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Maternal Preconception PFOS Exposure of *Drosophila melanogaster* Alters Reproductive Capacity, Development, Morphology and Nutrient Regulation

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Abstract

Perfluorooctanesulfonic acid (PFOS) is a persistent synthetic surfactant widely detected in the environment. Developmental PFOS exposures are associated with low birth weight and chronic exposures increase risk for obesity and type 2 diabetes. As an obesogen, PFOS poses a major public health exposure risk and much remains to be understood about the critical windows of exposure and mechanisms impacted, especially during preconception. Here, we leverage evolutionarily conserved pathways and processes in the fruit fly *Drosophila melanogaster* (wild-type *Canton-S* and *megalin*-UAS RNAi transgenic fly lines) to investigate the window of maternal preconception exposure to PFOS on reproductive and developmental toxicity, and examine receptor (megalin)-mediated endocytosis of nutrients and PFOS into the oocyte as a potential mechanism.

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Declaration of interests

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.

Preconception exposure to 2 ng PFOS/female resulted in an internal concentration of 0.081 ng/fly over two days post exposure, no mortality and reduced *megalin* transcription. The number of eggs laid 1–3 days post exposure was reduced and contained 0.018 ng PFOS/egg. Following heat shock, PFOS was significantly reduced in eggs from megalin-knockdown transgenic females. Cholesterol and triglycerides were increased in eggs laid immediately following PFOS exposure by non-heat shocked transgenic females whereas decreased cholesterol and increased protein levels were found in eggs laid by heat shocked transgenic females. Preconception exposure likewise increased cholesterol in early emerging wildtype F1 adults and also resulted in progeny with a substantial developmental delay, a reduction in adult weights, and altered transcription of *Drosophila* insulin-like peptide genes. These findings support an interaction between PFOS and megalin that interferes with normal nutrient transport during oocyte maturation and embryogenesis, which may be associated with later in life developmental delay and reduced weight.

Keywords

Drosophila melanogaster; Perfluorooctanesulfonic acid (PFOS); Preconception exposure; Reproduction, Development, Nutrient Regulation

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are synthetic surfactants used in pharmaceuticals, cosmetics, paper coatings, non-stick and stain-resistant products and firefighting foams. They are ubiquitous and persistent in the environment. For example, perfluorooctanesulfonic acid (PFOS) has an estimated 5 year half-life in humans, is detected in 98% of human urine samples and in numerous other tissues such as pancreas, and has no known environmental half-life (Calafat et al. 2007; Maestri et al. 2006; Olsen et al. 2007, OECD 2002). PFAS are obesogens where developmental exposures are associated with low birth weight and chronic exposures increase risk for obesity and type 2 diabetes in adolescents and adults (Apelberg et al. 2007; Bach et al. 2016; Chen et al. 2012; de Cock et al. 2016; Domazet et al. 2016; Fei et al. 2007; Karlsen et al. 2016; Lin et al. 2009; Lv et al. 2013; Mora et al. 2016; Su et al. 2016). PFAS also disrupt lipid metabolism (Chen et al. 2014; Cui et al. 2016; Shi and Zhou 2010) in zebrafish. Therefore, PFAS, such as PFOS, pose a major public health exposure risk and are associated with an increased risk for metabolic syndrome/dysfunction (Lin et al. 2009; Su et al. 2016). Although a number of genetic and behavioral factors contribute to metabolic syndrome, exposures to endocrine disrupting compounds, such as PFOS, have been shown to increase susceptibility to this disease (Thayer et al. 2012). The timing of these exposures is critically important, and early life exposures, including prior to conception, may play a role in the developmental origins of metabolic syndrome/dysfunction and truncation of healthspan (Chamorro-García et al. 2013; Inadera 2013; Lane et al. 2015; Manikkam et al. 2013; Schug et al. 2013; Simmons 2006; Skinner et al. 2013; Tracey et al. 2013).

Preconception exposures to environmental pollutants may alter the development of a maturing oocyte and cause subtle but significant changes that affect the early stage embryo. Oocyte nutrients are largely acquired through receptor-mediated endocytosis, regulated by a

complex comprised of three low density lipoprotein receptors (LDLRs): megalin (aka low density lipid receptor-related protein 2, Lrp2), cubilin and amionless. Collectively, these are referred to as the Multifunctional Endocytotic Receptor Complex (MERC), and are evolutionarily conserved in many animals, including insects (Fisher and Howie 2006b; Fisher and Howie 2006a). Nutrients endocytosed during oocyte maturation provide a required but limited energy source for the embryo and are critical for later life health (Luzzo et al. 2012; Wu et al. 2010). MERC is capable of transporting a diverse array of lipoprotein ligands and the uptake of vitamins and cholesterol (Fisher and Howie 2006b; Kozyraki and Gofflot 2007; Willnow et al. 1996). Loss of function mutations in orthologs of LDLR genes resulted in impaired nutrient deposition, structural defects, and reduced viability of embryos in humans, other vertebrates (Bujo et al. 1997; Geng and Oliver 2009; Kantarci et al. 1993; Renata and Francoise 2007) and insects (Schonbaum et al. 1995).

In addition to MERC's regulation of nutrient deposition into the oocyte via receptor-mediated endocytosis (Virant-Klun et al. 2013), this process can also serve as a "Trojan horse" for toxicants. The MERC protein megalin is highly conserved, especially in regulatory, ligand-binding, and cytoplasmic domains (Anzenberger et al. 2006) and its expression and function can be disrupted by chemical exposures (Gena et al. 2010; Kwekel 2008; Pedersen et al. 2010; Reisman et al. 2012; Sant 2014). Thus, it is plausible that maternal exposure to PFAS will disrupt the expression and/or function of MERC proteins and alter the loading of nutrients (and PFAS) into the maturing oocyte.

The fruit fly, *Drosophila melanogaster*, has served for many years a robust model organism for understanding the mechanisms controlling essential biological processes, such as energy regulation, growth, development and longevity, and various human disease states, including metabolic syndrome (hypertension, increased weight, elevated triglycerides, glucose and cholesterol), obesity, insulin resistance and type 2 diabetes (Musselman et al. 2011). The availability of genomic, transcriptomic and proteomic sequences and the ease of genetic manipulations, including RNAi fly lines, have made *D. melanogaster* an excellent system that has allowed a better understanding of human diseases at the molecular level (Niwa and Niwa 2011) and has the ability to dissect the relative contributions of both genes and the environment on metabolic dysfunction (Ruden et al. 2005).

The insulin signaling pathway couples growth, development and lifespan to nutritional conditions. Insulin and insulin-like growth factor are conserved systemic signals that regulate growth and metabolism in response to nutrient availability (Brankatschk et al. 2014). *D. melanogaster* has organs that are analogous to most of those involved in animal and human energy metabolism including; brain, heart, liver and adipose-like tissue (fat body), kidney (nephrocytes, Malpighian tubules), gastrointestinal track and blood (hemolymph). The regulation of metabolism in *D. melanogaster*, which is largely controlled by the insulin-/insulin-like growth factor pathway, shares a number of conserved regulators (FOXO, mTOR, Akt, etc.) and brain neuroendocrine cells (insulin-producing cells, IPC), a primordial pancreas producing insulin and glucagon (adipokinetic hormone, AKH). The eight *Drosophila* insulin-like peptides (DILPs), produced in a variety of tissues, have sequence, structure and function similarities with vertebrate insulin and insulin-like growth

factor, and have functional roles in lifespan, resistance to stress and starvation and in lipid transport (Ruden et al. 2005).

As in vertebrates, *D. melanogaster* has a requirement for cholesterol, which serves as a structural component of cell membranes and as a precursor for the synthesis of steroid hormones (Niwa and Niwa 2011). Unlike vertebrates, insects cannot synthesis cholesterol *de novo* but must obtain it either directly from their diet or from plant sterols, which are converted to cholesterol in the gut. The principal steroid hormones in insects are the ecdysteroids, including ecdysone and 20-hyroxyecdysone, which function in larval molting and metamorphosis. The biosynthesis of ecdysteroids is located in the prothoracic gland in the larval stages but occurs in the female ovary where cholesterol is then maternally deposited for embryogenesis (Niwa and Niwa 2011). Megalin/LPR2 is a type 1 LDLR usually located at cell surfaces and efficiently transports a number of low-density lipoproteins, including apolipoprotein E, which carries cholesterol (Fisher and Howie 2006b; Kozyraki and Gofflot 2007).

In this current work, we use wild-type *Canton-S* (*CS*) and *megalin*-UAS RNAi transgenic fly lines to investigate the reproductive capacity, development and morphological effects that PFOS causes following a non-lethal maternal preconception exposure. Our working hypothesis is that maternal preconception PFOS exposure results in alterations in nutrient loading into developing eggs and in changes in the transcript levels of *Drosophila* insulin-like peptides, which control nutrient utilization and leads to developmental and morphological changes in the progeny from exposed maternal females. We further present results indicating that megalin is involved in PFOS uptake into eggs and that the effects reported herein are dose-dependent.

