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Perfluorooctanoic acid induces transgenerational modifications in reproduction, metabolism, locomotor activity, and sleep behavior in *Drosophila melanogaster* and deleterious effects in human cancer cells

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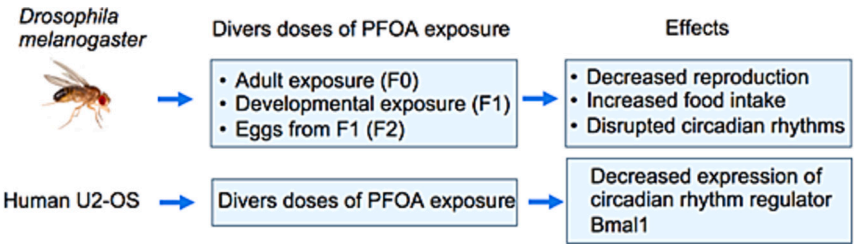
HIGHLIGHTS

- PFOA exposure disrupts the fitness, including reproduction and feeding behavior of *Drosophila*.
- PFOA exposure has transgenerational effects on locomotor activity and sleep behavior in *Drosophila*.
- PFOA affects the transcriptional and protein levels of BMAL1 in U-2 OS cell line.

GRAPHICAL ABSTRACT



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ABSTRACT

Perfluorooctanoic acid (PFOA) has been widely documented to affect various aspects of health, including development, metabolism and neuronal function in a variety of organisms. Despite numerous reports detailing these effects, a comprehensive mechanistic model remains elusive, especially with regard to the long-term effects of PFOA, as it bioaccumulates in food chains with a long half-life. In this study, we evaluated the impact of PFOA on several critical physiological states of *Drosophila melanogaster*. Our findings indicate that PFOA exposure significantly decreases reproductive capacity and induces alterations in starvation resistance and feeding behavior in flies. Interestingly, PFOA exposure also caused changes in locomotor activity and sleep patterns compared with flies receiving a standard diet. Notably, compared with controls, the F2 generation showed increased locomotion and shorter sleep duration during the dark phase, even without direct exposure to PFOA, indicating possible transgenerational effects. Transcriptomic analysis revealed that PFOA also disrupts fatty acid metabolism and alters the expression of immune-responsive genes in *Drosophila*. In the U-2 OS human osteosarcoma cell line, we examined the impact of PFOA on circadian rhythm regulatory proteins and discovered that, compared with controls, BMAL1 levels increased at concentrations from 10 nM to 10 μM. In summary, this

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research highlights the influence of PFOA on diverse biological processes, including reproduction, feeding behavior, starvation resistance, locomotion, and sleep activity in *Drosophila*. It also highlights the ability of PFOA to alter BMAL1 expression patterns in human osteosarcoma cells with deleterious effects.

1. Introduction

Perfluorooctanoic acid (PFOA) is one of the most extensively utilized perfluorinated chemicals (PFCs). Despite reductions in PFOA emissions across North America and Europe during the early 2000s, numerous countries and regions worldwide continue to employ PFOA across various industrial sectors (Du et al., 2023b). Its chemical stability renders PFOA ubiquitous in both environmental settings and wildlife habitats, with detectable traces also found in humans (Olsen et al., 2007; Zareitalabad et al., 2013). Human exposure to PFOA occurs through multiple environmental media, including air, water, food, and dust (Gustafsson et al., 2022; Zareitalabad et al., 2013). The longevity of PFOA in organisms, including humans, at low concentrations, poses challenges for traditional toxicological assessments aimed at understanding its long-term impacts. Consequently, much of our knowledge about the effects of PFOA on human health comes from epidemiological research. In this regard, several studies have found a close relationship between PFOA exposure and an increased risk of kidney and testicular cancer (Olsen et al., 2003; Steenland et al., 2010). Some studies have shown elevated blood levels of PFOA and thyroid dysfunction (Du et al., 2023a).

In vitro studies have discovered complex effects of PFOA exposure, including hormone disruption, gene expression modification, and cancer progression. Notably, PFOA exposure in human hepatocyte models has been linked to enhanced cellular proliferation through its interaction with the estrogen receptor α (ERα) and activation of the peroxisome proliferator-activated receptor γ (PPARγ) (Buhrke et al., 2015). Additionally, long-term exposure of rat liver epithelial cells to PFOA exhibited carcinogenic characteristics (Qu et al., 2023). PFOA activation of PPARα has also been associated with increased malignancy in breast cancer cells (Sakai et al., 2022; Sonthithai et al., 2016). Similarly, it has been shown to reduce the efficacy of chemotherapeutic drugs in acute lymphoblastic leukemia cells (Lagunas-Rangel et al., 2023; Lagunas-Rangel et al., 2022b). These *in vitro* findings underscore the multifaceted impact of PFOA on biological processes, highlighting the complexity of its mechanisms of action.

In vivo studies across various models have revealed diverse effects of PFOA on metabolism, hormonal balance, reproductive health, neurological function, and developmental processes. Studies utilizing models such as mosquito larvae have shown no discernible population-level effects or toxicity risks associated with PFOA exposure (Marziali et al., 2019). However, PFOA-induced obesogenic effects, spanning multiple generations, have been observed in *Caenorhabditis elegans* and medaka fish (Lee et al., 2017; Li et al., 2020). PFOA has demonstrated ER binding activity and altered testosterone levels, potentially leading to reproductive toxicity in rainbow trout (Benninghoff et al., 2011). Furthermore, evidence suggests that PFOA may act as a developmental neurotoxicant in mice, impacting nervous system development (Johansson et al., 2008). Human studies corroborate PFOA's metabolic effects, showing significant increases in cholesterol and lipid levels following exposure (Seyoum et al., 2020; Steenland et al., 2010). Despite the elucidation of various adverse effects in these models, inconsistencies exist, possibly due to variations in PFOA concentrations across different animal or cell models. Thus, it is essential to study the effects of chronic, dose-dependent exposure to PFOA on the body's physical state, as this research can provide valuable information on safe exposure levels and their potential impact on human health. Understanding these relationships will help to develop guidelines for minimizing the health risks associated with PFOA exposure.

Drosophila melanogaster serves as an important whole-organism model for investigating conserved physiological phenomena, including circadian rhythm, metabolism, reproduction, and behavior (Williams et al., 2014, 2016, 2020). The conservation of genes involved in human diseases makes *Drosophila* an invaluable model for investigating key aspects of mammalian biology, such as metabolism, circadian rhythms and neurological functions (Axelrod et al., 2015; van Dam et al., 2020). Leveraging its relatively simple nervous system, *Drosophila* presents an opportunity to dissect the intricacies of neural processes and behaviors (Ramdya et al., 2017). The fly has a unique advantage in studying the generational toxicity of compounds, as it is easy to cultivate and has a short life cycle (Demir, 2020; Kumar et al., 2022). Its amenability to high-throughput assays and genetic manipulation renders it particularly well-suited for toxicological studies and drug screening (Su, 2019).

Currently, knowledge about the chronic and transgenerational effects of PFOA is limited. Further research is needed to understand their long-term impact and possible consequences for future generations in order to develop strategies to prevent them. In this study, a *Drosophila* model is utilized to investigate the effects of varying concentrations and prolonged exposure to PFOA on key physiological functions, including feeding behavior, metabolism, sleep patterns, and reproductive health. In addition, it takes advantage of the *Drosophila* model to explore the dose-dependent transgenerational effects of PFOA exposure as well as transcriptomic changes. In addition, it examines physiological alterations related to circadian rhythms, complementing these findings by analyzing the expression of basic helix-loop-helix ARNT-like protein 1 (BMAL1) in a human osteosarcoma cell line and viability effects.

