under long-term exposure to RF-EMF, the body's oxidative stress systems may have a certain impact. Studies have suggested that the effectiveness of radiofrequency radiation-induced oxidative stress caused by cell phones and Wi-Fi frequencies can significantly increase the levels of intrabrain protein hydroxylization (PC), nitric oxide (NO) and propylene dialdehyde (MDA) in exposed rats and increase GSH-Px, T-AOC, SOD and CAT levels (Asl et al., 2020). At the same time, RF-EMF increases ROS levels in cells to reduce proliferation and increase ageing (Choi et al., 2020), but there is evidence that the increase in ROS does not occur immediately but with increased exposure time or cumulative dose (Liu et al., 2014). The increase in SOD can in turn reduce the increase in ROS (Consales et al., 2018). In our study, long-term RF-EMF exposure did not induce a significant increase in ROS levels in fruit flies, which may be because of an increase in CAT and SOD activity, enhancing the ability of the antioxidant stress defence systems in fruit flies to remove free radicals. In contrast, the GSH-PX level decreased with high-intensity radiation in the exposed fruit flies, which is consistent with the results of a former study (Esmekaya et al., 2011). The level of *Duox* gene expression involved in ROS production was also significantly decreased under high-intensity conditions, while the expression levels of the *sod1*, *sod2*, and *cat* genes were increased, and the change in enzyme activity is consistent. In addition, the host's immune response and interference may also lead to a decrease in microbial community diversity in fruit flies (Wong et al., 2011). In our study, at the Shannon index level, the diversity of microbial flora in the larvae exposed to RF-EMF was also relatively low. It is well known that compositional changes in the microbiome affect the nutrition and development of insects, especially through the number of host fat bodies (Martinson and Strand, 2021) (Newell and Douglas, 2014) (Ma et al., 2015), and microbiome evolution affects the host's feathering time (Arora, Mars Brisbin et al., 2020). It has been reported that *Citrobacter freundii* and *Klebsiella oxytoca* promote the development and metabolism of fruit flies (Gao et al., 2020).

Previous studies have found that symbiotic bacteria have effects on the physiology and development of fruit flies, and *Bacillus acetate* and *Lactobacillus* are the most common symbiotic bacteria (Storelli et al., 2011). Animals that can oxidize sugar or ethanol to produce acetic acid experience food corruption but enhanced growth and reproduction, and this ability can significantly promote the development of larvae (Li et al., 2017) (Lee et al., 2020). There are also related reports that both *Bacillus acetate* and *Lactobacillus* significantly shorten the development time of fruit flies, indicating that the two bacteria can improve the adaptability and promote the growth of fruit flies (Jin et al., 2020). In our study, long-term exposure to RF-EMF significantly increased *Bacillus acetate* and *Lactobacillus* in their microbiome. And *Bacillus oriental acetate* can also increase the number of cell divisions and promote the secretion of moulting hormones through the insulin signalling pathway (Li et al., 2017), which significantly promotes the growth of fruit flies. Both *Bacillus acetate* and *Lactobacillus* can be coupled with nutritional dependence by activating the interaction between the insulin/TOR and moult