2. Materials and methods

2.1. Drosophila strains

The *Canton-S* (*CS*) wild-type strain was obtained from Dr. Barry Pittendrigh (Department of Entomology, Michigan State University, East Lansing, MI, USA). A heat shock inducible Hsp70-GAL4 driver (RRID:BDSC_1799) line was purchased from the Bloomington *Drosophila* Stock Center (BDSC, Bloomington, IN, USA) and a upstream signaling sequence (UAS)-RNA interference (RNAi) transgenic line for *megalin* knockdown (-*megalin*; VDRC ID 105387) was obtained from the Vienna *Drosophila* Resource Center (VDRC, Vienna, Australia). All flies were maintained at 25°C, 50% relative humidity (RH), 12:12 h light:dark cycle and fed on the Jazz-Mix® *Drosophila* food (Fisher Scientific, Fair Lawn, NJ, USA) in culture bottles (Genesee Scientific, San Diego, CA, USA). Flies were transferred to new medium every week.

2.2. Knockdown of megalin using UAS-RNAi transgenic fly lines

Newly emerged virgin females of GAL4 driver 1799 line and males from *megalin*-UAS-RNAi lines were collected from the culture bottles (< 4–6 h old). After incubated for 48 h to confirm that the females do not lay fertilized eggs, the driver 1799 females were paired with the same number of males of *megalin*-UAS-RNAi line by transferring them to a new

individual culture vial. The mating pairs were removed from the vials after 24 h and the eggs from the mating pairs (F1) on the medium were monitored until the pupal stage. Matured black-colored pupae were randomly divided in two groups. One group was heat shocked (HS) at 37°C for 45 min and the other group of non-heat shocked (NHS) pupae was used as control flies. After F1 adults emerged, randomly selected mating pairs (the same number of females and males of F1) were transferred to new vials. Vial were changed every 24 h to harvest laid eggs and the number and hatchability of the eggs were determined. All experiments were replicated three times.

In parallel, ten F1 females were collected randomly to verify the reduction of *megalin* transcription after heat shock. Total RNA was extracted from the females using PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA) with in-column DNaseI digestion according to the manufacturer's instruction and cDNA was synthesized using iScript cDNA Synthesis Kit (BIO-RAD, Richmond, CA). RT-qPCR was conducted using Power SYBR Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany) and the *megalin* primer set (Forward primer-GAT GGC AGC AAA TGG ACA CC; Reverse primer-GGT GTC CAG TTT CGA GTC CA) or *ribosomal protein L32* (*RpL32*; a reference gene, Kim et al. 2018) primer set (Forward primer-GAA GCG CAC CAA GCA CTT CA; Reverse primer-ATA CTG TCC CTT GAA GCG GC) in a StepOne Plus Real Time PCR System (Applied Biosystems) with the following cycling conditions: initial hold for 95°C for 10 min, 40 cycles each of 95°C for 15 s(denature) followed by 60°C for 1 min (anneal and extend), and then sequential increases of 0.2°C/sec from 45 to 95°C for melting curve analysis. Quantification of the relative transcription level of a gene was calculated as $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001).

2.3. Preconception PFOS exposure and effect on fecundity and egg hatch

The potassium salt of heptadecafluorooctanesulfonic acid (perfluorooctane sulfonic acid, PFOS, 98% pure, CAS 2795-39-3) was purchased from Sigma-Aldrich (USA) and dissolved in 90% acetone: 10% acetonitrile to prepare 8 mM stock solution and serially diluted with 100% acetone to obtain 0.4, 0.8, 2, and 4 ng PFOS/ 200 nl acetone working solutions. Aliquots (200 nl) of each solution were topically applied to the ventral abdomen of 1-day-old females of the *CS* strain. Once the optimal dose of PFOS (2 ng/fly) was determined, 200 nl aliquots of a 20 μM PFOS solution were applied to CS females or HS/NHS F1 females (see 'Effects of *megalin*-knockdown in female flies on the offspring' section) of the 1799 × UAS-RNAi^{megalin} cross using a PB-600 Repeating Dispenser (Hamilton Company, Reno, NV, USA). Control flies were treated with the same amount of acetone without PFOS. The treated female flies and the same number of *CS* or F1 males were transferred to new vials with food to collect eggs and the vials were changed every 24 h. The number and hatchability of the eggs from the mating pairs were determined. All experiments were replicated three times. In parallel, 10 treated females were collected randomly to check the transcription level of *megalin* as described above.

2.4. Nutrient analysis of eggs from preconception exposed females

The four types of experimental flies described above; *CS* females/acetone-treated, HS/NHS F1 females/acetone-treated, PFOS (2 ng)/acetone-treated *CS* females and PFOS (2 ng)/

acetone-treated HS/NHS F1 females, were transferred to embryo collection cages (5.6 cm (D) X 7.6 cm(H); Genesee Scientific, San Diego, CA) with the same number of males. Each collection container had a grape agar plate (FlyStuff Grape Agar Premix; Genesee Scientific, San Diego, CA) supplemented with active yeast paste to collect laid eggs (Rothwell and Sullivan 2000). The plates were changed every 24 h and plated eggs were collected using a fine brush. Batches of 50 eggs (8100 total eggs) were washed with PBS three times and used to measure three major nutrients: cholesterol, proteins and triglycerides. All liquid was removed from the microcentrifuge tubes and replaced with 200 μL 0.05% Tween 20 in Reverse Osmosis water. Eggs were disrupted using a pestle and motorized pestle unit followed by three 10 sec intervals of sonication using a Branson SFX250 Sonifier. Cholesterol was measured using Infinity Cholesterol Liquid Stable Reagent (ThermoFisher, Middletown, VA, USA). Briefly, 10 μL of sample were loaded into a 96-well plate followed by 100 μL of reagent. The plate was incubated at 37 °C for 5 mins, and absorbance was read at 500 nm on Biotek Cytation plate reader (BioTek Instruments, Winooski, VT). Results were compared to a cholesterol standard curve. Protein was measured using BIO-RAD Protein Assay Dye Reagent (BIO-RAD, Richmond, CA). Briefly, 5 μL of sample or BSA standard was loaded into a 96-well plate with 50 μL of a 1:5 dilution of the reagent. The plate was incubated for 5 min at room temperature, and its absorbance then read at 595 nm. Triglycerides were measured using Infinity Triglycerides Liquid Stable Reagent (ThermoFisher, Middletown, VA, USA). Briefly, 25 μL of sample was added to a 96-well plate with 150 μL of reagent. The plate was incubated at room temperature for 15 min with slow orbital shaking. Absorbance was read at 540 nm and results compared to a glycerol standard curve.

2.5. Preconception exposure, mating and development studies

2.5.1 Preconception dosing and mating protocols.—

Stocks of the *CS* wild type strain of *D. melanogaster* (Bloomington Stock Center #64349) were maintained at 25°C with a 12:12 h light:dark cycle on Nutrifly Bloomington Formulation food (Genesee Scientific 66–112) with 6 mM propionic acid sodium salt (Genesee Scientific 20–271). 1–5 day old virgin females were topically dosed with 2 ng PFOS (Millipore-Sigma cat#33829) in acetone or acetone vehicle control. Females were placed into individual food vials and allowed to recover for 24 hours at 25°C with a 12:12 h light:dark cycle before introducing a single male (1–5 days old) for mating. Eggs laid on days 1–3 were pooled to establish the “early group” embryos, where PFOS was detectable, and eggs laid on days 4–6 were pooled to establish the “late group” embryos, where PFOS was no longer detectable.

2.5.2. F1 adult weight determination.—

Vials were stored at 25°C under a 12:12 h light-dark and visually assessed at 24 hour intervals for larval development. For the purpose of assessment, Day 1 was the day that the parental mating pair was placed into the vial. At Day 1 post-adults emergence, flies were placed at −80°C for five minutes to incapacitate them prior to being weighed by mating pair group (Mettler-Toledo dual range, XS 105, Columbus OH, USA). Data presented represents the average adult weight for the mixed male and female progeny of each mating pair.

2.5.3. Lifespan analysis of F1 adult progeny.—Progeny of control and PFOS exposed females were sorted into vials by sex on day 1 post eclosion (Sun et al 2013). The total number of adults was counted and vials were monitored daily for survival. Flies were moved to fresh food vials weekly without the use of carbon dioxide anesthesia, to prevent mixing generations of progeny. The comparison of survival curves (Kaplan-Meier method) were performed by using Log-rank (Mantel-Cox) test using GraphPad Prism (ver. 8, GraphPad Inc., San Diego, CA, USA).

2.6. Nutritional content of CS F1 adult progeny

For cholesterol determinations, two flies were washed in Danieau's solution pH 7.2 (17.4 mM NaCl, 0.21 mM KCl, 0.12 mM $MgSO_4$+H_20, 0.18 mM $Ca(NO)_3$, 1.5 mM HEPES) and homogenized in 200 μl of PBST containing 0.05% Tween 20 (Apex Bioresearch Products, Genesee Scientific El Cajon, CA #18–173). Total cholesterol was measured in triplicate using the Infinity Cholesterol Liquid Stable Reagent (Thermo Fischer Scientific Waltham, MA #T1213421). Cholesterol was quantified according to manufacturer's instructions and were read using a SpectraMax i3 plate reader (Molecular Devices, San Jose, CA). To determine triglycerides, single flies were washed in Danieau's solution and homogenized in 200 μL PBST. Samples were diluted 1:10 in PBST and total triglyceride was measured in triplicate using the Infinity Triglyceride reagent (Thermo Fischer Scientific Waltham, MA #TR22421). Triglyceride was quantified according to manufacturer's instructions using a SpectraMax i3 plate reader. Glucose was determined using single flies washed in Danieau's solution and homogenized in 500 μl of 100 mM maleate buffer, pH 5.5 (Alfa Aesar Haverhill, MA #J62049). Total glucose was measure in triplicate using the Glucose Oxidase Liquid Stable Reagent (Thermo Fischer Scientific Waltham, MA TR15221). Glucose was measured according to manufacturer's instructions and read using a SpectraMax i3 plate reader.