2. Materials and methods

2.1. Fly strains and husbandry

The wild-type fly lines, *CantonS*, and *Oregon-R-C*, obtained from the Bloomington Stock Center (Bloomington, Indiana, USA), were crossed to create the lab wild-type *CSORC* strain. *Drosophila* were maintained on Jazz-Mix Drosophila food (Thermo-Fisher Scientific, AS153) supplemented with 1.5 % yeast extract (Apex Bioresearch Products, 20-254), 0.3 % propionic acid (Sigma-Aldrich, 402907) and 0.05 % tegosept (Apex Bioresearch Products, 20-258). All flies were maintained at 25 °C and 60 % humidity under a 12:12 h light/dark cycle, unless otherwise specified.

2.2. Drosophila exposure to PFOA

Different concentrations of PFOA (CAS Number: 335-67-1) containing food (10 nM, 100 nM, 1 μM, 10 μM, 100 μM, 300 μM, 700 μM and 1 mM) were used in this study. The concentrations (100 μM, 300 μM, 700 μM and 1 mM) are similar to those used in previous studies in human cell lines and *Drosophila* (Pierozan et al., 2018; Wang et al., 2010). To prepare PFOA-containing food, the powder (Sigma-Aldrich, 171468) was first diluted in ethanol to create a 100 mM stock solution. This solution was then added to standard fly food to achieve the desired PFOA concentrations, ensuring that the volume of ethanol was kept constant in all preparations. To prepare flies for adult exposure to PFOA (F0), 1-day-old males that had developed on normal food were collected and then transferred to either PFOA-containing food or normal food. For flies exposed to PFOA development (F1), mated females laid eggs on food containing PFOA. These eggs developed in the PFOA-containing food until eclosion. One-day-old adults were then collected and transferred to fresh PFOA-containing food for the time required by the experiment. To prepare F2 flies, virgin females and males of the F1 generation were

collected within 3 h of eclosion and transferred to normal food to lay eggs. These eggs developed into adults that were maintained on normal food. Flies of the same age, fed only with vehicle (DMSO), served as a control group.

2.3. Reproduction

We determined the effects of PFOA exposure on reproductive capacity for F0 and F1 generation flies. Briefly, 20–30 pairs of flies were transferred to flasks containing food mixed with PFOA. They were allowed to mate and lay eggs for a period of 24 h. After this time, eggs were counted using a stereoscope. The vials were kept at 25 °C and 60 % humidity under a 12:12 h light/dark cycle to let the eggs develop. The total number of adult flies emerging from these vials was counted. The adult emergence ratio was then calculated as the number of adult flies/the number of eggs in each vial.

2.4. Starvation assay

Starvation resistance was carried out using the Drosophila Activity Monitoring System 2 (DAMS2) from TriKinetics (Waltham, MA, USA). Newly eclosed (0-day-old) or 7-day-old male flies were used for the DAMS2 assays. Thirty-two flies of the F1 generation were individually placed in tubes with agarose (Sigma-Aldrich, A4718) at one end to provide water and humidity, but no food. The tubes were sealed with a black plug at the agarose end, and flies were introduced through the open end, which was sealed with a cotton plug. Flies were maintained at 25 °C and 60 % humidity under a 12:12 h light/dark cycle and monitored until no activity counts were detected for another 12 h.

2.5. Feeding assays

Quantification of food intake was carried out using the FlyPAD to monitor sips per fly with normal food or food containing PFOA (100 μM to 700 μM). Three-day-old flies were collected and fasted for 18 h with access to water. Each fly was individually transferred to the FlyPAD and allowed to feed on 4 μL of 150 mM sucrose (Sigma-Aldrich, S9378) in 0.9 % agarose (Sigma-Aldrich, A4718) for 1 h. Throughout the experiments, flies were maintained at 60 % humidity and 22 °C.

The Capillary feeding (CAFE) assay was adapted from Ja et al., 2007 (Ja et al., 2007). Individual flies were gently placed in 1.5 mL Eppendorf microcentrifuge tubes, each containing a capillary tube (5 μL). To assess food intake, the capillary tube was filled with a liquid diet consisting of 5 % sucrose (Sigma-Aldrich, S9378), 2 % yeast extract (Apex Bioresearch Products, 20-254) and 0.1 % propionic acid (Sigma-Aldrich, 402907). Three food-filled capillaries were set as controls without flies. The final consumption of food was determined as the decreased food level (mm) minus the average diminishment in control capillaries (evaporated volume). Daily food consumption was measured every 24 h and calculated cumulatively over 4 consecutive days. These experiments were run in three replicates with 10 flies for each group.

2.6. Locomotion and sleep/activity behavior

Male flies 6–7 days old were used for locomotor activity and sleep analysis assays. Locomotor activity was monitored using DAMS (Trikinetics, Waltham, MA) for 4 days under a 12 h light/12 h dark cycle (12 L: 12D), followed by another 3 days in darkness (12D: 12D), at 25 °C and 60 % humidity. Total activity counts and sleep time were analyzed using a MATLAB toolkit (MathWorks, Natick, MA). Data collected from the 3 days of 12 L: 12D and the 3 days of 12D: 12D were used to calculate total activity counts and total sleep duration.

2.7. RNA-seq analysis

Ten 3-day-old male flies were fed for 5 days with PFOA or a control vehicle (ethanol) in their food. RNA extraction was carried out using the Trizol method (Sigma-Aldrich, T9424), following the manufacturer's instructions. RNA-seq analysis followed established protocols (Lagunas-Rangel, 2023, 2024). Sequencing was performed at the Uppsala Genome Center Sequencing Services using Illumina HiSeq2000. The quality of raw reads was assessed using the FastQC toolkit, and low-quality reads and adapters were trimmed using Trimmomatic (Bolger et al., 2014). HISAT2 (Kim et al., 2019) was used to align the processed reads to the BDGP6.46 reference genome (Ensembl release 109). Gene expression was quantified using featureCounts (Liao et al., 2014). Poorly expressed genes with no more than one count per million reads (1 CPM) in at least two samples were excluded from further analysis. Raw counts were normalized and differential expression analysis was conducted using Limma (Ritchie et al., 2015). Genes with log2 fold change (log2fc) > 1.0 and adjusted p-value (padj) < 0.05 were classified as upregulated, while those with log2fc < -1.0 and padj < 0.05 were classified as downregulated. Gene Ontology (GO) enrichment analysis was performed on all differentially expressed genes using the clusterProfile package (Yu et al., 2012), providing insights into the biological processes affected by PFOA exposure.

2.8. Cell culture

The U-2 OS human bone osteosarcoma cell line was obtained from ATCC (HTB-96). U-2 OS cells were cultured in DMEM supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS), 50 μg/mL streptomycin and 50 units/mL penicillin (ThermoFisher Scientific). Cell cultures were maintained in a humidified incubator with 5 % CO2 at 37 °C. The stable BMAL1 luciferase reporter cell line BMAL1, U-2 OS-Luc, was generated as previously described (Sun et al., 2022).

2.9. Cell viability assays

In 96-well plates, 10^4 cells per well were seeded and incubated overnight at 37 °C in an incubator with the appropriate CO_2 levels. The next day, the medium was replaced with a fresh medium containing various concentrations of PFOA, and the cells were incubated for 24 h. Cell viability was assessed using PrestoBlue HS Cell Viability Reagent (Invitrogen, P50200), following the manufacturer's instructions. Absorbance was measured at 570 nm, with 600 nm as the reference wavelength, using a Multiskan GO microplate spectrophotometer (Thermo Scientific). At least five independent biological replicates were performed for each group at each time point.

2.10. BMAL1 activity assay

For this assay, 5×10^3 U-2 OS-Luc cells were seeded in a 96-well plate and cultured overnight. PFOA was dissolved in DMSO to create a 100 μM stock solution and then added to the 96-well plate at final concentrations ranging from 0 nM to 1 μM, including vehicle (DMSO) control groups. After 48 h of exposure to PFOA, the medium was removed and cells were lysed with lysis buffer (20 μL/well) (Thermo Scientific, 16189). Subsequently, 100 μL of D-Luciferin (10 μM) (Nanolight Technology, 306) was added to each well and luminescence measurements were performed with a luminometer (EnSpire™ Multimode Plate Reader, United States).

