# Perflurooctanoic acid induces developmental cardiotoxicity in chicken embryos and hatchlings

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Running title: PFOA induces avian developmental cardiotoxicity

Key words: perflurooctanoic acid, chicken embryo, immunohistochemistry, cardiac ultrasound, cardiac myofibril ATPase.

Acknowledgments: We thank Dr. J. Brown, Dr. Abdel-Rahman, Dr. Virag, Dr. Capehart, Dr. Chalovich, Dr. Tulis, Dr. Wingard and Dr. D. Brown for guidance during the course of the experiments. We also thank Qing Hu, Jason Franklin, Ian Byran and Lalage Katunga for technical assistance. The MF20 antibody developed by Donald A. Fischman was obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by The University of Iowa. This work was supported in part by a faculty startup award from the Department of Pharmacology and Toxicology and the East Carolina University Division of Research and Graduate Studies.

**Competing financial interests declaration:** There are no competing financial interests to declare.

**Abbreviations:** PFOA, Perflurooctanoic acid. H&E, Hematoxylin and Eosin. Tris, Tris (hydroxymethyl) aminomethane. NaCl, Sodium chloride. KCl, Potassium chloride. EDTA, Ethylenediaminetetraacetic acid. EGTA, Ethylene glycol tetraacetic acid. K-acetate, Potassium acetate. TCDD, 2,3,7,8- tetrachlorodibenze-*p*-dioxin.

## Abstract

**Background:** Perfluorooctanoic acid (PFOA) is a widespread environmental contaminant that is detected in serum of the general U.S. population. The median human serum concentration in 2007-2008 was 4.3 ng/mL. PFOA is a known developmental toxicant that induces mortality in mammalian embryos, the causes of which are as yet unknown. As the cardiovascular system is crucial for embryonic survival, PFOA-induced effects on the heart may partially explain embryonic mortality.

**Objectives:** To assess the impact of PFOA exposure on the developing heart in an avian model.

**Methods:** Histopathology and immunohistochemical staining against myosin were used to assess morphological alterations after PFOA exposure. Echocardiography and cardiac myofibril ATPase activity assays were used to assess functional alterations following PFOA exposure.

**Results:** Overall thinning and a thinning of the myosin dense layer in the right ventricular wall were observed in PFOA-exposed chicken embryo hearts. Alteration of multiple cardiac structural and functional parameters, including left ventricular wall thickness, left ventricular volume, heart rate, stroke volume, and ejection fraction were detected with echocardiography in one-day-old hatchling chickens exposed to PFOA throughout development. An ATPase activity assay indicated that cardiac myofibril ATPase also was affected by developmental PFOA exposure.

**Conclusions:** The heart appears to be a developmental target of PFOA. Additional studies will investigate the mechanism of PFOA-induced developmental cardiotoxicity.

## Introduction

Perfluoroalkyl acids (PFAAs) are fluorinated compounds used to manufacture materials for myriad consumer and industrial products, including nonstick, stain-repellant, water repellant, and fire-retardant coatings. One PFAA that is of public health concern because of its presence in environmental media and biota is perfluorooctanoic acid (PFOA). PFOA is a polymerization aid used in the manufacture of fluorinated polymers and elastomers, the most well known of which is polytetrafluoroethylene (PTFE). According to the current major manufacturer of PFOA, a minimal amount of PFOA is still present in the end products (DuPont 2011), which can leach out and contribute to exposure levels. PFOA also is a breakdown product of certain fluorinated telomer alcohols and other precursor compounds of fluorinated polymers (Wang et al. 2005).

Increasing reports of PFOA-induced toxicity lead to a stewardship program between major flourochemical manufacturers and the U.S. Environmental Protection Agency (USEPA). The goal of the stewardship program is to eliminate PFOA and precursor products that can break down to PFOA by 2015 (USEPA 2011). However, as PFOA does not bio-degrade, it persists in the environment and in biota; it is already ubiquitous in environmental media as well as in serum of the general world population. The median serum concentration in the general U.S. population reported in 2007-2008 is 4.3 ng/mL (CDC 2011). In an area of West Virginia contaminated with PFOA by a manufacturing plant, an epidemiological study of the exposed populations reported that the median PFOA serum concentration was 26.6 ng/mL, with a high value of 17,556 ng/mL (Steenland et al. 2009).