2.7. Drosophila-like insulin peptides (dilps) expression in CS F1 adults

A single fly was homogenized in squishing buffer (10 mM-Tris-Cl pH 8.2, 1 mM EDTA, 25 mM NaCl and 200 μg/mL proteinase K) to extract nucleic acids as previously described (Cherbas et. al., 2015). Total RNA extraction and cDNA synthesis were performed by using Monarch® Total RNA Miniprep Kit (New England Biolabs Inc., Ipswich, MA #T2010) and iScript cDNA synthesis kit (Bio-Rad, Hercules, CA #1708890), respectively, following their respective manufacturer's protocols. RT-qPCR was performed by using primer sequences (Supplemental Table S1) from Integrated DNA Technologies (Coralville, IA) and SsoAdvanced Universal SYBR Green Supermix (BIO-RAD, Hercules, CA #1725270). Fluorescence signal of PCR products was detected by using BIO-RAD CFX96 Touch Real-Time PCR System (Bio-Rad, Hercules, CA). Cycle threshold (Ct) of housekeeping gene (tubulin) and target genes was used to calculate fold change of gene expression ($2^{-\Delta\Delta Ct}$) relative to the control.

2.8. Statistical analyses

2.8.1. Fecundity, egg hatchability and egg nutrient content—All statistical analyses were performed using GraphPad Prism (ver. 6, GraphPad Inc., San Diego, CA,

USA). Mean and standard deviations were calculated for each data and statistical significance was determined by ANOVA followed by Bonferroni post hoc tests and Student's t-tests.

2.8.2. Time to adulthood and adult weights—The distribution of each data set was analyzed for normal Gaussian distribution using the D'Augostino & Pearson Omnibus test. As these data sets were normally distributed, the means of each PFOS group was compared to their corresponding acetone control group using an unpaired Student's t-test. Statistical analysis was carried out using GraphPad Prism software (ver. 8, GraphPad Inc., San Diego, CA, USA).

2.8.3. Dilp expression—The distribution of each data set was analyzed for normal Gaussian distribution using the D'Augostino & Pearson Omnibus test. As these results were not normally distributed, the distribution of each PFOS dataset was compared to the corresponding acetone control group using the Mann-Whitney U Test. This analysis was suggested in: A-Z Quantitative PCR Edited by Stephen A. Bustin ISBN-10: 0963681788. Statistical analysis was carried out using GraphPad Prism software (ver. 8, GraphPad Inc., San Diego, CA, USA).

2.9. Quantitative analysis of PFOS

Analytical standard of perfluorooctanesulfonic acid (PFOS, purity: 98%) was obtained from Sigma Aldrich (St. Louis, Mo). The internal standard ($^{13}C_8$ PFOS, 50 μg/mL in methanol) was purchased from Cambridge Isotope Laboratories (Andover, MA). Individual stock solutions were prepared at the concentration of 1.0 mg/mL in acetonitrile. Purity and molecular weight (acid form) were used to calculate the concentration of the stock solutions. PFOS was diluted to make a solution containing 0.1 mg PFOS/mL in acetonitrile. Working solutions of 0.001 – 0.1 μg/mL were prepared by serial dilution with acetonitrile. The internal standard solution 250 ng PFOS/mL was prepared in acetonitrile.

For sample extraction, either fifty eggs or thirty adult flies of the *CS* strain were collected in a 2 mL polypropylene tube. 50 μL of the internal standard solution (250 ng/mL) was added, followed by 100 μL of deionized water and 200 μL of acetonitrile and vortexed for 1 min. After sonication for 30 min, 50 mg of sodium chloride was added, the tubes were vortexed for 1 min and centrifuged at 4 °C for 5 min at 8,600g, (Centrifuge 5418R, Eppendorf, Hamburg, Germany). Lastly, the acetonitrile (upper) layer was filtered using a PTFE syringe filter (13 mm, 0.2 μM, Pall Corp., Port Washington, NY) and transferred to a liquid chromatographic (LC) vial (12 × 32 mm, Waters Corp., Milford MA).

PFOS was analyzed using LC-MS/MS on an Acquity UPLC H-class system equipped with Acquity triple quadrupole mass spectrometer (Waters, Milford, MA). The chromatographic separation was performed on reversed-phase analytical column Atlantis T3 (2.1 mm × 100 mm and 3.0 μm particle size, Waters, Milford, MA). The column oven temperature was set at 40 °C. The mobile phase consisted of (A) 2 mM ammonium acetate and (B) 100% acetonitrile. The flow rate of mobile phase was 0.25 mL/min and the following gradient program was used: Initial at 5% B, held for 0.5 min and increased to 95% B over 4.5 min then held for 2 min. The gradient returned to the initial conditions and held for 2.5 min. The

total analytical run-time was 10 min and the injection volume was 5 μL. For MS/MS detection, electrospray ionization (ESI) in negative ion mode was used with the following MS parameter: Capillary voltage 4.5 kV, source temperature 150 °C, desolvation line temperature 250 °C and the desolvation flow rate of 500 L/hr (nitrogen) was used for. The tandem MS detection was achieved by multiple reaction monitoring (MRM) and the MRM transitions (quantitation ion and identification ion) and parameters including the retention time (t_R) are listed in Supplement Table S2.

For the standard injections, 350 μL of acetonitrile and 50 μL of the internal standard solution (250 ng/mL) were mixed with 100 μL of the various concentration of the working solution (2.5 – 250 ng/mL). The ratios of signal response between PFOS and ISTD were used to calculate the detected amount of PFOS in samples, based on internal standard calibration.

3. Results

3.1. Selection of preconception PFOS dose

Increasing amounts of PFOS topically applied to *CS* females reduced both fecundity (Fig. 1A) and mortality (Fig. 1B) over time. At the dose of 2 ng/fly and above, a significant reduction in fecundity was determined at day 3 post exposure, which persisted to day 7 (Fig. 1A; $P<0.05$). There was no significant reduction on fecundity at lower doses. There was a highly significant increase in mortality at the 4 ng dose beginning at day 3 post exposure and continued to day 7 (Fig. 1B; $P<0.001$). At the 2 ng dose, there was no significant motility over the first 6 days following PFOS exposure ($P>0.05$). From these results, a maternal preconception exposure to PFOS was set at 2 ng/fly and effects evaluated over a 5 day post exposure duration.

3.2. Preconception exposure reduces fecundity and megalin transcription

The average number of eggs laid by *CS* females treated with 2 ng PFOS were significantly decreased compared with acetone-treated control females, with a 57% reduction apparent at 5 days post exposure (Fig. 2A, $P<0.001$). It should be noted that most of the reduction seen occurred in the first 2 days post exposure. There were, however, no significant differences in the hatchability of eggs laid by PFOS-treated versus acetone-treated females (Fig. 2B). Using RT-qPCR, the level of mRNA of the *megalin* gene, a component of MERC, was significantly reduced (~50%) in PFOS-treated versus acetone-treated females, with most of the reduction occurring in the first 3 days post exposure (Fig. 2C).

3.3. Reduced megalin transcription reduces fecundity of transgenic F1 females

Using the *megalin*-Gal4/UAS-RNAi transgenic fly lines, the transcript level of the *megalin* gene was significantly and substantially reduced (~80% reduction) upon heat shock treatment of the F1 females (HS, megalin(−)) versus non-heat shocked F1 females (NHS, megalin(+)) (Fig. 3A). Additionally, *megalin* knockdown females (HS, megalin(−)) produced a significantly reduced number of eggs post emergence versus the NHS megalin(+) females (Fig. 3B). By day 5 post emergence, megalin(−) females produced 50% less eggs than the megalin(+) females. The hatchability of eggs that were laid by megalin(−) females, however, were not significantly different from those laid by megalin(+) females (Fig. 3C).

These findings are similar to those reported above for PFOS-treated *CS* females and their eggs (Fig. 2).

3.4. Preconception exposure alters nutrients in eggs laid by megalin(+) or megalin(−) transgenic females

There were no significant differences in levels of major nutrients (cholesterol, protein, triglycerides) in eggs laid by acetone-treated *CS* females, NHS megalin(+) F1 females or HS megalin(−) females (data not shown) so only the data from eggs laid by NHS megalin(+) and HS megalin(−) F1 females are shown (Fig. 4).

Preconception PFOS treatment resulted in a significant 62% increase in cholesterol in eggs laid by PFOS-treated NHS megalin(+) F1 females compared with those from PFOS-untreated NHS megalin(+) F1 females (Fig. 4A, $P<0.05$). As seen in Fig. 4D, cholesterol was increased in early laid eggs (1–3 days post oviposition) and not in later laid eggs (day 4 post-oviposition). In eggs from HS megalin(−) F1 females, preconception PFOS treatment resulted in a 26% decrease in cholesterol, albeit not significant ($P>0.05$), versus eggs from PFOS-untreated HS megalin(−) F1 females (Fig. 4A). Again, reduced cholesterol was seen only in early laid eggs (1–2 days post oviposition, Fig. 4D).