2.11. Western blots

For protein level analysis, 1×10^5 U-2 OS cells were seeded in a 6-well plate and cultured overnight. The medium was then replaced with fresh medium containing varying concentrations of PFOA (from 0 nM to 1 μM). After an incubation period of 48 h, the cells were washed with PBS and lysed with RIPA buffer (Cell Signaling, 9806) supplemented with a protease inhibitor cocktail (Roche, 11836153001). Protein concentration was determined with the Bradford assay (Thermo Fisher Scientific, 23200). For each sample, 20 μg protein was resolved in 4 %–15 % SDS-

PAGE gel (Bio-Rad, 4561023) and transferred onto PVDF membranes (Bio-Rad, 1620174). The transferred membranes were blocked in EveryBlot Blocking Buffer for 5 min (Bio-Rad, 12010020) and incubated with the BMAL1 primary antibodies (Invitrogen, PA1-523) at 4 °C overnight. Then, the membrane was washed trice with TBST for 7 min each time and moved to horseradish peroxidase-coupled isotype-specific secondary antibodies (Sigma-Aldrich, A0545) for 1 h at room temperature. Chemiluminescence detection was performed by ChemiDoc MP Imaging System (BioRad, Sweden) using SuperSignal™ West Atto Ultimate Sensitivity Substrate according to the manufacturer' s instructions (ThermoFisher Scientific, 16425284).

2.12. Data analysis

GraphPad Prism version 9 (La Jolla, CA, United States) was used for statistical analysis and graph generation. All data were expressed as mean ± SEM. The normality of the data was assessed using the Shapiro-Wilk test. One-way analysis of variance (ANOVA) followed by Tukey's or Dunnett's multiple comparisons test was used to compare differences between groups, as appropriate. Starvation survival data were subjected to survival analysis using Log-rank tests with Mantel-Cox posttest and presented as survival curves. Immunoblot analysis was performed with ImageJ 1.53q.

3. Results

3.1. PFOA decreased Drosophila reproductive capacity

CSORC flies were exposed to a series of concentrations of PFOA either during adult stages (F0), developmental stages (F1) and eggs from F1 but kept in normal food (F2) (Fig. 1A). The effects of PFOA on the reproductive capacity of fruit flies were extensively evaluated. Initially, adult flies (F0) exposed to various concentrations of PFOA (0 μM, 100 μM, 300 μM, 700 μM and 1000 μM) showed consistent egg-laying behavior, with no significant alterations in the number of eggs laid over a 24-h period (Fig. 1B). However, when examining the developmental outcomes of the eggs laid by F0, a dose-dependent response to PFOA exposure was observed. In particular, exposure to 700 μM PFOA caused a significant decrease in the emergence of adult flies from F1 eggs, with complete developmental failure observed at 1000 μM

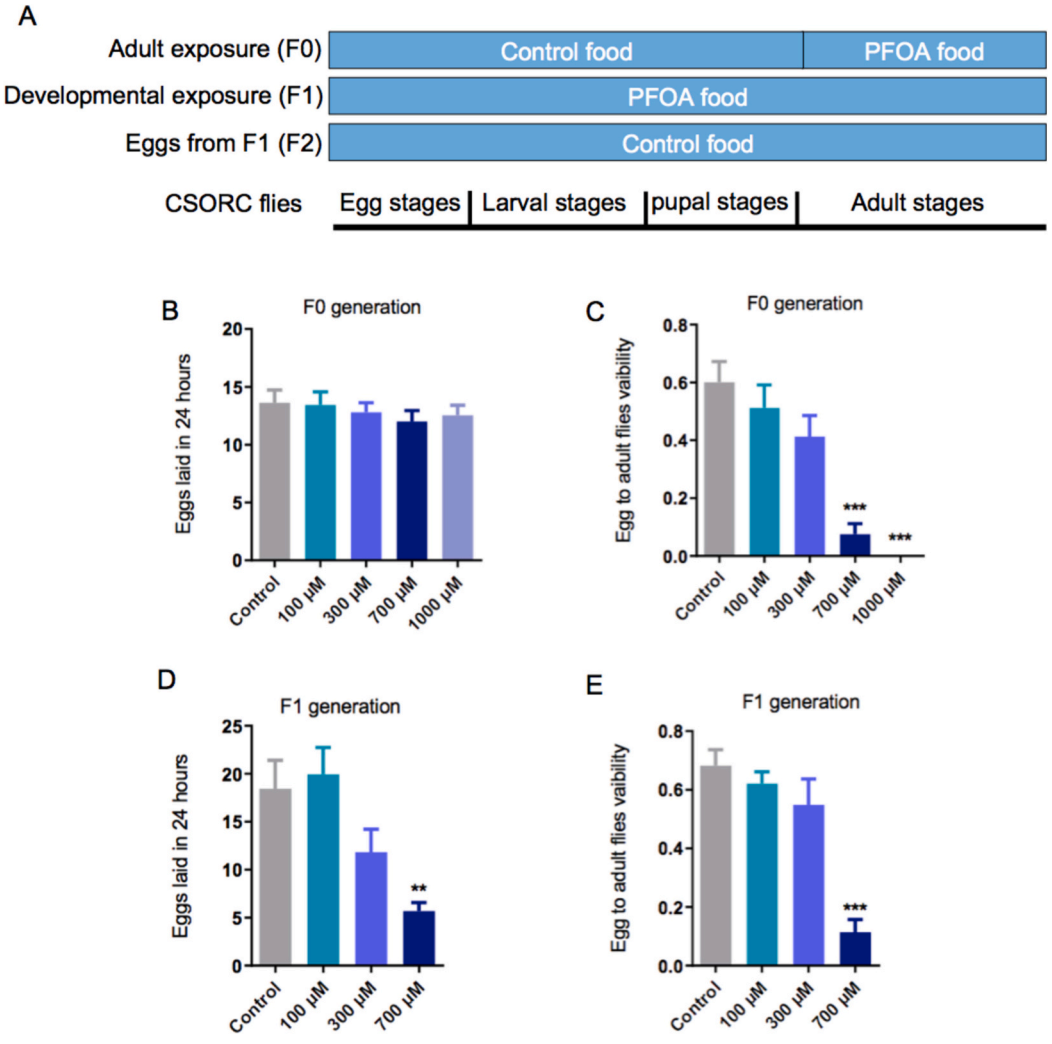

Fig. 1. PFOA exposure affects the reproduction of flies. (A) CSORC flies were exposed to PFOA food either during adult stages (F0), developmental stages (F1) and eggs from F1 but raised in normal food (F2). The concentrations of PFOA were set at 0 μM, 100 μM, 300 μM, 700 μM and 1000 μM as provided in food. (B) PFOA exposure during the adult stage (F0) for 5 days has no effects on the number of eggs laid. (C) The eggs of PFOA adult exposure (F0) flies have decreased ability to develop into adult. At the concentration of 1000 μM, no eggs developed into adult flies. (D) Flies that were developed in PFOA food (F1 flies) laid less eggs than controls. (E) The eggs of F1 flies have decreased egg to adult flies ability with a dose-response relationship. Data in graphs are presented as means ± SEM. For (B) and (C), $n = 30$ pairs of flies for each treatment from three replicates. For (D) and (E), $n = 20$ pairs of flies for each treatment from two replicates. $^{**}p < 0.01$, $^{***}p < 0.001$, compared to normally fed flies, as assessed by one-way ANOVA followed by Tukey's multiple comparisons.

(Fig. 1C). Interestingly, flies exposed to 700 μM PFOA during developmental time (F1) showed a marked reduction in egg-laying ability compared to the control group, indicating a sustained impact on reproductive function (Fig. 1D). Similarly, we observed that eggs from F1 generation (F2) also displayed decreased ability to develop into adult stages following exposure to 700 μM PFOA (Fig. 1E). These results provide detailed insight into the detrimental effects of PFOA on fruit fly reproduction, highlighting the importance of assessing its multigenerational impact to understand its reproductive toxicity profile more comprehensively.