PFOA has demonstrated multisystem toxicity in laboratory models and data from studies of such models suggest that it is an agonist of the peroxisome proliferator activated receptor alpha (PPARα). In laboratory models, exposure to PFOA induces liver, pancreas and testicular

cancer (Biegel et al. 2001); endocrine disruption (Olsen et al. 1998) and immunotoxicity (DeWitt et al. 2008) also have been observed. In addition, PFOA has been reported to induce developmental effects, including retarded development, decreased fetal survival and increased deformities in animal models (Wolf et al. 2007). In humans, epidemiology studies have revealed that PFOA exposure is associated with elevated serum cholesterol and uric acid levels (Steenland et al. 2010).

As the general human population has measurable serum concentrations and accompanying changes in cholesterol levels, investigation of additional health effects of PFOA is warranted, especially for those effects related to the cardiovascular system. In addition, reasons for increases in fetal mortality associated with developmental PFOA exposure (Wolf et al. 2007, Lau et al. 2004, and DeWitt et al. 2009) are not known. For embryo survival, a properly functioning cardiovascular system is crucial. Development of the cardiovascular system is a complex and delicate process, with numerous signaling activities that are vulnerable to exogenous disruptions from exposure to pathogens, drugs and environmental pollutants. For example, lipopolysaccharide exposure leads to cellular hypertrophy in H9c2 myocardiac cells and is associated with alteration to the calcineurin/NFAT-3 signaling pathway (Liu et al. 2008). Adiramycin, an antitumor drug used to treat leukemias, lymphomas and neoplasms, was reported to induce ventricular septal defects, dextropostion of the aorta, and aortic arch anomalies in a dose-related manner when topically administered to five day old chicken embryos (Takaqi et al. 1989). The authors suggested that adiramycin decreased embryonic cardiac blood flow and inhibited rapidly exchangeable calcium within cardiac cells (Takaqi et al. 1989). An example of an endogenous compound that can induce cardiotoxicity when its signal is extended during development is retinoic acid (RA). Although essential for cardiac development, when additional

RA was administered to 7.5 day old mouse embryos, the outflow tract was absent and ventricles were reduced (Xavier-Neto et al. 1999); therefore, agents that interfere with RA signaling may affect cardiac development. It also was reported that exogenous RA disrupts primary heart tube identity via alteration of atrial-specific gene expression in chickens (Yutzey et al. 1994). In addition, a well known environmental pollutant, 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD) has been reported to enlarge left and right ventricles, thicken ventricular septa and thin the left ventricle wall in chicken embryos (Walker et al. 1997). 3,3',4,4',5-pentachlorobiphenyl (PCB-126) exposure also lead to decreased myocyte proliferation in zebra fish, possibly by affecting hemodynamics (Grimes et al. 2008). Taken together, these studies indicate that the developing heart is sensitive to perturbations by exogenous agents. Given PFOA's developmental toxicity in laboratory models, presence in human and wildlife serum, and possible effects on endogenous compounds associated with heart disease, we chose to investigate PFOA's effect on the developing cardiovascular system. As avian cardiovascular development is similar to mammalian cardiovascular development and lacks a direct maternal influence, avian embryos are ideal models for developmental toxicity studies. This is the first study to assess both morphological and functional changes in avian hearts developmentally exposed to PFOA.

## Methods

### Animals

Fertile chicken (*Gallus gallus*) eggs were purchased from the North Carolina State University Poultry Research Center (Raleigh, NC). Prior to injection and incubation, eggs were cleaned in a 20% povidone iodine solution and candled so that the air cell could be marked with pencil on the shell. Eggs were weighed, given ID numbers, and assigned to different doses so that weights

were evenly distributed. Due to size limitations in the egg incubator, eggs were incubated in batches of 40-58; eight batches of eggs were used for the entire study. Uninjected eggs were included in each batch as environmental controls.

**Chemicals**

Sunflower oil was purchased from Spectrum Organic Products, LLC (Boulder, CO). PFOA and other chemicals (if not otherwise mentioned) were purchased from Sigma-Aldrich (St. Louis, MO).

