Methods:

Animals. Male Sprague Dawley rats ([H1a: (SD) CVF, n = 6 rats/group (N=60); 6-7 weeks of age, 200 – 250 g) were obtained from Hilltop Lab Animals, Inc., Scottdale, PA. Females were not included in the initial studies to determine if there was an effect of inhaling PC-emissions. Once it is demonstrated that there is an effect, future studies may include females. All animals were free of viral pathogens, parasites, *mycoplasma*, *Heliobacter* and cilia-associated respiratory (CAR) bacillus. The rats were acclimated to the facilities for 1 week after arrival and housed in cages ventilated with HEPA-filters under controlled temperature and humidity conditions and a 12-h light/12-h dark cycle. Food (Teklad 7913; Envigo Madison, WI) and tap water were provided *ad libitum*. The animal facilities are specific pathogen-free, environmentally controlled, and accredited by AAALAC International. All procedures were approved by the NIOSH Animal Care and Use Committee and were in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the NIH Guide for the Care and Use of Laboratory Animals.

Exposure. After 1 week of acclimation to the facility, animals were exposed to filtered-air (controls) or emissions generated by 3DP. Briefly, animals were placed in separate compartments of a whole body exposure chamber that could hold up to 12 animals. There was no food or water available during the exposure. The cage rack with the animals rested on top of cage support beams which contained 1 cm outside diameter stainless steel tubes with small holes. Each hole was placed in the center of the cage partition so that aerosols would be drawn into the breathing space. Three desktop 3D-printers (LulzBot Mini, Fargo Additive Manufacturing Equipment 3D LLC, Fargo, ND) were placed in an airtight chamber. Black polycarbonate filament was fed into each printer and the printers were operated continuously during the 4 h

exposure period. Emissions generated in the exposure chamber were discharged into the chamber with the animals. Exposures were monitored as described in (Farcas et al. 2020). At the beginning of each exposure, animals were placed into the designated chamber (for air or 3D emission exposure). Emissions from the printing chamber or filtered air were pumped into the whole-body inhalation chamber. At the end of each exposure, animals were placed into their home cages and returned to the colony room.

The measured particulate emissions inside the exposure chamber averaged 592 $\mu g/m^3$ of particulate for a 4h exposure when the average was calculated over all days of the exposure. Particulate was collected every 5 seconds using a fast mobility particle sizer. The mean aerodynamic equivalent diameter of particles measured inside the exposure chamber during the print jobs was 40 nm. The PC particles were also collected on filters and imaged with a scanning electron microscope. Typical physical diameters for PC particulate ranged from 40 nm up to 90 nm (Farcas et al. 2019).

Analysis of volatile organic compounds by GC/MS. The volatile component of the emissions was analyzed by GC/MS from samples collected over a 4-h collection period. The levels of acetaldehyde, acetone, ethanol, and ethylbenzene were the highest among the detected compounds. However, concentrations of these chemicals were much lower than the OSHA permissible exposure limits (PELs). The method used for collecting and measuring volatile compounds can be found in (National Institute for Occupational Safety and Health 2018).

Bisphenol A and bisphenol A diglycidyl ether were also measured in emissions. Emissions were generated in a manner similar to that used to generate the exposure, and samples of particulate were collected from a chamber with no animals. The total concentration of particulate in each sample was approximately 600 $\mu g/m^3$. The emissions were collected onto

glass fiber filters (SKC lot #21600-7E5-274; n = 6 samples) and sent to BVNA labs (Novi, MI). Once received by the lab, the filters were place in separate glass test tubes and each sample cassette was wiped with a glass fiber filter wetted with 100% ethanol. Sample filters and wipes extracted in 3 ml of acetonitrile and then placed on a mechanical flatbed shaker for 30 min. An aliquot (18 µl) was transferred to individual auto-sampler vials for analysis by HPLC with ultraviolet detection following the parameters listed below. The instrument used for HPLC was a Thermo Fisher Vanquish UHPLC with a Zorbax ODS C18, 5 µM 250 mm x 4.4 mm internal diameter. The average level of bisphenol in the samples was 5.30 ± 0.18 µg/m^3. Using a mass particle distribution model to determine the particle in the nose, trachea and alveola (where the tidal vol is 1.7 ml, the breathing rate is 120 breaths/min and the exposure time is 240 min), exposure to bisphenol on day 1was 0.036g, on day 15 was 0.536 g and day 30 was 1.072 g.