3.2. PFOA affects the metabolism of adult fruit flies

We next asked whether PFOA adult exposure on fruit fly affects metabolism of flies by monitoring starvation resistance and feeding behavior. Newly eclosed and 7-day-old F0 adult flies were collected. Subsequently, starvation resistance and feeding behavior were investigated. The results (Fig. 2A and B) revealed that exposure for 3 days to these PFOA concentrations significantly increased the starvation resistance of newly eclosed adult flies. However, among the 7-day-old adult flies, only those exposed to 300 μM of PFOA showed a significant increase in starvation resistance.

To investigate the effects of PFOA on fruit fly feeding behavior, FlyPAD and CAFÉ assays were used to measure the number of sips and total food intake, respectively. Prior to the experiments, flies were deprived of food, but not water, for 18 h. The results of the FlyPAD assay (Fig. 2C) indicated that exposure to PFOA tends to increase the number of sips ingested by fruit flies, although this increase was not statistically significant. The duration of fly sipping showed no significant differences between these groups. Results from the CAFÉ assay (Fig. 2D) revealed that flies exposed to food containing 700 μM of PFOA showed significantly higher total food intake after starvation compared to the control group.

These results illustrate the intricate relationship between PFOA exposure and fruit fly starvation resistance and feeding behavior. Flies developed in concentrations (from 10 nM to 10 μM) of PFOA have no effects on starvation resistance for both newly eclosed and 7 days old adult flies (Fig. S1A). Flies developed in relatively higher concentrations (from 100 μM to 700 μM) of PFOA significantly increased starvation resistance compared to the control group in the newly eclosed flies, but not when they were 7 days old (Fig. S1B).

3.3. PFOA alters fruit fly activity rhythms

To explore the effects of PFOA on locomotor activity rhythms and sleep patterns of adult fruit flies (F0), 3-day-old adult males reared on

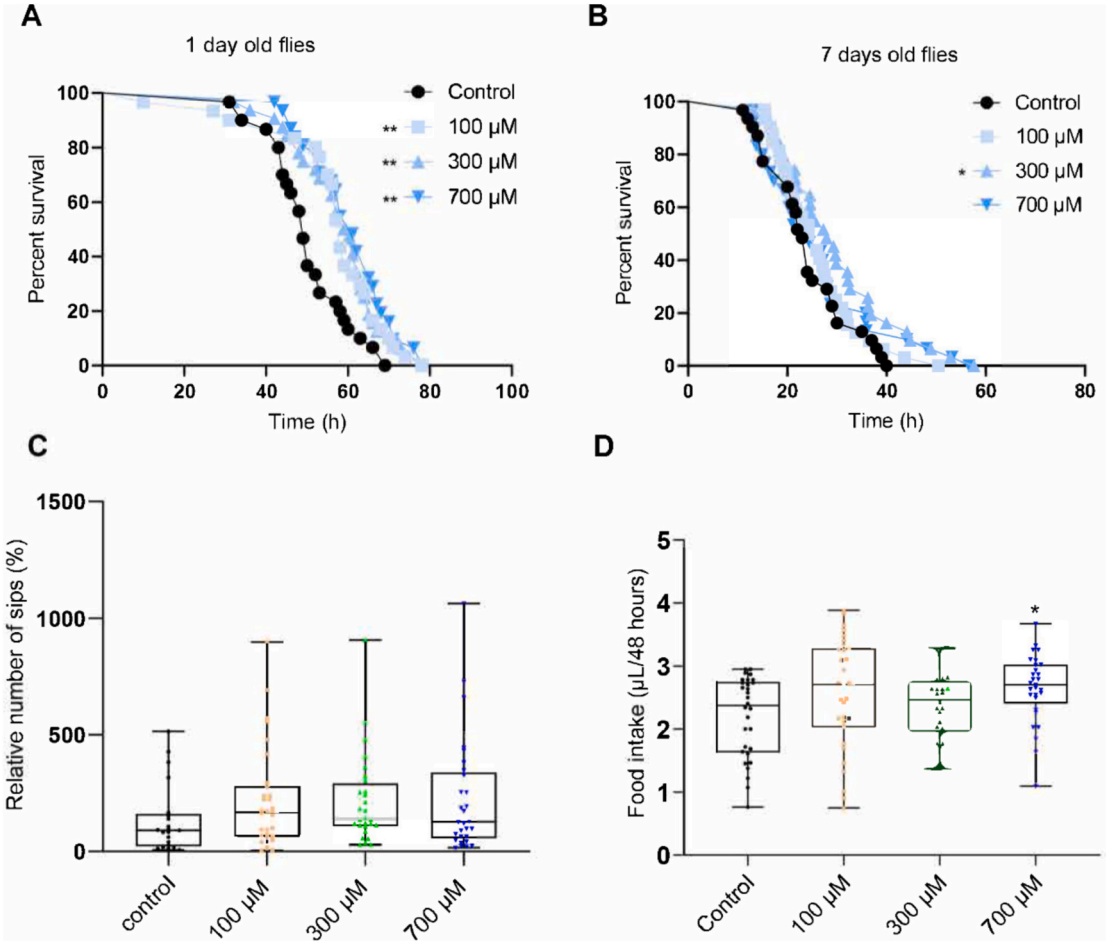

Fig. 2. PFOA altered the starvation resistance and feeding behavior of adult flies in a dose-dependent effect. (A) and (B) Flies were developed in control food or food containing PFOA at concentrations of 100 μM, 300 μM and 700 μM, then newly eclosed and 7 days old flies were used for starvation resistance assay. 30–32 flies from two replicated experiments. $^{**}P < 0.01$, data are presented in survival curves, as assessed by log-rank (Mantel-Cox) test. (C) Food intake quantifications based on the FlyPAD-monitored sips per flies which developed from 100 μM, 300 μM and 700 μM PFOA food. Flies were starved for 18 h before the experiments were performed. All of the data points were shown, $n = 21–28$ flies for each treatment from four replicates. (D) Total food intake in flies developed in 700 μM PFOA comparing to controls using Capillary Feeding assay (CAFE). All of the data points were presented in the graph, $n = 29–31$ flies for each treatment from three replicates ($^{*}p < 0.05$, compared to normally fed flies, as assessed by one-way ANOVA followed by Tukey's multiple comparisons).

normal food were transferred to normal food or food containing PFOA at concentrations of 100 μM, 300 μM, 700 μM and 1 mM and cultured for 6 days. Subsequently, their locomotor activity rhythms and sleep patterns were monitored under 12 L:12D and 12D:12D configurations using the DAMS system (Trikinetics, Waltham, MA). Fruit flies exposed to food containing 100 μM, 300 μM and 700 μM of PFOA showed no significant changes in locomotor activity rhythms or sleep patterns, compared with control flies (Fig. 3A-D). However, flies exposed to food containing 1 mM PFOA showed a significant increase in locomotor activity, observed mainly during the second 12-h dark period, while their sleep patterns were not affected (Fig. 3E-H).