**Egg Injection**

Injection procedures were as described in Henshel et al. (2003). Briefly, PFOA was suspended in sunflower oil and vortexed before injection into each egg. An awl was used to drill a 1 mm hole into the middle of the air cell area. A gel loading pipette tip was used to inject the PFOA mixture into eggs. Doses were 0, 0.5, 1 and 2 mg/kg of egg weight and the volume of injection was 0.1 μL of oil per gram of egg weight. After injection, a drop of melted paraffin was used to seal the injection hole.

**Embryo incubation**

Injected eggs were incubated in a Lyon Roll-X incubator (Chula Vista, CA), with temperature of 99.5-100ºF and a humidity of 87-88ºF (about 60%). Eggs were candled every 2-3 days, and infertile/undeveloped/dead eggs were removed and opened to assess the age of embryo death. Chickens that were allowed to hatch were transferred from the Lyon Roll-X incubator to a larger incubator (G.Q.F. Manufacturing Co., Savannah, GA) after external pipping. Pipped eggs were

placed individually into small containers large enough for the hatched chickens. Hatchling chickens were kept in a warmed brood box until euthanasia. All procedures were approved by the East Carolina University IACUC.

**Histology on D19 chicken embryo heart**

At embryonic day 19 (D19), which is two days prior to hatch, eggs were removed from the incubator and embryos were removed from eggs and quickly decapitated. Whole embryo weight, yolk weight, heart weight, and liver weight were recorded. Livers were frozen at -80ºC for later use in other studies. Hearts were excised, rinsed in ice cold saline to induce full dilation of ventricles, fixed in 10% phosphate buffered formalin for 24h, and then cut transversely with a microtome blade under a Motic SMZ-168 dissection microscope (Redding, CA). The cuts were made approximately 60% of the length of the heart from the ventricular apex (shown in Figure 1A). The ventricular tissues were routinely processed (Thermo Scientific Shandon Citadel 1000, Waltham, MA), embedded in paraffin, and sliced on a rotary microtome (Thermo Scientific, HM 315 Waltham, MA) at six μm per section. A tissue indicator within the heart was used to maintain a relatively constant position in each heart to ensure that measurements on ventricular wall thickness were made at the same location within each heart (shown in Figure 1D-F).

**Hematoxylin & Eosin (H&E) staining**

Harris modified hematoxylin (Fisher Scientific, Fair Lawn, NJ) and Eosin Y solution (Harleco, Gibbstown, NJ) were used for H&E staining. Adobe Photoshop (San Jose, CA) with rulers (made in Berkeley Logo, Berkeley, CA) was used to measure the average thickness of the right ventricular wall (Figure 1B). Average right ventricular wall thickness was normalized to the

whole heart weight to minimize potential PFOA-induced effects on embryo body weight or developmental stage.

**Immunohistochemistry**

Antigen Unmask Solution, Vectastain ABC Kit (Mouse IgG), and DAB Kit were purchased from Vector Labs (Burlingame, CA) and the MF-20 antibody against myosin was purchased from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa city, IA). Briefly, sections were deparaffinized, blocked in serum albumin for one hour, and then incubated with 1:100 MF-20 in phosphate buffered saline with 0.5% tween-20 for 30 minutes at room temperature. Secondary antibody was applied at a 1:75 dilution and then DAB was added to the sections for color development. Sections were then counter stained with hematoxylin. The myosin dense layer was highlighted in the right ventricular wall (Figure 1C). After staining, measurements similar to those done on the H&E stained sections were collected, with an additional measure to evaluate the thickness of the myosin dense layer. Myosin dense layer thickness also was normalized to whole heart weight.