Tissue collection. Animals were exposed for 4 h/day 4 days/week. Groups of animals (6 air control and 6 treated) were euthanized by injection of pentobarbital (100 mg/kg i.p.) 24 h after 1, 4, 8, 15 or 30 days of exposure. The brain was dissected and cut into right and left hemispheres. The olfactory bulb and hypothalamus were dissected from the right hemisphere of the brain and frozen at -80°C until used for PCR. The left hemisphere was placed in OCT compound in a cryomold and frozen at -80°C until sectioned and processed for immunohistochemistry and histology.

Tissue preparation: Histology. The left hemisphere of the brain was sectioned (20 µm) using a cryostat set at -20°C. Five slides were collected from the olfactory bulb and hypothalamus. The first section was put on slide 1, the second section on slide 2 etc. Using this procedure there was 100 µm between consecutive sections on each slide. There were 5-6

sections/slide from the olfactory bulb and 3-4 sections/slide from the hypothalamus of each animal. Using this method, each slide contains a section through the rostral to caudal portions of a brain region. For histological or immunohistochemical analyses, one slide from each animal and each region was processed.

The right testis was also dissected from each animal and placed in 10% buffered formalin. Each testis was paraffin embedded and sections (10 μm) were cut on a microtome. Five slides were collected from each animal, with each slide having 1 or 2 tissue sections. One set of sections was stained with Harris hematoxylin and eosin for histological analyses. The others were stored at rt until used for immunohistochemistry.

Histology and Immunohistochemistry. Immunohistochemistry was performed using a previously described protocol with slight modifications. Briefly, slides were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) for 5 minutes, rinsed 3 x 5 min in PBS and incubated in primary antibody diluted in 0.1 M PBS plus 0.3% Triton-x 100 and 10% normal serum overnight at 4°C. The primary antibodies were obtained from Santa Cruz Biotech. The following day the slides were rinsed, sections were incubated with the secondary antibody (either Cy3 or fluorescein labeled antibodies; Jackson Immunolabs) diluted 1:500 in 0.1M PBS-Tx at RT for one hour. Sections were then rinsed, air dried and cover-slipped using Prolong Gold with DAPI (Invitrogen, Waltham, MA).

Another set of sections was labeled using Fluoro-jade which identifies damaged neurons. A stock solution of Fluro-jade (3.0 % in dimethyl sulfoxide (DMSO)) was aliquoted and frozen at -20°C until use. For staining, sections were fixed as described above, incubated in a 0.06% potassium permanganate solution in 0.1M PBS for 10 min, rinsed and then incubated in the Fluoro-jade solution (0.003% in 0.1M acetic acid) for 1 h, in the dark, at room temperature.

Sections were then rinsed in water and cover-slipped as described above. Staining in the periventricular/arcuate nuclei was measured because the neurons that regulate pituitary function are primarily located in these regions. The percent area labeled was measured using Image J (National Institutes of Health). Labeling was measured in 3-5 sections per animal and the average labeled area was calculated and used for analyses. Gonadotropin releasing hormone (GnRH) and tyrosine hydroxylase (TH) neurons were also counted in these nuclei.

To label mitochondria Mitotracker Orange CMTMRos (Invitrogen, Waltham, MA) was used. This specific Mitotracker stain enters the cell and is oxidized to its fluorescent form which is then sequestered in active mitochondria. To perform staining, stock solution supplied by the manufacturer was thawed and diluted to 500 nM using 0.1 M phosphate buffered saline (PBS). Slides were incubated in diluted Mitotracker for 30 min at 37°C, rinsed 3 x 5 min in PBS, incubated in 4% paraformaldehyde in 0.1 M PBS for 10 min, dried, cover-slipped using Prolong Gold with DAPI, and stored in a light tight container at 4°C. Mitotracker staining was quantified by visualizing the sections using fluorescent microscopy at a wavelength of 554/576 nm (excitation/emission). Both the percent area labeled and the intensity of the labeling were measured using Image J (NIH). Labeling was measured in 3-5 sections per animal and the average labeled area and intensity were calculated and used for analyses.