There was no significant difference in the protein content of eggs from PFOS-treated NHS megalin(+) F1 females when compared to PFOS-untreated NHS megalin(+) F1 females (Fig. 4B). The protein content of eggs from HS megalin(−) F1 females was significantly increased (+64%) by preconception exposure to PFOS compared with eggs from PFOS-untreated HS megalin(−) F1 females (Fig. 4B, $P<0.01$). The increase in protein occurred over the entire test period (1–5 days post oviposition, Fig. 4E).

Triglyceride levels were significantly increased (+35%) in eggs laid by PFOS-treated NHS megalin(+) F1 females versus PFOS-untreated NHS meaglin(+) F1 females (Fig. 4C, $p<0.05$). The increase in triglycerides was more pronounced in early laid eggs than in later laid eggs (Fig. 4F). There was no significant difference in the triglyceride content of eggs from PFOS-treated HS megalin(−) F1 females when compared to PFOS-untreated HS megalin(−) F1 females (Fig. 4C).

3.5. Preconception exposure results in developmental delay, reduced weight and increased longevity of progeny from early laid eggs

The development of early laid eggs (1–3 days following PFOS treatment of *CS* females) was slowed at all developmental stages, starting with 1^{st}–2^{nd} instar and continuing until adulthood when compared to the early group eggs from acetone-treated control females (Supplemental Fig. S1A). A similar developmental delay was not seen in the late group eggs (4–6 days post PFOS treatment, Supplemental Fig. S1B). The developmental delay seen in early laid eggs from PFOS-treated females was significantly and substantially different from eggs of acetone-treated control females with development being delayed approximately 1 –2 days (Fig. 5A).

The weights of adults that developed from early laid eggs from PFOS-treated females were significantly less than early laid eggs from acetone-treated control females (40% reduction

from 1.32 to 0.76 mg on average for pooled males and females, Fig. 5B). The weights of adults from later laid eggs of PFOS-treated females were not significantly different from those laid by acetone-treated females.

The lifespan of F1 progeny from early laid eggs of PFOS-treated mothers was significantly increased compared with progeny of acetone-treated control females (P=0.003, Fig. 5C) or males (P= 0.049, Fig. 5D). Neither F1 female nor male progeny from later laid eggs of PFOS-treated mothers had significantly increased lifespans when compared to their respective acetone-treated controls (Fig. 5 E&F).

3.6. Preconception exposure altered nutrients in adults from early laid eggs

The levels of triglycerides, cholesterol and glucose were determined for young (1 day post emergence), peak reproductive age (5–7 days post emergence) and mature (14 day post emergence) adult flies that developed from early laid eggs from PFOS-treated *CS* females (Fig. 6).

Significant differences in the level of triglycerides following preconception PFOS treatments were seen in 1 and 5 day old adult progeny where triglycerides were significantly increased by 51% in PFOS-treated 1-day females but were significantly decreased by 47% in PFOS-treated 1 day males compared to their respective controls (Fig. 6A). Triglycerides were significantly increased by 2-fold in 5 day PFOS-treated males, while no significant change was measured in 5 day females.

There were significant and consistent differences, however, in cholesterol levels at all three age groups: where 1) PFOS exposure increased cholesterol in day 1 and 5 female and male progeny (with a significant 58% increase in PFOS-exposed day 1 males and a 47% increase in PFOS-exposed day 5 females) (Fig. 6B); and 2) PFOS exposure decreased cholesterol in 14 day female (a significant 50% decrease) and male (a significant 85% decrease) progeny (Fig. 6B).

There were no significant differences in the levels of total sugar in female or male progeny at 1 or 14 days following preconception PFOS treatments (Fig. 6C). An increase in total sugar was observed in 5 day old female and male progeny, with female progeny showing a statistically significant 39% increase in total sugars (Fig. 6C).

3.7. Preconception exposure altered dilp transcription in adults from early laid eggs

Preconception PFOS exposure of *CS* females resulted in significant changes in the expression levels of all *dilps* tested (2, 3, 5, 6 and 8) but were inconsistent between 1 and 14 day old progeny and between female and male progeny (Fig. 7). PFOS exposure affected *dilp* expression in 1 day old progeny more than 14 day old progeny and generally effected males more than females. All five *dilps* tested were significantly up-regulated by PFOS exposure in 1 day old male progeny but none remained up-regulated by day 14. However, *dilp* 2 was consistently and significantly down-regulated (49% in 1-day progeny and 80% in 14-day progeny) and *dilp* 6 was consistently up-regulated in both 1 and 14 day old female progeny (40% in 1-day progeny and 41% in 14-day progeny) (Fig. 7A&D, respectively).

3.8. PFOS is detected in maternal females and eggs following preconception exposure and megalin knockdown reduces PFOS uptake into eggs

3.8.1. CS females—PFOS was detected using LC-MS/MS at 0.081±0.018 ng/fly in "early" females (1–2 days following application of preconception PFOS treatment) but was under detection limit in "late" females (3–4 days post-exposure (Fig. 8A). PFOS was not detected in any acetone-treated control females. The percent of the topically-applied PFOS dose detected in 1–2 day old females following preconception PFOS treatment was 4.1% (0.081 ng/2 ng × 100).

PFOS was detected at 0.018±0.002 ng/egg in eggs from early females (1–2 days following PFOS treatment) but was not detected in eggs from late females (3–4 post-treatment) (Fig. 8B). PFOS was not detected in any eggs from vehicle (acetone)-treated control females. The percent of total PFOS detected in maternal females following preconception PFOS treatment that was subsequently detected in early eggs (eggs from 1–2 day old exposed females) was 22% (0.018 ng/0.081 ng × 100)). On average, females had 4.5-fold more PFOS than eggs (0.081 ng/0.018 ng) (Fig. 8AB).

3.8.2. Transgenic megalin (+/−) F1 females—In eggs from NHS-megalin(+) females, PFOS was detected in 1–2 day old eggs (0.0317±0.0087 ng/egg) but was not detected in 3–4 day old eggs (Fig. 8C, left side) following exposure. In eggs from HS-megalin(−) females, PFOS was consistently detected in 1–3 day old eggs (0.0227±0.0045 ng/egg) but not detected in 4 day old eggs (Fig. 8C, right side) following exposure. Using this data, there was a 28.4% reduction in total PFOS in megalin(−) versus megalin(+) eggs ((0.0317–0.0227)/0.0317 × 100), however, the difference was not statistically significant ($P>0.05$, Student's t-test). Day 1 following exposure was always the peak amount of PFOS found in either megalin(+) or megalin(−) eggs. Using only Day 1 data, there was a 55.1% reduction in PFOS in megalin(−) versus megalin(+) eggs ((0.0263–0.0118)/0.0263 × 100) and this difference was statistically significant ($P<0.05$, Student's t-test).

4. Discussion

Drosophila adults and larvae have been previously shown to be highly sensitive to PFASs, including PFOS and perfluorooctanoic acid (PFOA). The number of offspring from *Drosophila hydei* adults and larvae fed on increasing concentrations of PFOS (5 to 5000 ng PFOS/ml diet) were significantly reduced in a concentration-dependent manner (Van Gossum et al. 2010). The number of second generation offspring from flies exposed to 50 ng PFOS/ml were also significantly reduced even though the second generation was not directly exposed to PFOS, indicating that maternal exposure to PFOS may affect both oogenesis and embryogenesis (Van Gossum et al. 2010). Developmental exposure of *D. melanogaster* to increasing concentrations of PFOA likewise resulted in decreased weights and in a reduced longevity of adults (Wang et al. 2010). Additionally, larvae showed reduced body volume, altered foraging behavior, molting arrest and dose-dependent mortality. Interestingly, nutrient supplementation of sucrose diet with yeast attenuated the effects of PFOA, suggesting that PFOA was interfering with nutrient utilization leading to the disruption of metabolic pathways responsible for larval development (Wang et al.2010). PFOS has also

been reported to be highly toxic to the bumblebee *Bombus terrestris* and cause detrimental reproductive effects, which were correlated with a significant reduction in ovary size. Additionally, PFOS reduced electron transport activity, lipid levels and was strongly antagonistic in an ecdysteroids-inducible luciferase reporter assay, suggesting an endocrine disrupting action by way of the molting hormone and/or the ecdysteroid receptor in *D. melanogaster* S2 cells (Mommaerts et al. 2011).

In this current research, topical preconception PFOS exposures resulted in a dose- and time-dependent increase in *CS* female mortality and a reduction in the number of eggs they laid. A preconception topical dose of 2 ng PFOS/fly was selected for further study as it produced no significant female mortality but significantly reduced the number of eggs laid over a post-exposure interval of 5 days. It should be noted that the effect of PFOS on reducing egg numbers occurred primarily in the early stages of exposure (1–3 days post exposure).

Megalin (Lrp2), a low density lipoprotein receptor (LDLRs) and component of the MERC, functions to carry nutrients into the egg during oocyte maturation, thereby providing a required but limited energy source for the embryo, which is critical for later life health. Megalin transports a low density lipoprotein known as apolipoprotein E, which has cholesterol as one of its substrates. Given the importance of cholesterol in insect development and maturation and because insects cannot *de novo* synthesize sterols, functional maintenance of this transporter is critical for insect survival, development, particularly during embryogenesis, and the extension of healthspan. Preconception PFOS exposure resulted in a significant and substantial reduction in the transcription level of *megalin* in treated *CS* females and the effect of PFOS occurred primarily in the earlier stages (Days 1–3) following exposure. The reduction in *megalin* transcript level in HS-megalin(−) females lead to a similar reduction in the numbers of eggs laid but the hatchability of laid eggs was unaffected, which mimic the results seen in PFOS-treated *CS* females.