**Cardiac ultrasound on hatchling chickens**

Cardiac ultrasound data were collected on hatchling chickens to evaluate potential functional changes induced by developmental PFOA exposure. Within 24 hours post hatch, hatchlings were evaluated with an ultrasound instrument (Visualsonics Vevo 2100, Toronto, Ontario, Canada) for direct cardiac function measurement. Hatchlings were enclosed in stretchy, breathable gauze ("stockinette") so that they could be affixed to the operation table with surgical tape without damaging their feathers. A hole was cut in the torso portion of the stockinette and ultrasound gel

was applied directly to the torso of each hatchling. Heart rate, stroke volume, ejection fraction, fraction shortening, left ventricular posterior wall dimension, left ventricular volume and mass were measured and analyzed; the person performing the analysis was blind to the dose group assignments. Histology was evaluated on the right ventricular wall, whereas cardiac ultrasound was performed on the left ventricle. For evaluation of histology, the right ventricular wall is a better target as the wall is clearly indicated and the lumen is large and obvious. The left ventricle is a better target for echocardiography. Technically, the left and right ventricles should have the same output to maintain circulation stasis; therefore both right and left ventricle function should reflect cardiac function as a whole.

**Cardiac myofibril ATPase assay**

To supplement the ultrasound data and to provide an additional functional measure, animals were euthanized shortly after ultrasound measurements and a cardiac myofibril ATPase assay was performed on hearts to determine if calcium activated magnesium dependent ATPase activity was altered. The hearts were removed as described for the morphology study and stored at -80ºC until the day of the assay.

*Preparation of cardiac myofibril*

Hearts were dissected to exclude vessels and atrial tissue and 200 mg of ventricular tissue were used per animal. The tissue was homogenized in 1 mL homogenization buffer (Tris 50mM, EDTA 5mM, NaCl 100mM, KCl 2mM, protease inhibitor (Thermal Scientific, Rockford, IL), 0.5% Triton, pH 7.2-7.4). The resulting tissue suspension was centrifuged at 2000g (4ºC, 15 minutes). Supernatant was discarded, the pellet was resuspended in 1 mL wash buffer (KCl 0.1M + protease inhibitor) and then centrifuged again at 2000g (4ºC, 15 minutes). The wash was

repeated a total of four times. Immediately prior to the last centrifugation, suspensions were filtered through 70 μm nylon cell strainers (BD Falcon, Bedford, MA) to exclude tissue debris. The final pellets were then resuspended in storage buffer (Tris 50mM, K-acetate 100mM, KCl 5mM and protease inhibitor, pH 7.2-7.4) and protein concentration was determined (Thermo Scientific, Rockford, IL).

Protein concentration was adjusted to 50 μg/mL, and every sample was divided into a calcium-dependent ATPase group (storage buffer plus 3mM magnesium chloride and 0.1mM calcium chloride) and a calcium-independent ATPase group (storage buffer plus 3mM magnesium chloride and 10mM EGTA). The samples were equilibrated at room temperature for 5 minutes and then ATP (final concentration 0.5mM) was added to initiate the reaction. After 30 minutes at room temperature, PiGoldLock (Innova Biosciences, Babraham, Cambridge, UK) with 1:100 accelerator was added to stop the reaction and to visualize free inorganic phosphate. Two minutes later, stabilizer (Innova Biosciences, Babraham, Cambridge, UK) was added to each well to stop ATP hydrolysis. Samples were incubated at room temperature for 30 minutes to develop color completely and then read at 630nm (Biotek Synergy HT plate reader, Winooski, VT).

**Serum PFOA concentration**.

Samples were prepared as described in Reiner et al. (2009). In brief, serum samples were thawed by placement in cool water and vortexed 30 seconds prior to sampling. An aliquot of serum (25 μL) was placed in a 5 mL polypropylene tube (BD Falcon, Franklin Lakes, NJ) and denatured with 100 μL of 0.1 M formic acid containing ~5 ng $^{13}C_2$-PFOA. Samples were vortexed for 1