3.4. Developmental exposure to PFOA alters Drosophila activity and sleep patterns

To evaluate the influence of PFOA exposure at the developmental

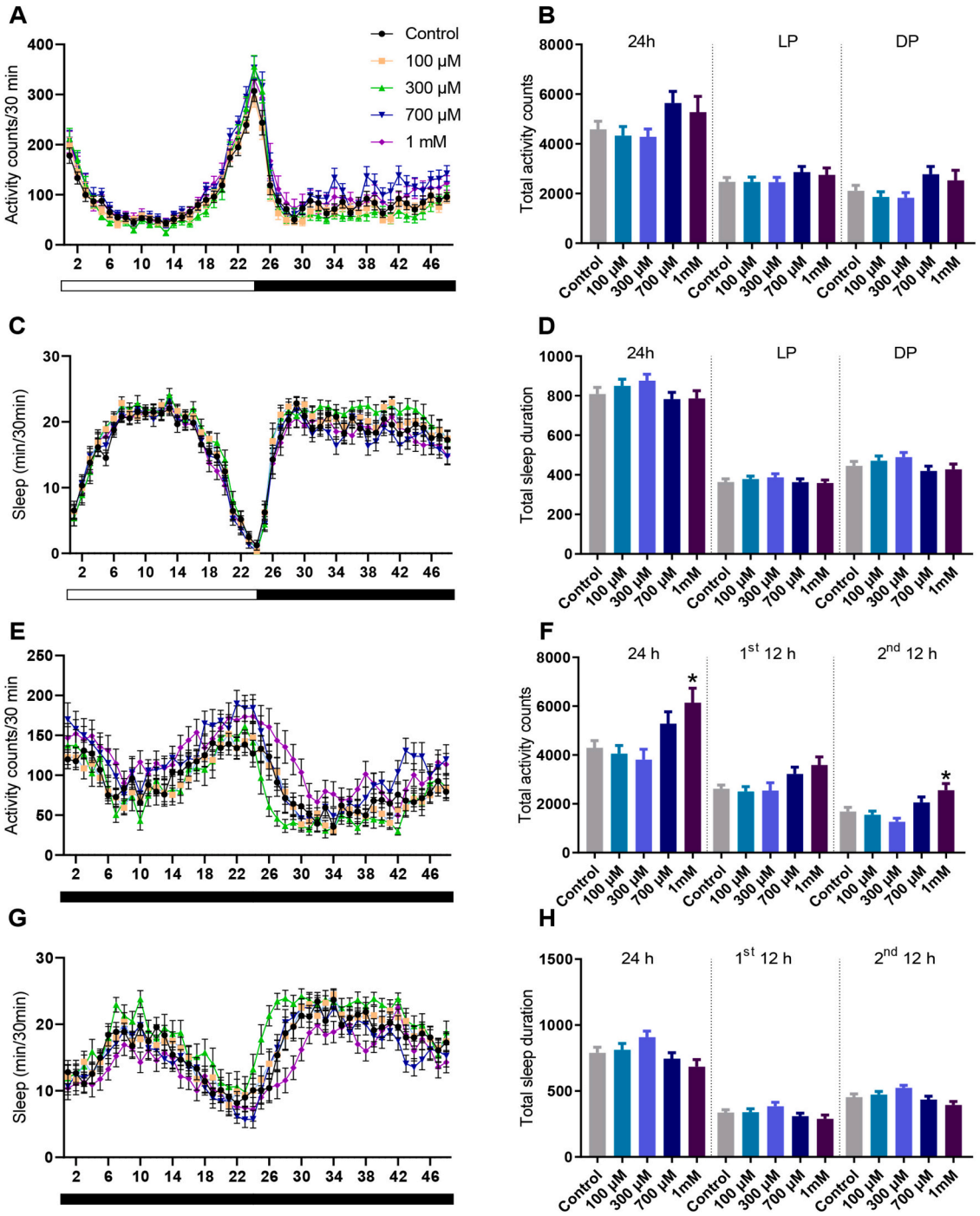

Fig. 3. PFOA adult exposure (F0) flies shown disturbed sleep/activity pattern in the Dark phase of 12D: 12D condition. 1-day old adult males developed in normal food were collected and then transferred to normal food or food containing PFOA at concentrations of 100 μM, 300 μM, 700 μM and 1 mM for 6 days. After that, locomotor activity rhythms and sleep patterns of these flies were monitored. (A-D) The average locomotor activity and sleep pattern were measured over 3 days in normal 12 L: 12D cycles for 3 days. (*E*-H) The average locomotor activity and sleep pattern measured over 3 days in 12D: 12D cycles. Data in graphs are presented as means ± SEM, $n = 17–30$ flies for each treatment from two replicates. *$P < 0.05$, as assessed by one-way ANOVA followed by Tukey's multiple comparisons. Data average from 3 days were used for analysis.

stage on total activity and sleep patterns of fruit flies (F1), flies were reared from the egg stage to the adult stage on normal food or on food containing 100 μM, 300 μM and 700 μM PFOA. Next, 6–7 day old male flies were used to measure locomotor activity rhythms and sleep patterns. Using the DAMS system, locomotor activity rhythms and sleep patterns of fruit flies were monitored in 12 L:12D and 12D:12D configurations. The results indicate that flies exposed during development to food containing 700 μM PFOA showed a significant decrease in total activity and a significant increase in sleep duration (Fig. 4A-D).

Under 12D:12D dark conditions, fruit flies exposed to food containing 700 μM PFOA during development showed a significant increase in sleep duration, while total activity tended to decrease compared to the control group (Fig. 4E-H). Supplementary Fig. 2 illustrates that F1 flies exposed to 1 μM and 100 μM PFOA significantly increased their locomotor activity during the 12D:12D cycle ($p < 0.05$).

3.5. Generational effects of PFOA exposure on locomotor activity in Drosophila

To evaluate the impact of PFOA exposure on the activity and sleep patterns of fruit fly F2 generation, parental flies were reared from the egg to adult stage on normal food or food containing 100 μM, 300 μM and 700 μM PFOA. Subsequently, F1 virgin females and males were collected and allowed to lay eggs on normal food. The eggs layed by F1 flies were reared on normal food (F2) and 6–7 days old F2 male flies were collected to measure locomotor activity and sleep patterns under 12 L:12D cycle conditions. The results indicate that F2 generation flies from parents exposed to 700 μM PFOA showed a significant increase in total activity and a significant decrease in sleep duration, with the main impact observed during the dark phase (Fig. 5A-D).

We further observed that under 12D:12D cycle conditions, F2 generation flies from parents exposed to 700 μM PFOA showed a significant