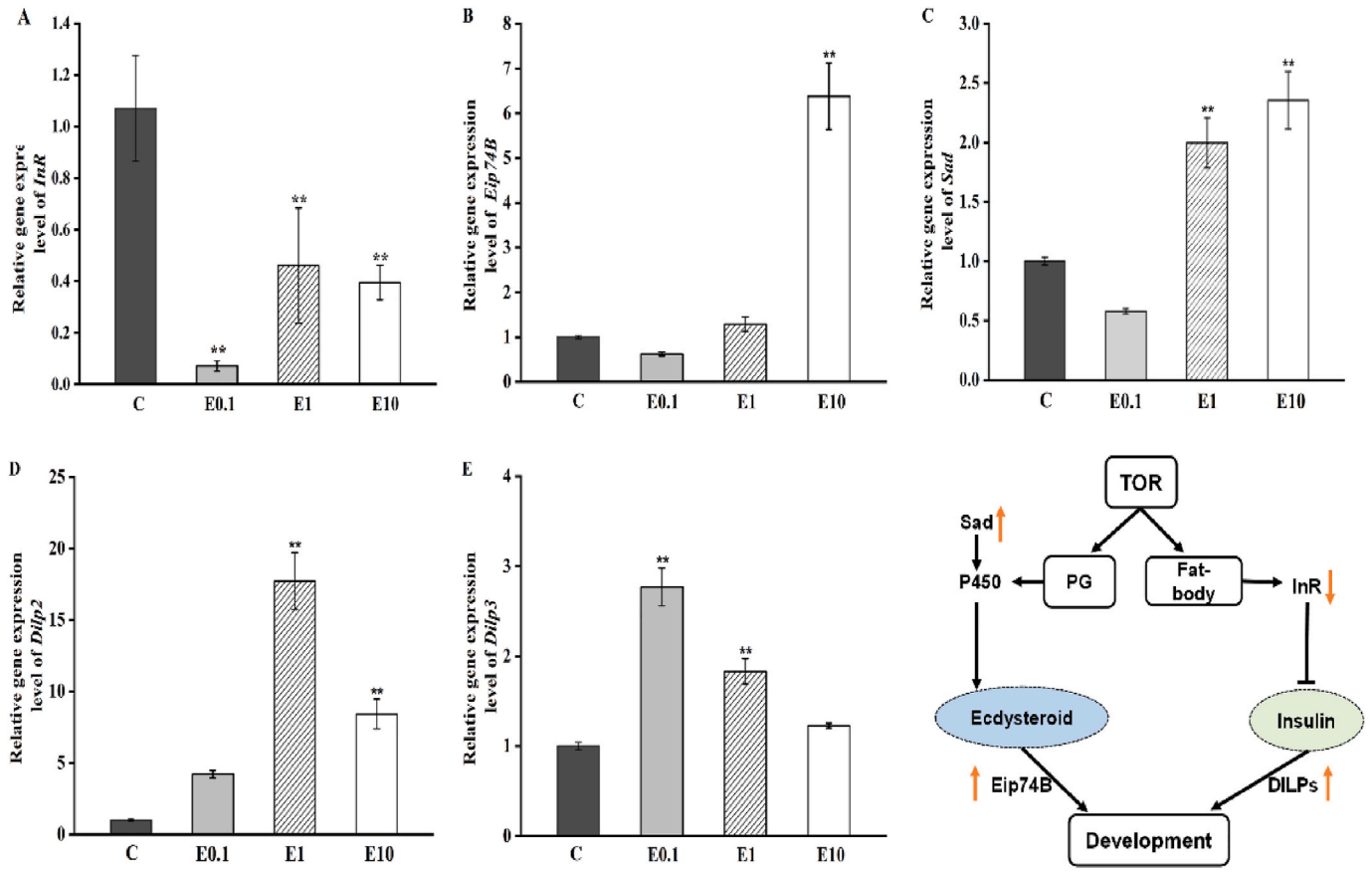


Fig. 6. Effects of long-term exposure to 3.5 GHz (0.1 W/m^2, 1 W/m^2 and 10 W/m^2) RF-EMF on growth and development in L3 larvae. (A) The change of *InR* gene expression in F1 L3 larvae. (B) The change of *Eip74B* gene expression in F1 L3 larvae. (C)The change of *Sad* gene expression in F1 L3 larvae. (D) The change of *Dilp2* gene expression in F1 L3 larvae. (E) The change of *Dilp3* gene expression in F1 L3 larvae.Values represent mean ± SEM. (*, $0.01 < p < 0.05$; **, $p < 0.01$).

hormone signalling pathways, and both insulin and TOR play a role in the production and entry of moulting steroids through direct coupling of nutritional perception in PGs (Layalle et al., 2008).