Preconception PFOS exposure likewise caused significant alterations in the nutrient content of eggs laid by both NHS-megalin(+) and HS-megalin(−) females, indicating that PFOS may be targeting megalin and altering nutrient uptake by eggs. PFOS significantly increased cholesterol and triglyceride levels in eggs from NHS-megalin(+) females and these increases were primarily associated with the early laid eggs. However, preconception PFOS exposure significant decreased cholesterol and increased protein in eggs laid by HS-megalin(−) females and these effects were again associated with early laid eggs.

Preconception PFOS exposure of maternal *CS* females also altered the nutrient content of F1 adult progeny from early laid eggs. Cholesterol was increased in both female and male progeny at Day 1 and Day 5 post-emergence but was significantly reduced in both females and males by Day 14.

Given the importance of megalin in nutrient transport during egg production and embryogenesis, particularly involving the transport of cholesterol, the above findings strongly suggest an interaction between PFOS and megalin leading to the alteration of normal nutrient transport during oocyte maturation and embryogenesis.

Preconception PFOS exposure of maternal *CS* females also resulted in a significant and substantial developmental delay, reduction in adult weights and an increased female lifespan in F1 progeny from early laid eggs that were not seen in progeny from later laid eggs. Interestingly, the results obtained following preconception PFOS exposure are similar to those obtained by feeding *D. melanogaster* on a high-sugar diet, which produces obesity (as indicated by triglyceride accumulation) and insulin resistance (as indicated by a developmental delay and size/weight reduction) (Musselman et al. 2011). Recently, the epidemiologic findings of PFAS and obesity and type 2 diabetes were reviewed. Of the 22 studies on obesity and the 32 studies on diabetes identified from the literature, approximately 2/3 reported a positive association between PFAS and these disease states (Qi et al. 2020).

The insulin/insulin-like growth factor signaling (IIS) pathway is a key regulator in energy metabolism in *Drosophila* where it couples nutrient uptake to growth (Underwood et al.1994), development (Engelman et al. 2006), stress resistance (Tatar et al. 2003) and lifespan (Hwangbo et al. 2004). The *Drosophila* insulin-like peptides (DILPs) are highly related to vertebrate insulin and insulin-like growth factor, and also have functional roles in nutrient utilization, lipid transport, stress resistance, and lifespan (Ruden et al. 2005). Preconception PFOS exposure significantly affected *dilp* expression in F1 progeny from early laid eggs. Most notable was the significant and consistent down-regulation of *dilp* 2 and the consistent up regulation of *dilp* 6 seen in 1 and 14 day old F1 female progeny from PFOS-treated mothers. It is well established that *dilp* 6 expression from fat body extends lifespan, in part, by down regulating *dilp* 2 in the brain, which allows the stimulation of glycogen phosphorylase (Post et al. 2019). Taken together, these findings are consistent with the highly significant increase in lifespan seen in female F1 progeny from early laid eggs of PFOS-treated mothers.

It is also apparent from the above that there is a strong correlation between the action/effect of preconception PFOS exposure in early exposed maternal females (1–2 days post exposure) and in their early laid eggs (1–2 days post oviposition) that is not seen or reduced in 3–4 day post exposure maternal females or in their later laid eggs, including reduction in the number of eggs laid, reduction in the transcript levels of *megalin* in treated females, increased levels of cholesterol and triglycerides, a slowed development, reduced adult weight and increased female lifespan, indicating a dose-effect relationship. PFOS was detected in both topically-exposed maternal *CS* females and in their eggs (preconception exposure) but only at 1–2 days post exposure. PFOS was never detected in either 3–4 day females or their eggs and was never found in either acetone-treated control females or their eggs. These data show that PFOS is only subsequently detected in eggs when first detected in preconceptionally-exposed females, which is primarily at 1–2 days post exposure, the time interval were most of the PFOS effects occur, indicating dose-dependent effects of PFOS when administered preconceptionally to *D. melanogaster*.

The RNAi knockdown of megalin in HS-*megalin*(−) F1 females resulted in a significant decrease in the transfer of PFOS (−55%) to their early laid eggs compared to early laid eggs from NHS-megalin(+) F1 females. Because megalin is part of the MERC complex in *D. melanogaster* and functions as a low-density lipoprotein transporter, it is possible that it is

acting as a Trojan horse mechanism bringing PFOS into the developing egg. One of the potentially most harmful ramification of the increased uptake of PFOS into early laid eggs by females that have a full complement of megalin (*CS* and NHS-megalin(+) flies) is that megalin also transports cholesterol into developing eggs in the ovaries of females. Because the biosynthesis of ecdysteroids, including ecdysone and 20-hydroxyecdysone, from cholesterol is located in the female ovary, the possibility exists that excess PFOS may inhibit or alter this process. Indeed, PFOS was shown to be strongly antagonistic in an ecdysteroids-inducible luciferase reporter assay, suggesting an endocrine disrupting action by way of the molting hormone and/or the ecdysteroid receptor in *D. melanogaster* S2 cells (Mommaerts et al. 2011). Therefore, the increase in cholesterol seen in both NHS-megalin(+) eggs and *CS* F1 flies from early laid eggs of PFOS-treated females may be the result of cholesterol not being converted into ecdysteroids in the ovary, even though the transcription of megalin appears to be reduced by PFOS treatment.

5. Conclusions

A sub-lethal preconception PFOS exposure resulted in a reduced number of eggs laid and decreased transcription of *megalin* in treated maternal females. Although the hatchability of laid eggs from PFOS-treated females was not significantly different from non-treated females, the nutrient levels measured in early laid eggs and the F1 adult progeny from these eggs differed significantly compared with those from either control (acetone-treated) females or females 3–4 days post exposure. Additionally, F1 progeny from early eggs laid by PFOS-treated females had a significant developmental delay, reduced weight, increase female lifespan and altered *dilp* expression pattern compared with those from either acetone-treated control females or females 3–4 days post PFOS exposure. It is also shown that PFOS was only detected in treated females and in their eggs at the early time intervals following preconception PFOS exposure, indicating a dose-effect/response relationship existed. The knockdown of megalin in transgenic flies resulted in a substantial and significant decrease in the amount of PFOS taken up by the developing egg, indicating that megalin is acting as a Trojan horse mechanisms for the transport of PFOS. Therefore, it appears that an interaction between PFOS and megalin exists and leads to the alteration of normal nutrient transport during oocyte maturation and embryogenesis, which may be associated with later in life developmental delay, reduced adult weight and increased female lifespan that may be due to the altered expression of DILPs and/or a disruption in ecdysteroid function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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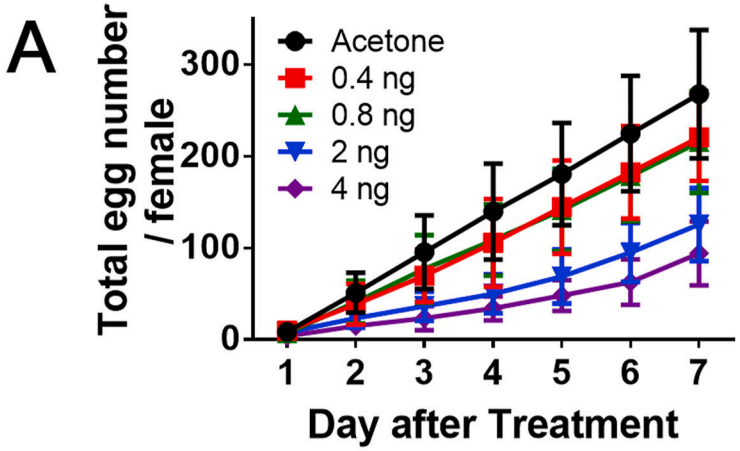
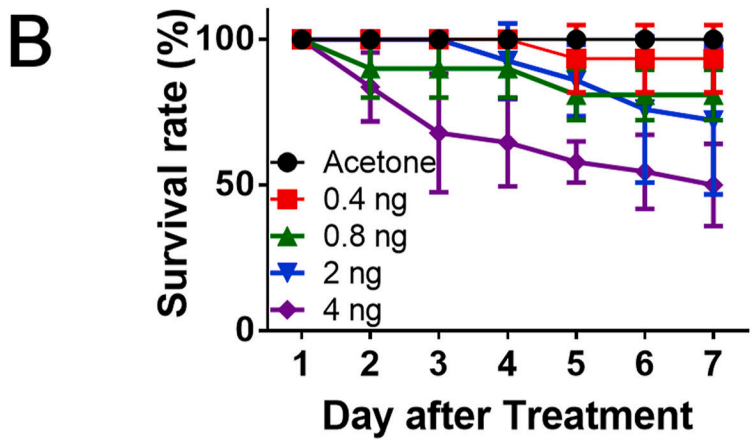
- Preconception PFOS exposure of females reduced eggs laid and the transcription of *megalin*.
- Nutrients in early eggs and in F1 adults from treated flies differed from untreated flies or later eggs.
- F1 flies from early eggs of treated females had developmental delay and reduced weight.
- F1 flies from early eggs of treated females had altered *dilp* expression.
- PFOS is only detected in treated females and their early laid eggs, indicating a dose-response.
- Megalin knockdown decreased PFOS uptake into eggs and may act as a Trojan horse mechanism.