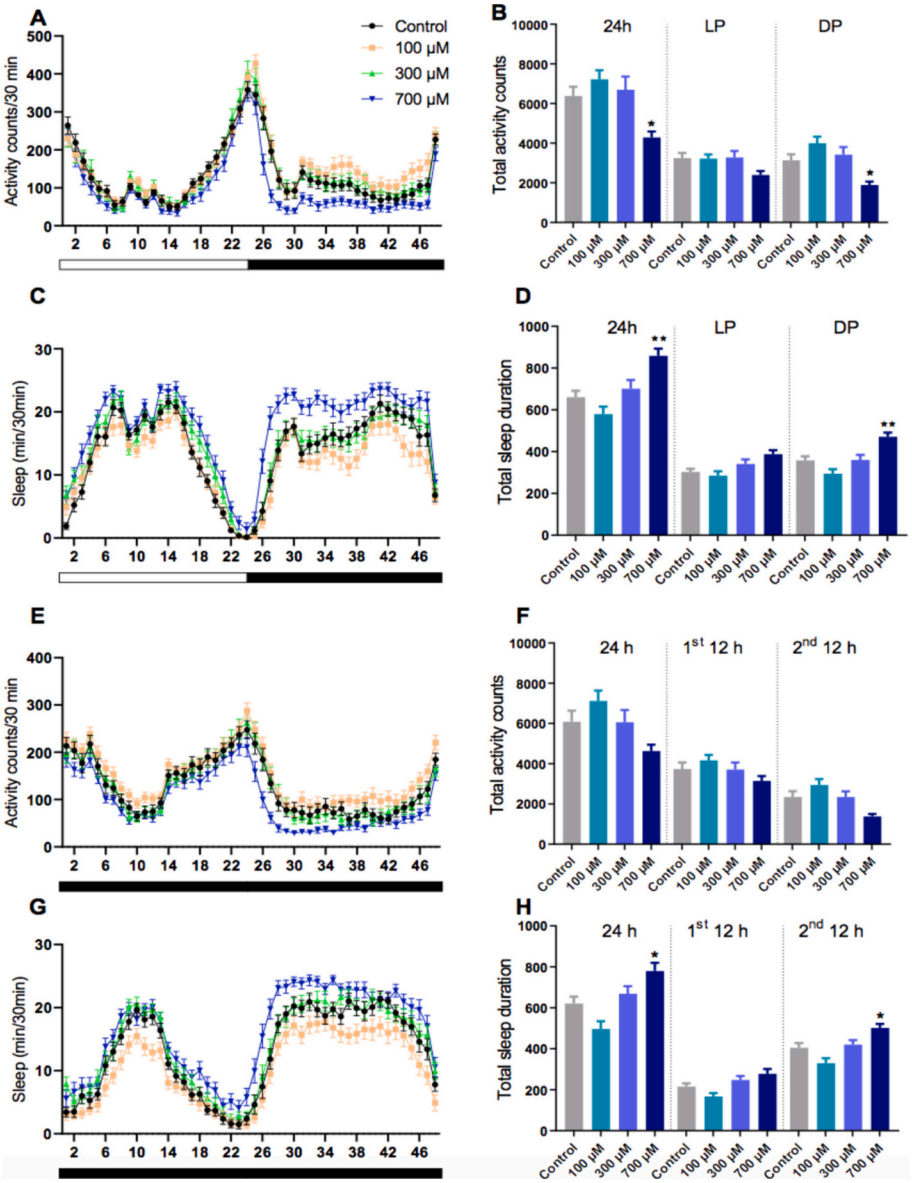

Fig. 4. PFOA developmental exposure (F1) flies displayed altered total activity and sleep time. Flies were raised in normal food or food containing PFOA at concentrations of 100 μM, 300 μM and 700 μM through the egg to the adult stage. 6–7 days old male flies (F1 generation) were used for measuring locomotor activity rhythms and sleep patterns. The average locomotor activity (A, B) and sleep pattern (C, D) were measured over 3 days in normal 12 L: 12D cycles for 3 days. The average locomotor activity (E, F) and sleep pattern (G, H) were measured over 3 days in normal temperature at 12D: 12D cycles for 3 days. Data in graphs are presented as means ± SEM, n = 30–32 flies for each treatment from two replicates (*$p < 0.05$, **$p < 0.01$, compared to normally fed flies, as assessed by one-way ANOVA followed by Tukey's multiple comparisons). Data average from 3 days was used for analysis.

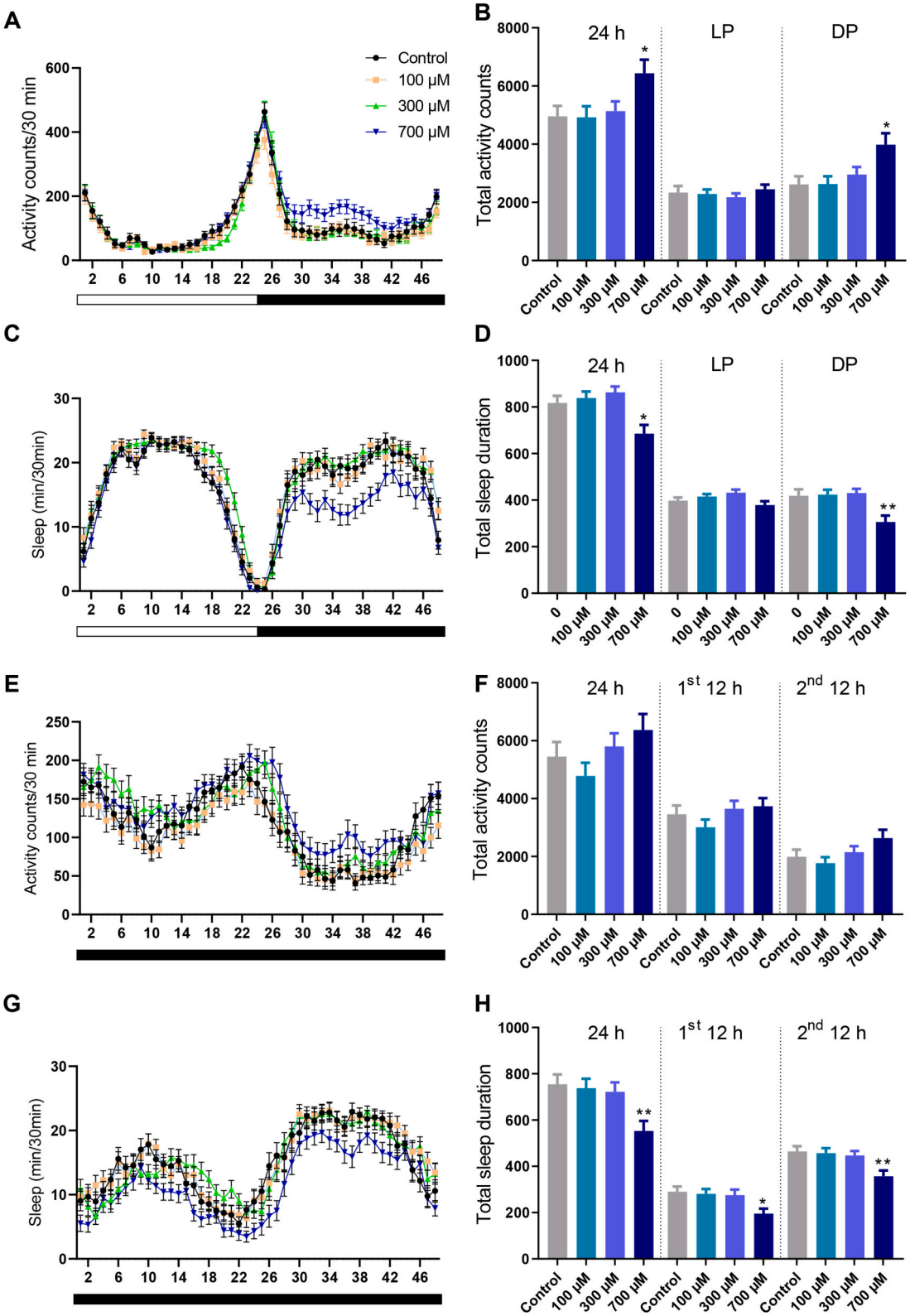

Fig. 5. The eggs derived from F1 generation that developed in normal food (F2) flies have changed locomotioin activity and sleep behaviro. Flies were raised in normal food or food containing PFOA at concentrations of 100 μM, 300 μM and 700 μM through the egg to the adult stage. Then virgin females and males of F1 generation flies were collected and allowed to lay eggs in normal food. 6–7 days of male flies developed from these eggs were used for measuring locomotor activity and sleep patterns. The average locomotor activity (A, B) and sleep pattern (C, D) were measured over 3 days in normal 12 L: 12D cycles for 3 days. The average locomotor activity (E, F) and sleep pattern (G, H) were measured over 3 days in normal temperature at 12D: 12D cycles for 3 days. Data in graphs are presented as means ± SEM, n = 30–32 flies for each treatment from two replicates (*$p < 0.05$, **$p < 0.01$, compared to normally feed flies, as assessed by one-way ANOVA followed by Tukey's multiple comparisons). Data average from 3 days was used for analysis.

decrease in sleep duration (Fig. 5E-H). Although a trend toward increased total activity was observed, this effect was not statistically significant.

3.6. PFOA modifies the metabolism of flies

To further investigate the effects of PFOA, flies were exposed to a concentration of 10 μM for 5 days, after which RNA was extracted and sequenced. We identified a total of 102 differentially expressed genes (DEGs) in PFOA-feded flies compared to control flies. Among them, 66 genes were up-regulated, whereas 36 genes were down-regulated. GO results revealed that PFOA significantly alters genes related to fatty acid transport and metabolism and increases the expression of immunoreactive genes in *Drosophila* (Fig. 6).

3.7. PFOA exposure decrease viability and altered BMAL1 expression in OS U-2 cells

Based on experiments with fruit flies, it is evident that PFOA exposure during the developmental and adult stages affects locomotor activity and sleep patterns, which are connected to circadian rhythms. Consequently, we set out to explore the effects of PFOA on circadian rhythm. For this purpose, we used our stable U-2 OS BMAL1 luciferase reporter cell line. Cell viability assays performed by PrestoBlue detection revealed no significant impact on the viability of U-2 OS cells with PFOA concentrations ranging from 0.1 nM to 10 μM (Fig. 7A). Luminescence assays were performed with PFOA concentrations ranging from 1 nM to 100 nM. The results indicated a significant increase in BMAL1 transcript expression levels following exposure to PFOA concentrations of 10 nM and 100 nM (Fig. 7B).