Ecdysone has multiple effects on development, including the larval moulting period and the metamorphosis period, which control the transition period of development. Developmental transformations, such as metamorphosis, are controlled by the release of PTTH by the brain under the influence of JH, which stimulates PG to produce and release ecdysone, which is synthesized in thymus PG cells at the front end of larvae or the ring glands of fruit flies. The interaction between the ketone and insulin signals explains why the increase in the level of ketone inhibits the development of larval tissue, leading to overall decay at the end of larval life. *Sad*, a cytochrome P450-encoding gene involved in moulting steroid biosynthesis, can increase the activity of ecdysone 20E (Petryk et al., 2003). The *InR* gene acts as a negative feedback insulin receptor that plays a crucial role in the insulin/TOR signalling pathway and promotes the production of insulin-like peptide, and the *Eip75B* gene encodes a nuclear receptor whose product regulates eating behaviour, fat deposition, and developmental time by responding to the NO signalling pathway. Changes in gene expression in larvae stimulate growth hormone insulin-like peptides and moulting hormones to increase system signals, which in turn increases the growth rate of larvae, consistent with previous findings (Tower, 2011). Therefore, RF-EMF may act as an environmental stress trigger and lead to microbiome changes and further participate in the production of PG moulting steroids through the insulin/TOR signalling pathway, thereby regulating the development of fruit flies.

5. Conclusion

In summary, this study shows that long-term exposure to simulated 3.5 GHz RF-EMF increases the pupation rate in the first 3 days and the eclosion rate in the first 2 days, shortens the mean development time, and advances the development of fruit flies. Environmental stress in the form of radiofrequency radiation will enhance the stress response and immune system of Drosophila (including thermal stress, the antioxidant stress system and humoral immune system) and affect the structural diversity, abundance and function of the microbial community in larvae. Furthermore, the systemic signals and ecdysteroid increases in the insulin/TOR and ecdysteroid signalling pathways are stimulated to promote the development of fruit flies. This study reveals the effects of long-term radiofrequency radiation on the development of fruit flies and can provide a reference with which to understand the health effects of electromagnetic radiation.

Credit author statement

Yahong Wang: Methodology, Writing- original draft, Writing-review & editing, Data curation, Software. **Zhihao Jiang:** Experimental operation $ Information collection. **Lu Zhang:** Literature retrieval $ Information collection. **Ziyan Zhang:** Methodology, Writing - review & editing. **Yanyan Liao:** Writing - review & editing. **Peng Cai:** Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.118646.

References

Altun, G., et al., 2018. Effects of mobile phone exposure on metabolomics in the male and female reproductive systems. Environ. Res. 167, 700–707.

Arora, J., et al., 2020. Effects of microbial evolution dominate those of experimental host-mediated indirect selection. PeerJ 8, e9350.

Asl, J.F., et al., 2020. The radio-protective effect of rosmarinic acid against mobile phone and Wi-Fi radiation-induced oxidative stress in the brains of rats. Pharmacol. Rep. 72 (4), 857–866.

Atli, E., Unlu, H., 2006. The effects of microwave frequency electromagnetic fields on the development of Drosophila melanogaster. Int. J. Radiat. Biol. 82 (6), 435–441.

Balaguru, S., et al., 2012. Investigation of the spinal cord as a natural receptor antenna for incident electromagnetic waves and possible impact on the central nervous system. Electromagn. Biol. Med. 31 (2), 101–111.

Balakrishnan, K., et al., 2014. Hsp70 is an independent stress marker among frequent users of mobile phones. J. Environ. Pathol. Toxicol. Oncol. 33 (4), 339–347.

Balmori, A., 2015. Anthropogenic radiofrequency electromagnetic fields as an emerging threat to wildlife orientation. Sci. Total Environ. 518, 58–60.

Balmori, A., 2021. Electromagnetic radiation as an emerging driver factor for the decline of insects. Sci. Total Environ. 767.

Bandara, P., Weller, S., 2017. Cardiovascular disease: time to identify emerging environmental risk factors. European J. Prevent. Cardio. 24 (17), 1819–1823.

Carlberg, M., et al., 2020. Is the increasing incidence of thyroid cancer in the nordic countries caused by use of mobile phones? Int. J. Environ. Res. Publ. Health 17 (23).

Choi, J., et al., 2020. Continuous exposure to 1.7GHz LTE electromagnetic fields increases intracellular reactive oxygen species to decrease human cell proliferation and induce senescence. Sci. Rep. 10.

Colinet, H., et al., 2010. Temporal expression of heat shock genes during cold stress and recovery from chill coma in adult Drosophila melanogaster. FEBS J. 277 (1), 174–185.

Consales, C., et al., 2018. 50-Hz magnetic field impairs the expression of iron-related genes in the in vitro SOD1(G93A) model of amyotrophic lateral sclerosis. Int. J. Radiat. Biol. 95 (3), 368–377.