Day	PFOS conc. (ng)			
	0.4	0.8	2	4
1	ns	ns	ns	ns
2	ns	ns	ns	ns
3	ns	ns	*	**
4	ns	ns	**	**
5	ns	ns	*	**
6	ns	ns	*	**
7	ns	ns	*	***



Day	PFOS conc. (ng)			
	0.4	0.8	2	4
1	ns	ns	ns	ns
2	ns	ns	ns	ns
3	ns	ns	ns	***
4	ns	ns	ns	***
5	ns	**	ns	***
6	ns	**	ns	***
7	ns	**	*	***

Fig 1.

(A) Cumulative egg numbers from PFOS-treated females and acetone-treated control for 7 days (left) and statistical significance of the number of eggs compared to that from control females analyzed by two-way ANOVA (right; ns, not significant; *, P<0.05; **, P<0.01; ***, P<0.001). (B) Survival rate of PFOS-treated females and control (left) and statistical significance of survival rate compared to control analyzed by two-way ANOVA (right). Error bars indicate standard deviation (SD).

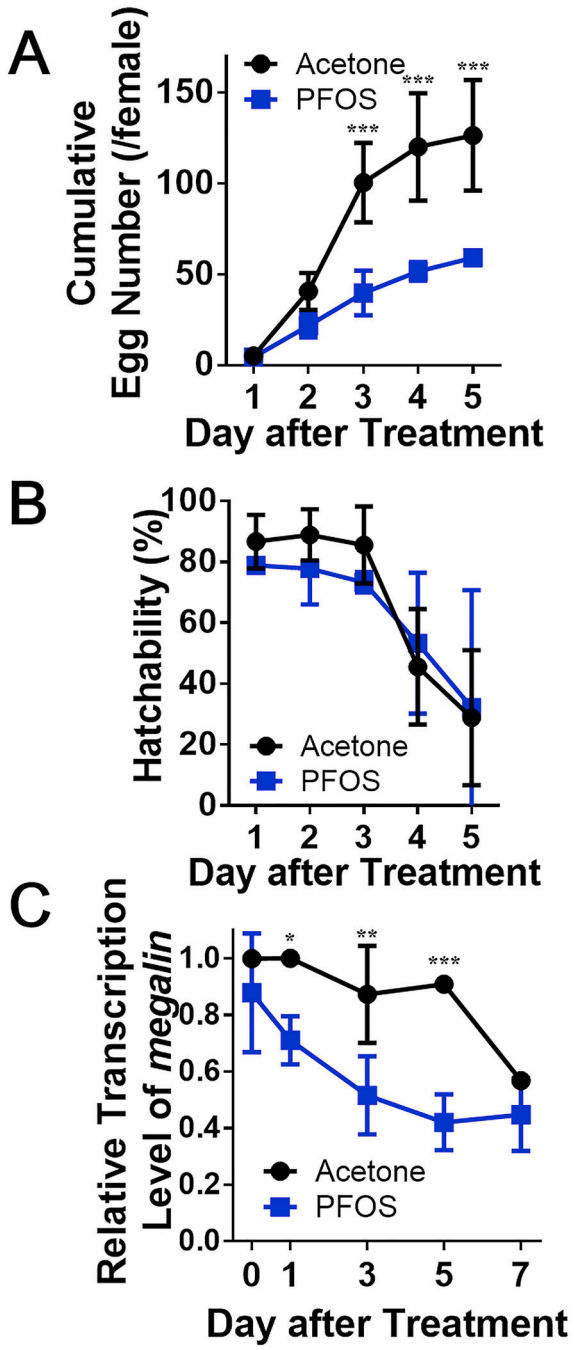


Fig. 2.

(A) Cumulative egg numbers from 2 ng PFOS-treated females and acetone-treated control for 5 days; (B) Hatchability of the eggs collected for every 24 h from PFOS-treated females and acetone-treated control. Asterisks (***) indicate statistical differences using two-way ANOVA (P <0.001); (C) Decreased transcription levels of *megalin* following PFOS treatment with transcripti on levels of *megalin* compared to acetone-treated control flies every 24 h. An asterisk (*) indicat es statistical differences using two-way ANOVA (*, P<0.05; **, P<0.01; ***, P<0.001). Error bars indicate standard deviation (SD).

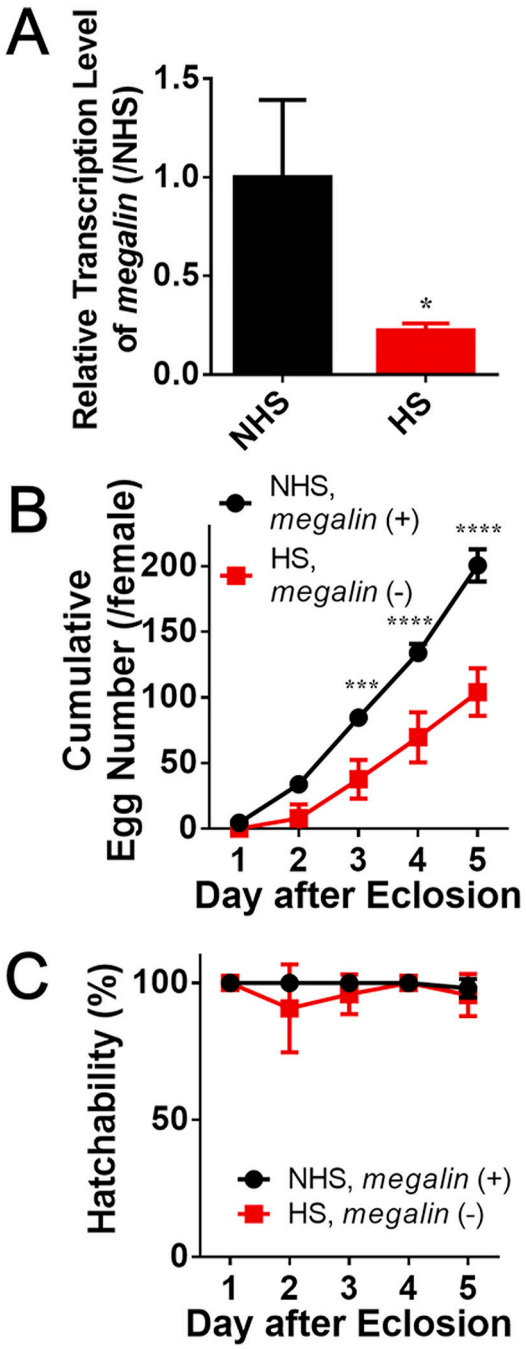


Fig. 3.

(A) Validation of *megalin* RNAi in transgenic flies. One group was heat shocked (HS) to induce RNAi of *megalin*. The other group (non-heat shocked; NHS) was used as control; (B) Cumulative egg numbers from HS females and NHS control for 5 days; (C) Hatchability of the eggs collected for every 24 h from HS females and NHS control. Asterisks (*) indicate statistical differences using two-way ANOVA (***, P<0.001; ****, P<0.0001). Error bars indicate standard deviation (SD).