To analyze the impact of PFOA exposure on BMAL1 protein expression in U-2 OS cells, we performed western blot experiments. The results revealed a significant increase in BMAL1 protein expression levels after 48 h of exposure to PFOA with concentrations of 10 nM and 100 nM (Fig. 6C and D). These findings suggest that PFOA exposure affects BMAL1 expression levels at both the transcriptional and protein levels in U-2 OS cells, which could lead to changes in circadian rhythm.

4. Discussion

We are increasingly exposed to environmental pollutants, including complex mixtures, which have significant effects on our health (Cedergreen, 2014; Goodson et al., 2015; Lagunas-Rangel et al., 2022a, 2022b). PFOA is a widespread pollutant that has been identified in human serum at levels ranging from 0.85 to 16.4 ng/mL (Cheng et al., 2022; Raymer et al., 2012). Environmental concentrations are also of concern, as PFOA has been detected in sediments at 0.27 ng/g, in sewage sludge at 37 ng/g, in surface water at 3 ng/L and in wastewater treatment effluents at 24 ng/L (Zareitalabad et al., 2013). These levels highlight the widespread nature of PFOA pollution and its potential impact on both human health and the environment. In addition, PFOA in sediments can become a potential source of secondary contamination (Fagbayigbo et al., 2022). It can also affect the sorption of nutrients available to plants and sediment-dwelling organisms or be released into the environment due to extreme weather conditions, as has been shown in several studies (Ahmadireskety et al., 2021). The estimated elimination half-life of PFOA in humans ranges from 1.2 to 14.9 years, while in the environment it is considered a “forever” pollutant due to its persistence (Dourson and Gadagbui, 2021).

In this study, we found that PFOA exposure significantly altered sleep behavior and activity in fruit flies. In addition, PFOA-fed flies showed reduced reproductive capacity and increased resistance to starvation. Notably, PFOA has a much longer half-life than other pollutants known to disrupt circadian rhythms. For example, dibutyl phthalate (DBP) has a half-life of 48 h in the human body, 7.4 h to 3.1 days in air, 1 to 14 days in surface water, and 2 to 23 days in groundwater and soil (Xu et al., 2022). Di(2-ethylhexyl)phthalate (DEHP) has a half-life of 12 h in the human body, 50 days in surface water, and 300 days in soil (Yuan et al., 2022), while benzene's half-life ranges from 9 to 24 h in the human body and 3 to 10 days in air (Hattemer-Frey et al., 1990). Remarkably, our long-term monitoring revealed that the behavioral changes induced by PFOA persisted across generations, with altered sleep patterns and activity evident in the F2 generation. Since circadian rhythms regulate sleep (Matenchuk et al., 2020), reproductive efficacy (Peterlin et al., 2019), and metabolic processes (Poggiogalle et al., 2018), the multigenerational effects of PFOA observed in this study could be strongly associated with alterations in circadian rhythms.

Supporting the idea that the effects of PFOA could involve a

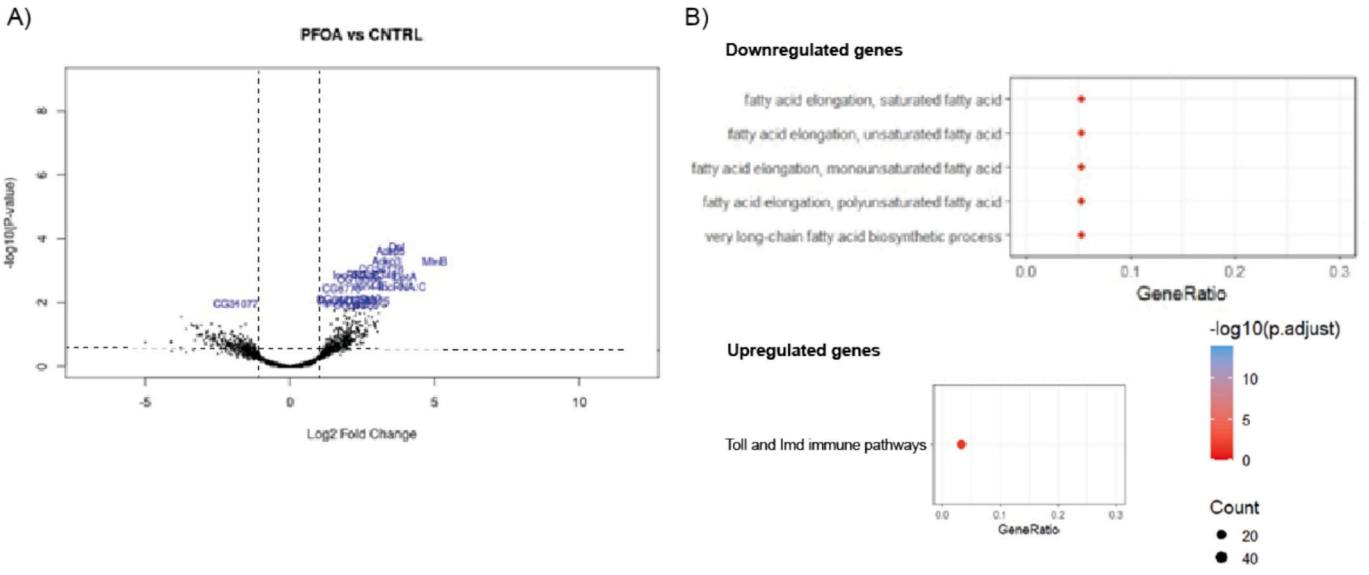

Fig. 6. Transcriptomic analysis reveals harmful effects of PFOA on fatty acid transport and metabolism. A) The volcano plot illustrates the comparison between flies fed a diet containing 10 μM of PFOA for 5 days and the control group, highlighting differentially expressed genes. B) Gene Ontology (GO) enrichment analysis was performed on all differentially expressed genes using the clusterProfile package, providing insights into the biological processes affected by PFOA exposure.

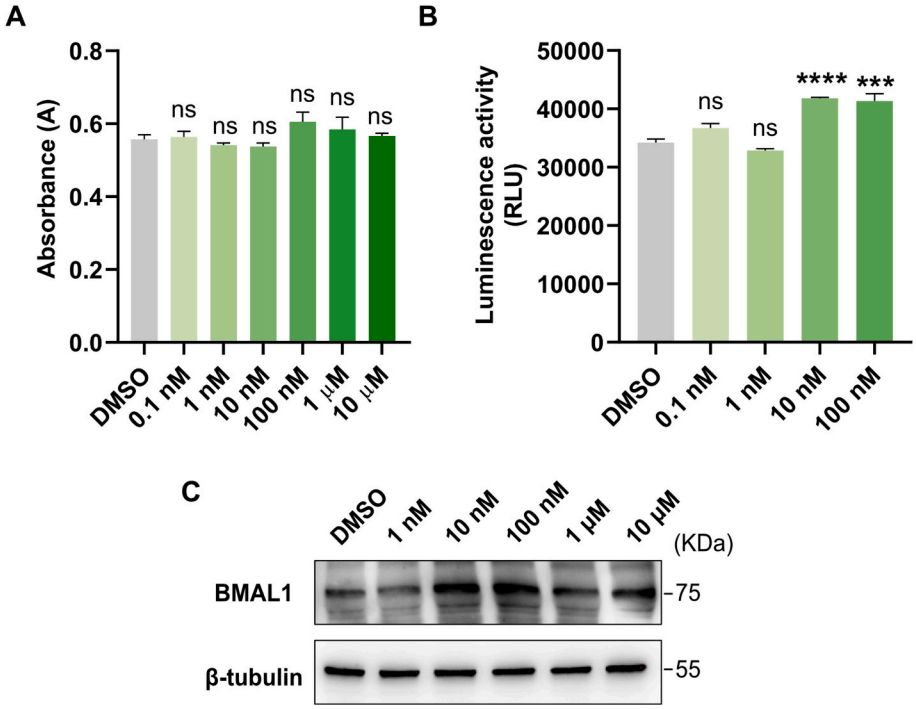

Fig. 7. Effect of PFOA exposure to circadian rhythm in U-2 OS cells. (A) Cell viability of U-2 OS cells exposed to PFOA performed by PrestoBlue Assay (n = 3). The concentrations of PFOA were set from 0.1 nM to 10 μM. (B) BMAL1 endogenous promoter driven luciferase activity of U-2 OS-Luc cells after PFOA treated (n = 3). ***$p < 0.001$, ****$p < 0.0001$ compared to DMSO control, ANOVA with Dunnet's poshoc was used. (C) Western blot results of BMAL1 in U-2 OS cells with PFOA exposure (n = 3).