min. Samples then received 1.0 mL of cold acetonitrile to precipitate proteins, were vortexed for 1 min and then centrifuged at 10,000 rpm for 2 min to pelletize proteins. An aliquot of the acetonitrile extract (200 μL) was combined with 200 μL of 2 mM ammonium acetate for LC-MS/MS analysis. All unknowns, replicates, method and matrix blanks, and QA/QC samples were prepared in this fashion. The standard curve preparation was matrix matched. Standards were prepared by spiking 25 μL of control Pel-Freez CD1 mouse serum into a 5 mL tube and then spiking in a corresponding mass of PFOA in methanol (0.250 – 1,250 ng) relating to 10 to 50,000 ng PFOA/mL of serum. This was covered by two standard curve ranges (10 – 500 ng/mL) and (500 – 50,000 ng/mL). Standards were likewise treated as all other samples. Samples that did not fall in the lower standard curve range were re-run with a slightly modified method. The 25 μLserum sample was diluted with 2.0 mL of 0.1 M formic acid, 200 μL was sampled and it was added to 2.0 mL of acetonitrile containing ~62.5 ng of $^{13}C_2$-PFOA. This acetonitrile extract was then used to prepare the samples further as described above.

Samples were quantitated using a Waters Acquity Ultrahigh Pressure Liquid Chromatography (UPLC) coupled to a Quatro Premier XE tandem mass spectrometer (MS/MS) (Watersd Corp, Milford, MA). Samples were run in a batch as to include double blanks (solvent blank), a method blank, matrix blank (blank serum), standards, quality control (QC) samples, replicates, and unknowns in sequence. Standards were run at the beginning and end of the analytical batch, and QC samples interspersed in the analytical batch. Quality control samples consisted of pooled mouse serum spiked at 6 concentrations over the analytical range. Avearge accuracy of QC pool samples was 94.1% with a % relative standard deviation (RSD) of less than 10% for all samples (n = 18). PFOA was monitored via the transition 413-369 m/z and 413-169 m/z and for the $^{13}C_2$-PFOA 415-370 m/z. Samples were run using an isocratic (50:50) mobile

phase consisting of 2 mM ammonium acetate aqueous solution with 5% methanol (solvent A) and 2 mM ammonium acetate in acetonitrile (solvent B). Samples were integrated using the equipment software and corrected if necessary by the operator.

### Statistical analysis

All data were analyzed by one way analysis of variance (ANOVA) by dose. Statistical significance was determined when $P < 0.05$. When ANOVA revealed a statistically significant model, post-hoc t-tests were performed to determine statistical significance between dose groups.

## Results

### H&E staining

Thinning of the right ventricular wall (corrected for whole heart weight) was observed in all dosed groups relative to the vehicle control group (Figure 2A). The 1 mg/kg and 2 mg/kg dose groups were statistically thinner relative to vehicle control group (20.2% and 22.7% thinner, respectively).

### Immunohistochemistry

Thinning of the myosin dense layer of the right ventricular wall was observed in all dosed groups relative to the vehicle control group (Figure 2B). The 1 mg/kg and 2 mg/kg groups were statistically significantly thinner relative to the vehicle control group (27.07% and 28.59% thinner, respectively).

### Cardiac ultrasound

All morphological parameters collected by cardiac ultrasound demonstrated statistical significance relative to the vehicle control group. The left ventricular posterior wall dimension at diastole (Figure 3A) increased by 5.7% (0.5 mg/kg), 29.6% (1 mg/kg), and 71.6% (2 mg/kg). The left ventricular posterior wall dimension at systole (Figure 3B) was increased 4.9% (0.5 mg/kg), 11.5% (1 mg/kg), and 26.0% (2 mg/kg). Left ventricular volume at diastole (Figure 3C) was increased by 6.7% (0.5 mg/kg) and decreased by 14.1% (1 mg/kg) and 40.4% (2 mg/kg). At systole, left ventricular volume (Figure 3D) was decreased by 12.2% (0.5 mg/kg), 48.5% (1 mg/kg), and 76.0% (2 mg/kg). Finally, left ventricle mass (Figure 3E) was increased by 5.4% (0.5 mg/kg), 8.8% (1 mg/kg), and 24.7% (2 mg/kg).

Functional parameters collected by cardiac ultrasound were statistically significant, too. Heart rate (Figure 4A) was decreased by 7.9% (0.5 mg/kg) and increased by 55.3% (1 mg/kg) and 52.9% (2 mg/kg). Stroke volume (Figure 4B) was elevated in the 0.5 mg/kg group (12.6%) and decreased in the 1 mg/kg group (3.4%) and 2 mg/kg group (29.5%). The ejection fraction (Figure 4C) was elevated by 5.4% (0.5 mg/kg), 9.0% (1 mg/kg), and 17.0% (2 mg/kg). Fraction shortening (Figure 4D) was elevated by 6.3% (0.5 mg/kg), 12.6% (1 mg/kg), and 30.0% (2 mg/kg).