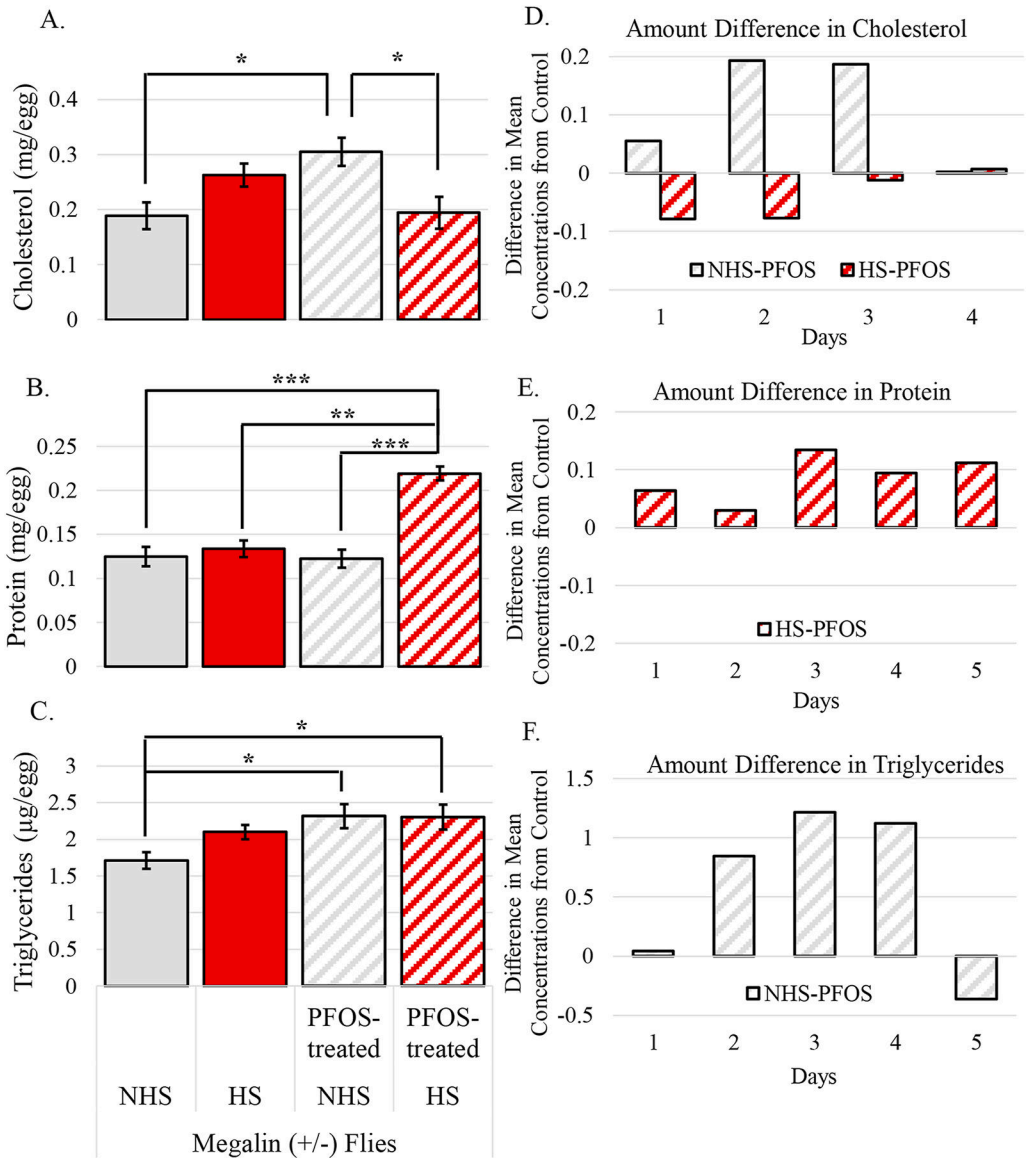


Fig. 4.

Comparison of three major nutrients in the eggs of control (NHS) and *megalin*-knockdown (HS) in non-exposed and PFOS-exposed females. Measurements of (A) cholesterol, (B) protein, and (C) triglycerides were determined in samples collected over a 5 day period. The average difference (D) cholesterol, (E) protein, and (F) triglycerides in NHS-PFOS or HS-PFOS from the NHS non-exposed eggs were calculated at each day after maternal treatment. Bars represent mean ± SEM. *p<0.05 ** p<0.01, **p<0.001, Two Way ANOVA, Tukey's post hoc. N = 15–21 pooled samples.

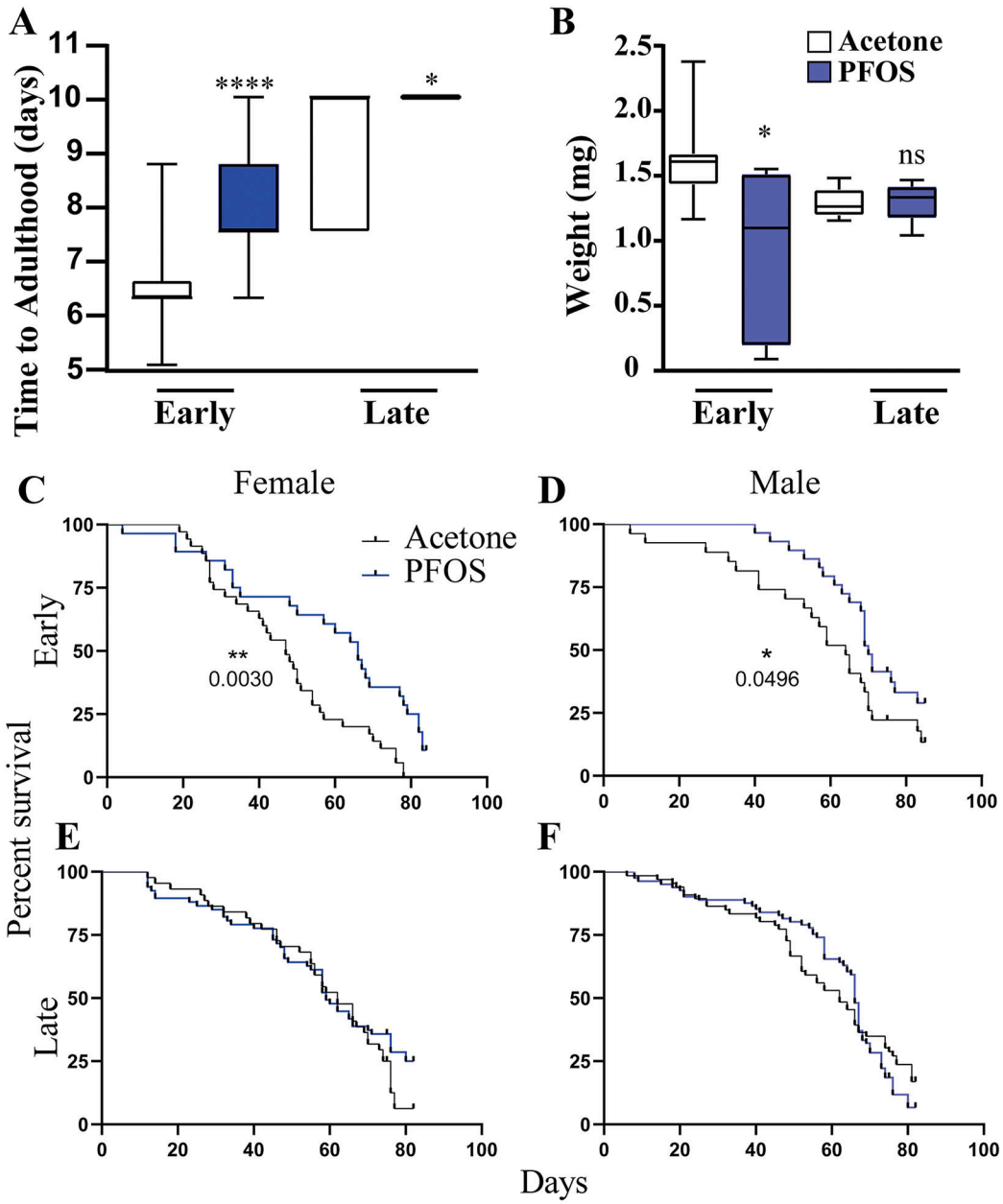


Fig. 5.

Preconception PFOS exposure delays development, decreases body weight and increases lifespan of early group F1 progeny: (A) Average time to adulthood for progeny of the groups indicated. n=20–26 mating pairs/treatment. D'Augostino & Pearson Normality test, Unpaired t-test *p<.05, ****p<.0001. (B) Weight of mixed gender adult flies 1–3 days post-eclosion. Weights are averaged for progeny of each mating pair, n=10–15 mating pairs/treatment, 16–25 flies/mating pair. D'Augostino & Pearson Normality test, Unpaired T-test *p<.05. Boxes indicate the 25th and 75th percentiles, horizontal bars indicate mean and error bars indicate min-max values. (C-F) Survival curves for early group female (C), early group male (D), late group female (E) and late group male (F) progeny of acetone control (black) and PFOS exposed (red) females. N= 6–10 vials of progeny/treatment group/sex.

The comparison of survival curves (Kaplan-Meier method) was performed by using Log-rank (Mantel-Cox) test using GraphPad Prism (ver. 8, GraphPad Inc., San Diego, CA, USA).

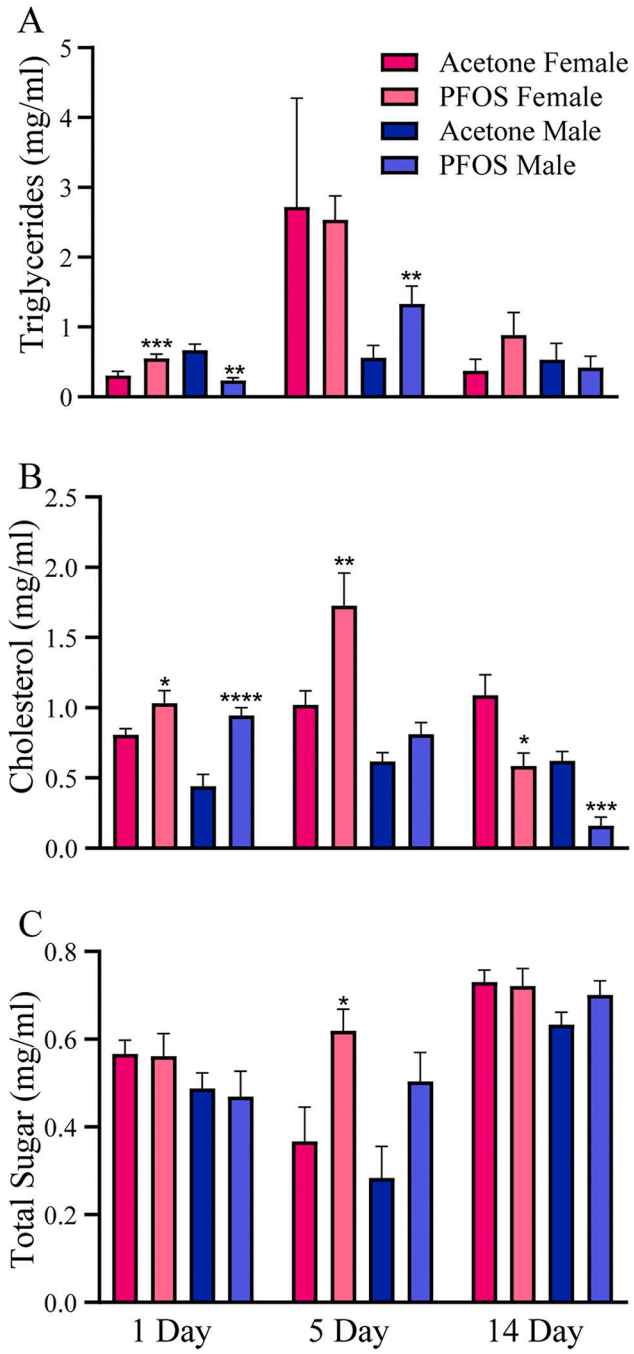


Fig. 6.