For paraffin embedded sections of the testes, slides were first heated at 60ºC for 20 min and then incubated in 3 rinses of xylene (20 min each at room temperature room temperature) to remove the paraffin. Sections were then dehydrated using descending concentrations of ethanol (100 – 75%) and rehydrated in water followed by 0.1 M PBS. One slide was stained with Harris hematoxylin and eosin (H&E) stain, and the number of Sertoli cells, Leydig cells and spermatogonium were counted in 4 photomicrographs/animal. The number of mature sperm was

estimated by measuring the density of the sperm tails in the seminiferous tubules. Other slides were used for immunohistochemistry, which was performed as described above.

Microscopy. Fluorescent-labeled slides were examined using an Olympus microscope photomicrographs were taken at a magnification of 20x using DP73 camera and CellSense version 510 (B&B Microscopes, Pittsburgh, PA). Densitometry was performed on photomicrographs using ImageJ. To perform densitometry, a threshold was set to identify regions that were immuno-stained. The area to be quantified was outlined and the total area of the outlined structure was measured along the with area of the immunolabeled cells that were at or above the set threshold. Depending on the region, 3-5 sections were analyzed. The average immune-stained area was calculated and used for analyses.

To quantify structures in the testes, a stereographic procedure utilizing a grid was used to count the number of Sertoli cells, spermatocytes at different stages of development and Leydig cells. The grid used for counting was a 12.5 mm square consisting of 100 smaller squares. The number of cells or structures within each box of the grid was counted, the number of cells or structures that intersected with either a vertical or horizontal line of the grid was counted. Three to 5 sections were analyzed from each animal. An average number of cells/section was calculated and used for analyses.

ELISAs. ELISAs for estradiol, prolactin, thyroid stimulating hormone (TSH) and follicle stimulating hormone (FSH) were performed using plasma samples collected from animals after 1, 4, 8, 15 or 30 days of exposure. Blood was collected by cardiac puncture, 24 h after the last exposure, allowed to clot and room temperature for 1 h and centrifuged at 1500 cpm for 15 min at 4ºC. Plasma was collected stored at -80 ºC until assayed. ELISAs for estrogen and prolactin were purchased from Calbiotech (El Cajon, CA), and ELISAs for TSH and FSH were purchased

from E-Lab Sciences (Houston, Tx). All assays were run according to the manufacturer's protocols. We chose to measure these hormones because previous studies have demonstrated that these hormones can be altered by endocrine disrupters such as bisphenol A. The measured coefficients of variation for each assay were as follows: estrogen 11.5%; FSH 3.6%; prolactin 2.33%; TSH 6.46%, and the lowest detectable levels for each assay were 0.25. A zero (0) in a dataset means that there was an inadequate amount of tissue or that the measured value that was below the lower limit of detection for the assay (limits of detection were 3.5 ng/ml for estradiol, 2.5 ng/ml for FSH, 2.5 ng/ml for TSH and 6.5 ng/ml for prolactin).

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). qRT-PCR was performed to determine if exposure to 3D printing fumes resulted in changes in transcript levels in the olfactory bulb and hypothalamus as previously described. Briefly, RNA was isolated from the tissue using RNAeasy lipid Miniprep kits (cat # 74804; Qiagen, Valencia, CA), and first strand cDNA was synthesized from 1 μg of total RNA using a Reverse Transcription System (Invitrogen; Carlsbad, CA). Melt curves were run for each transcript using each tissue. Samples that did not show a single defined melt peak in the 80ºC range were not included in the data set. To determine if the treatment resulted in a change in transcript levels, fold changes from the same day controls were calculated. This was done by calculating the average response for the control group and then subtracting the individual CT values for each sample from the average of the controls. Transcripts were measured in the olfactory bulb and hypothalamus and included immediate early genes, glial cell, oxidative stress and inflammatory markers and markers of proteins involved in neurotransmission.