disruption of the circadian rhythm, similar effects have been described for other pollutants. These include polystyrene nanoplastics (Kang et al., 2023), DBP (Liu et al., 2021), DEHP (Currie et al., 2005), ozone (Sundar et al., 2023), airborne particles (Daiber et al., 2022), benzene (Park et al., 2008), and cigarette smoke (Vasu et al., 2009), among many others (Lagunas-Rangel et al., 2022a). These findings highlight the broader context in which environmental pollutants can alter biological clocks, suggesting a common underlying mechanism by which various toxicants affect circadian regulation. Here, in an osteosarcoma cell line, PFOA was shown to affect the transcript and protein levels of BMAL1, which is an important regulator of circadian rhythms. Notably, these effects occur at concentrations similar to those found in the environment (Zareitalabad et al., 2013) and in human serum (Cheng et al., 2022; Raymer et al., 2012). These findings suggest that even at concentrations currently considered safe (according to the U.S. Environmental Protection Agency of 70 ng/L or 0.169 nM) (Cordner et al., 2019), PFOA could have biological effects, potentially altering circadian rhythms.

One of the advantages of the *Drosophila* model is its ability to assess effects across multiple generations due to its short generation time (Yamaguchi and Yoshida, 2018). This makes it an ideal model to study the transgenerational impact of environmental pollutants, such as PFOA. In this study, we found that PFOA has complex effects. For example, we observed a significant reduction in the number of eggs that became adult flies (F1) when reared on food containing 700 μM PFOA (Fig. 1B). Moreover, these effects were compounded in the next generation by limiting the number of eggs (from F1 generation) that became adults (F2), even when reared on normal food (Fig. 1D). It has been reported that PFOA exposure can adversely affect mice ovarian function by causing mitochondrial dysfunction, inducing apoptosis, and impairing follicular development, all of which contribute to reproductive toxicity (Zhou et al., 2022). In addition, PFOA disrupts spindle assembly and chromosomal alignment during the first division of early mouse embryos, affecting early embryonic division and blastocyst formation (Zhou et al., 2022). Similarly, exposure of pregnant mice to PFOA has detrimental effects on the development of the testes of male offspring. This damage may be due to reduced testosterone levels, inhibition of Sertoli cell activation, alterations in structural functions and impairment of the ability of these cells to perform their supportive functions in spermatogenesis (Bao et al., 2021; Song et al., 2018).

Our transcriptional analysis also highlights the impact of PFOA on fatty acid transport and metabolism in *Drosophila* (Fig. 6). Previous studies have shown that PFOA exposure early in life induces lipid accumulation in *Caenorhabditis elegans* and rats (Haughom and Spydevold, 1992; Lin et al., 2022), characterized by elevated triglyceride and reduced cholesterol levels (Louisse et al., 2020). This effect is largely attributed to the activation of PPARα by PFOA (Vanden Heuvel et al., 2006). Cholesterol reduction could also contribute to decreased levels of reproductive hormones, such as estrogen and testosterone, which could explain the decreased reproductive capacity observed. We also observed an increase in the expression of immune-responsive genes in flies, particularly those associated with the Toll and Imd pathways, which are the primary regulators of the immune response in *Drosophila* (De Gregorio, 2002).

A study reports that exposure to PFOA during pregnancy negatively affects the growth and development of offspring (Li et al., 2019). Moreover, prenatal PFOA exposure has been associated with small to moderate effects on neurobehavioral development in children, manifested primarily in increased hyperactive behavior (Høyer et al., 2015). These findings are consistent with our observations in fruit flies, where PFOA exposure caused changes in locomotor activity and sleep patterns. In F1 flies, exposure to 700 μM PFOA significantly reduced total activity (Fig. 4A and B) and increased sleep duration (Fig. 4G and H) in both the 12 L:12D and 12D:12D cycles. In contrast, F2 flies reared on normal food showed increased locomotor activity during the 12 L:12D, but not during 12D:12D dark conditions (Fig. 5A and B). Furthermore, in F2 flies, sleep time decreased in both the 12 L:12D and 12D:12D cycles (Fig. 5G and H). These results indicate that PFOA exposure has the potential to cause multigenerational toxicity, resulting in complex alterations of sleep patterns and locomotor activity in successive generations.

It has been described that central or peripheral administration in

mice of PFOA can decrease food intake through PPARα activation (Asakawa et al., 2007, 2008). In addition, studies have indicated a negative relationship between human prenatal PFOA exposure and waist circumference later in life (Frigerio et al., 2023). This is consistent with our findings that developmental exposure to PFOA-containing food significantly increased starvation resistance, with a greater effect observed in newly eclosed males compared to 7-day-old male flies (see Fig. 2A and B).

PFOA activates several signaling pathways, including PPARα and constitutive androstane receptor (CAR) which directly regulate key components of the circadian clock (Li et al., 2017). For example, PPARα and PPARγ are direct regulators of core clock components, BMAL1 and REV-ERBα, and, conversely, PPARα is also a direct BMAL1 target gene (Chen and Yang, 2014). CAR can coactivate the aryl hydrocarbon receptor repressor (AhR), which competes with CLOCK for dimerization with BMAL1. While the CLOCK/BMAL1 dimer functions as an activator at E box promoters, the AhR/BMAL1 dimer behaves differently, acting as a repressor (Lagunas-Rangel et al., 2022a). These two phenomena may be interconnected with our observations regarding the impact of PFOA on flies, including the potential transgenerational effects.

In conclusion, PFOA exhibits multiple and generational effects in *Drosophila*, altering reproduction, locomotor activity, sleep patterns and feeding behavior. These alterations may be related to the deleterious effects on lipid metabolism and interference with circadian rhythms caused by PFOA. For further validation of the effect of PFOA on sleep and activity in flies, we demonstrated that PFOA interferes with the circadian rhythm of U-2 OS cells at low concentrations, comparable to those found in the human body and the environment (Cheng et al., 2022; Raymer et al., 2012; Zareitalabad et al., 2013). In view of these considerations, we believe that further research evaluating the effects of PFOA on living organisms and the environment is warranted. Particular attention should be paid to studies examining the impact of trace pollutants with confirmed hormonal activity. PFOA, in particular, may enhance the release of these pollutants from their sources due to its unique range of octanol-water partition coefficient (Kow) values. Furthermore, studies are needed to elucidate the precise mechanisms linking the effects of PFOA on locomotor activity, sleep, feeding behavior and reproduction to circadian rhythm regulations. It is crucial to explore additional factors, such as possible synergistic effects with other pollutants, as well as the living conditions of individuals. This includes populations with altered circadian rhythms, such as night shift workers or frequent travelers who experience significant time differences from their place of origin. Such investigations would provide deeper insight into the global effects of PFOA exposure in a variety of real-world settings.

CRediT authorship contribution statement

Sifang Liao: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Chengxi Sun:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Francisco Alejandro Lagunas-Rangel:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Wen Liu:** Supervision, Investigation. **Shiyao Yi:** Writing – original draft. **Dalia Browne-Johnson:** Investigation. **Filippa Eklund:** Investigation. **Yi Zhang:** Writing – review & editing. **Błażej Kudłak:** Writing – review & editing, Supervision. **Michael J. Williams:** Writing – review & editing, Supervision, Methodology. **Helgi B. Schiöth:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.177472.

Data availability

Data will be made available on request.

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