Nutritional content of adult F1 progeny that developed from early laid eggs of *CS* mothers following either preconception PFOS or acetone exposure: (A) triglycerides, (B) cholesterol, (C) total sugar. Error bars indicate standard deviation (SD). N= 10–15 animals/treatment group. D'Augostino & Pearson Normality test, Mann-Whitney U test **p<0.01, ***p<0.001, ****p<0.000.

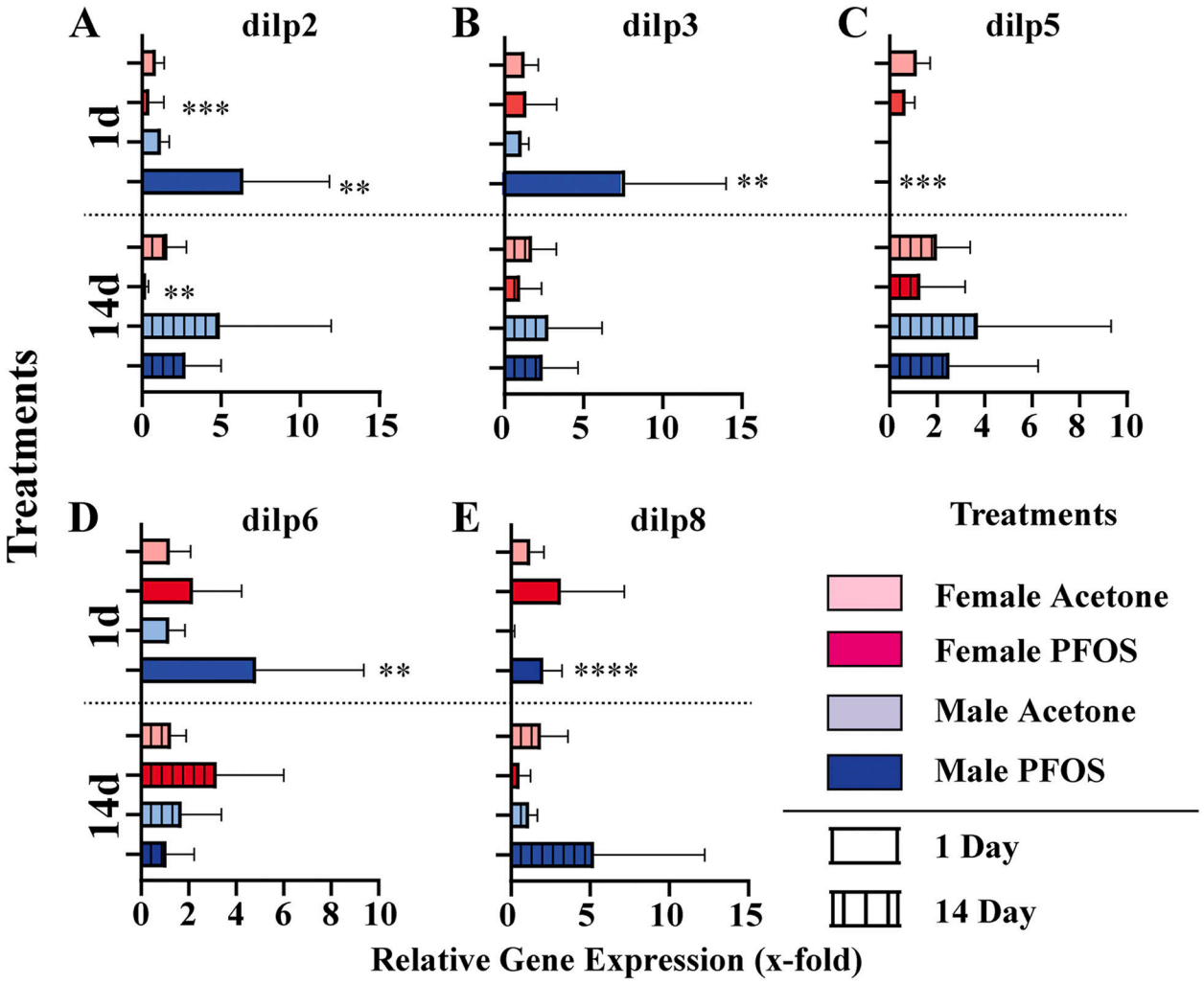


Fig. 7.

Preconception PFOS exposure alters *Drosophila* insulin-like peptide (*dilp*) expression levels in early group F1 progeny. Fold-change in expression of *dilp* 2 (A), 3 (B), 5 (C), 6 (D) and 8 (D) for female or male progeny from either preconception PFOS or acetone exposed mothers at 1 day and 14 days post-eclosion (see treatment legend). Fold-change in expression is calculated as $2^{-\Delta\Delta ct}$ using tubulin as a reference gene. Error bars indicate standard deviation (SD). N=8–10 animals/treatment. D'Augostino & Pearson Normality test, Mann-Whitney U test **p<0.01, ***p<0.001, ****p<0.000.

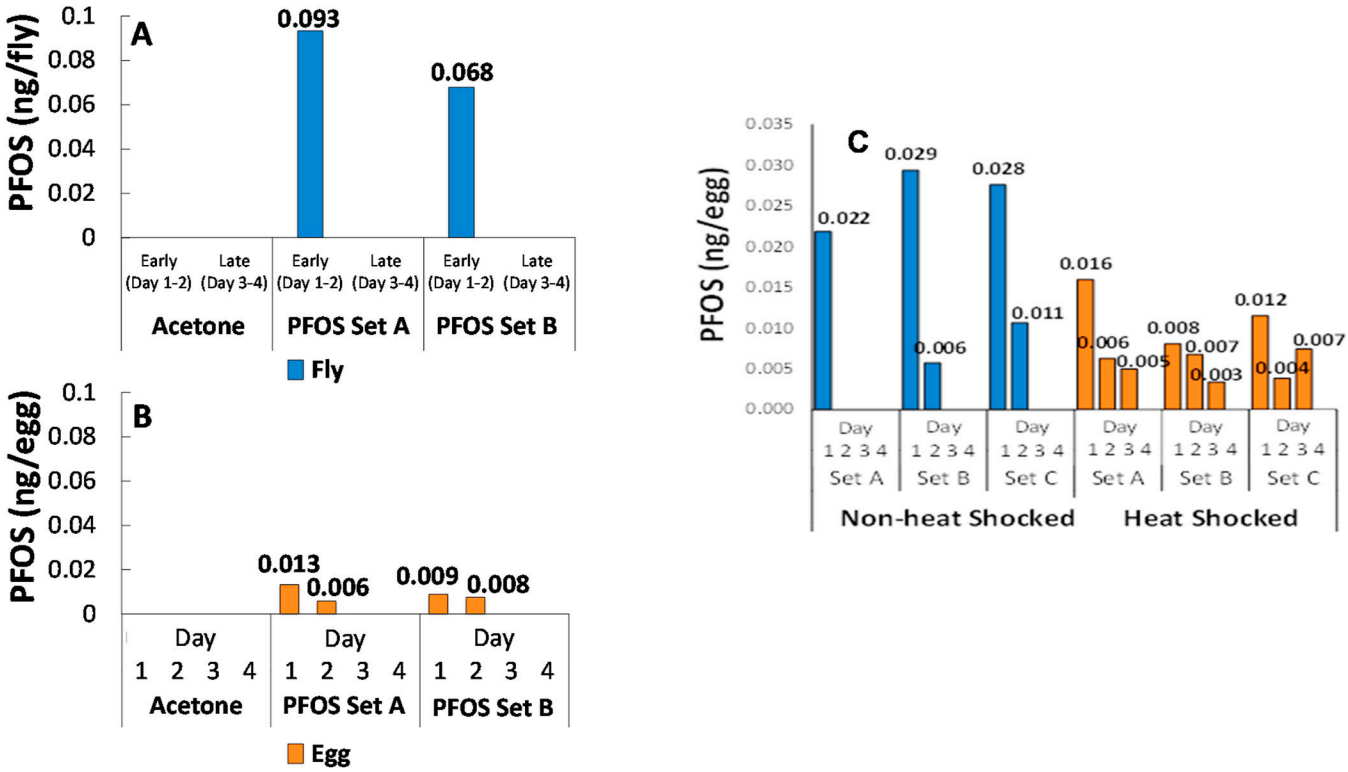


Fig. 8.

Detected amount of PFOS in maternal *CS* female flies (A) and their F1 eggs (B) following preconception exposure to PFOS. PFOS levels in eggs from non-heat shocked (NHS-megalin(+) or heat shocked (HS-megalin(−) transgenic female flies following preconception exposure to PFOS (C). Eggs were collected every 24 h from PFOS- treated female.