Dalio, J. S. "Effect of Electromagnetic (cell phone) radiations on Apis mellifera".

Danker-Hopfe, H., et al., 2016. Effects of mobile phone exposure (GSM 900 and WCDMA/UMTS) on polysomnography based sleep quality: an intra- and inter-individual perspective. Environ. Res. 145, 50–60.

Dasgupta, S., et al., 2020. Impacts of high dose 3.5 GHz cellphone radiofrequency on zebrafish embryonic development. PLoS One 15 (7).

De Giudici, P., et al., 2021. Radiofrequency exposure of people living near mobile-phone base stations in France. Environ. Res. 194.

Eggert, T., et al., 2020. RF-EMF exposure effects on sleep - age doesn't matter in men. Environ. Res. 191.

Ekengren, S., Hultmark, D., 2001. A family of Turandot-related genes in the humoral stress response of Drosophila. Biochem. Biophys. Res. Commun. 284 (4), 998–1003.

Erkut, A., et al., 2016. The effect of prenatal exposure to 1800 MHz electromagnetic field on calcineurin and bone development in rats. Acta Cir. Bras. 31 (2), 74–83.

Esmekaya, M.A., et al., 2011. 900 MHz pulse-modulated radiofrequency radiation induces oxidative stress on heart, lung, testis and liver tissues. Gen. Physiol. Biophys. 30 (1), 84–89.

Gaestel, M., 2010. Biological monitoring of non-thermal effects of mobile phone radiation: recent approaches and challenges. Biol. Rev. 85 (3), 489–500.

Gao, H., et al., 2020. Effects of intestinal bacteria Citrobacter freundi and Klebsiella oxytoca on the development and substance metabolism of Drosophila suzukii (Diptera:Drosophilidae). Acta Entomol. Sin. 63 (4), 462–469.

Gokcek-Sarac, C., et al., 2021. Possible effects of different doses of 2.1 GHz electromagnetic radiation on learning, and hippocampal levels of cholinergic biomarkers in Wistar rats. Electromagn. Biol. Med. 40 (1), 179–190.

Gorski, R., et al., 2021. Morphological and cytophysiological changes in selected lines of normal and cancer human cells under the influence of a radio-frequency electromagnetic field. Ann. Agric. Environ. Med. 28 (1), 163–171.

Guxens, M., et al., 2016. Outdoor and indoor sources of residential radiofrequency electromagnetic fields, personal cell phone and cordless phone use, and cognitive function in 5-6 years old children. Environ. Res. 150, 364–374.

Jin, H., et al., 2020. Symbiotic bacteria regulate Drosophila oviposition preference by oxygen concentration. Weishengwu Xuebao 60 (5), 1010–1022.

Layalle, S., et al., 2008. The TOR pathway couples nutrition and developmental timing in Drosophila. Dev. Cell 15 (4), 568–577.

Lee, J., et al., 2020. Taxon-specific effects of Lactobacillus on Drosophila host development. Microb. Ecol. 79 (1), 241–251.

Lee, J.S., et al., 2005. Subchronic exposure of hsp70.1-deficient mice to radiofrequency radiation. Int. J. Radiat. Biol. 81 (10), 781–792.

Li, Y., et al., 2017. Isolation of Acetobacter orientalis and their promotion of the growth and development of Drosophila melanogaster. Acta Microbiol. Sin. 57 (10), 1536–1545.

Liu, K.J., et al., 2014. The protective effect of autophagy on mouse spermatocyte derived cells exposure to 1800 MHz radiofrequency electromagnetic radiation. Toxicol. Lett. 228 (3), 216–224.

Ma, D., et al., 2015. Studying host-microbiota mutualism in Drosophila: harnessing the power of gnotobiotic flies. Biomed. J. 38 (4), 285–293.

Mahapatra, C.T., Rand, M., 2012. Methylmercury tolerance is associated with the humoral stress factor gene Turandot A. Neurotoxicol. Teratol. 34 (4), 387–394.

Martin, S., et al., 2021. Health disturbances and exposure to radiofrequency electromagnetic fields from mobile-phone base stations in French urban areas. Environ. Res. 193.

Martinson, V.G., Strand, M.R., 2021. Diet-Microbiota interactions alter mosquito development. In: Frontiers in Microbiology, vol. 12.

Meo, S.A., et al., 2015. Association of exposure to radio-frequency electromagnetic field radiation (RF-EMFR) generated by mobile phone base stations with glycated hemoglobin (HbA1c) and risk of type 2 diabetes mellitus. Int. J. Environ. Res. Publ. Health 12 (11), 14519–14528.

Newell, P.D., Douglas, A.E., 2014. Interspecies interactions determine the impact of the gut microbiota on nutrient allocation in Drosophila melanogaster. Appl. Environ. Microbiol. 80 (2), 788–796.

Odemer, R., Odemer, F., 2019. Effects of radiofrequency electromagnetic radiation (RF-EMF) on honey bee queen development and mating success. Sci. Total Environ. 661, 553–562.

Ohtani, S., et al., 2016. Exposure time-dependent thermal effects of radiofrequency electromagnetic field exposure on the whole body of rats. J. Toxicol. Sci. 41 (5), 655–666.

Organization, W.H., 2011. Electromagnetic Fields and Public Health: Mobile Phones. Issues.

Othman, H., et al., 2021. Exposure to 2.45 GHz radiation triggers changes in HSP-70, glucocorticoid receptors and GFAP biomarkers in rat brain. Int. J. Mol. Sci. 22 (10).

Panagopoulos, D.J., et al., 2004. Effect of GSM 900-MHz mobile phone radiation on the reproductive capacity ofDrosophila melanogaster. Electromagn. Biol. Med. 23 (1), 29–43.

Peleg, M., et al., 2018. Radio frequency radiation-related cancer: assessing causation in the occupational/military setting. Environ. Res. 163, 123–133.

Petryk, A., et al., 2003. Shade is the Drosophila P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. Proc. Natl. Acad. Sci. U.S.A. 100 (24), 13773–13778.

Piccinetti, C.C., et al., 2018. Measurement of the 100 MHz EMF radiation in vivo effects on zebrafish D. rerio embryonic development: a multidisciplinary study. Ecotoxicol. Environ. Saf. 154, 268–279.

Rafique, N., et al., 2020. Effects of mobile use on subjective sleep quality. Nat. Sci. Sleep 12, 357–364.

Ridley, E.V., et al., 2012. Impact of the resident microbiota on the nutritional phenotype of Drosophila melanogaster. PLoS One 7 (5).

Shin, S.C., et al., 2011. Drosophila microbiome modulates host developmental and metabolic homeostasis via insulin signaling. Science 334 (6056), 670–674.

Storelli, G., et al., 2011. Lactobacillus plantarum promotes Drosophila systemic growth by modulating hormonal signals through TOR-dependent nutrient sensing. Cell Metabol. 14 (3), 403–414.

Tower, J., 2011. Lactobacillus plantarum gives Drosophila the grow signal. Cell Metabol. 14 (3).

Verbeek, J., et al., 2021. Prioritizing Health Outcomes when Assessing the Effects of Exposure to Radiofrequency Electromagnetic Fields: A Survey Among Experts, vol. 146. Environment International.

Vijver, M.G., et al., 2014. Investigating short-term exposure to electromagnetic fields on reproductive capacity of invertebrates in the field situation. Electromagn. Biol. Med. 33 (1), 21–28.

Wang, Y.H., et al., 2020. Enhancement in the ATP level and antioxidant capacity ofCaenorhabditis elegansunder continuous exposure to extremely low-frequency electromagnetic field for multiple generations. Int. J. Radiat. Biol. 96 (12), 1633–1640.

Weisbrot, D., et al., 2003. Effects of mobile phone radiation on reproduction and development in Drosophila melanogaster. J. Cell. Biochem. 89 (1), 48–55.

Wong, C., et al., 2011. Low-diversity bacterial community in the gut of the fruitfly Drosophila melanogaster. Environ. Microbiol. 13 (7), 1889–1900.

Zhang, H.Y., et al., 2021. Alterations in the activity and sleep of Drosophila melanogaster under simulated microgravity. Npj Microgravity 7 (1).

Zhou, X.F., et al., 2021. Serratia symbiotica enhances fatty acid metabolism of pea aphid to promote host development. Int. J. Mol. Sci. 22 (11).