**Cardiac myofibril ATPase**

No difference was observed in calcium ATPase activity among groups. Increased calcium-independent ATPase activity, along with the ratio of calcium-independent ATPase activity to calcium-dependent ATPase activity was observed, but these differences were not statistically significant.

## PFOA Serum concentration

Mean serum concentrations of PFOA in the vehicle control and uninjected controls were below the limits of quantitation (LOQ) of 50 ng/mL. Mean PFOA serum concentrations in the dosed hatchlings (mean ± standard deviation) were 1230.8 ± 363.9, 2055.7 ± 1577.5, and 5670.0 ± 3394.1 ng/mL for the 0.5, 1, and 2 mg/kg dose groups, respectively. Quality control standards were all within +/- 20% of spiked values, with coefficients of variation of less than 5%.

## Discussion

Cardiovascular diseases are one of the major threats to human health and the complexity of their etiology prevents them from being fully understood and therefore prevented. Environmental contaminants are capable of directly inducing acute cardiac events as well as increasing chronic coronary heart disease incidence (Fang et al. 2010). Congenital heart disease is the most prevalent cause of infant death, accounting for more than 24% of total infant mortality (Roger et al. 2011). The establishment of the primary heart tube, looping of the developing heart to the right, and septation to form a four-chamber heart are under the control of multiple signaling pathways, the most important of which include bone morphogenic protein (BMP), fibroblast growth factor (FGF) and TGF-beta (Kirby et al. 2002). Exogenous agents are capable of disrupting these processes to alter heart development, resulting in malformation. Even if disruption in development does not directly lead to heart malformation, the effects of delayed development or altered function could still contribute significantly to heart diseases later in life. Previous studies reported that developmental delays and the subsequent catch up in development could increase the incidence of coronary heart disease, obesity, hypertension and type II diabetes (Langley-Evans et al. 2006 and Eriksson et al., 2006). PFOA is capable of mimicking

endogenous ligands and blocking or activating certain receptors, which are known to include PPARα, retinoid X receptor (RXR), and other receptors in the steroid hormone receptor superfamily (DeWitt et al. 2009). PPARα is known to be involved in heart development (Steinmatz et al. 2005). Moreover, we cannot eliminate the possibility of other unknown interactions of PFOA with other endogenous ligands. Thus, PFOA is potentially capable of disrupting signaling pathways in heart development and inducing developmental cardiotoxicity by its putative interaction with PPARα.

Normal ventricle wall thickness is a prerequisite to normal cardiac function and cardiac remodeling, either primary (cardiomyopathy) or secondary (hypertensive heart), is one of the most important factors in heart diseases. Thickening, thinning, or stiffening of ventricular walls could lead to disturbances in electricity-contraction coupling, induce ischemia and affect hemodynamics (Dukanović et al. 2009). Alteration of cardiac morphology is observed in many cardiovascular diseases, such as hypertrophic cardiomyopathy (Olivotto et al. 2009), hypertensive heart (Koren et al. 1991) and congenital heart diseases (Buchhorn et al. 2003). All of these diseases are reported to have ventricle dimension and ventricle mass alterations. In our study, similar alterations of cardiac morphology were observed: histopathology demonstrated that the right ventricular wall, especially the myosin dense layer, was affected by developmental exposure to PFOA. Echocardiography showed increased left ventricle dimension and mass, which looked like an early stage hypertensive heart (Koren et al. 1991). Whether these alterations were due to primary alteration of the contractile machinery or secondary to volume/pressure overloading is unknown; the similarity between human diseases and our observations in experimental animals suggests that developmental PFOA exposure might

357 contribute to human cardiovascular diseases, especially congenital heart diseases and/or later
358 cardiovascular diseases including hypertensive heart and cardiomyopathy.
359 Cardiac ultrasound allowed us to directly measure cardiac functional parameters,
360 including heart rate, stroke volume, ejection fraction and fraction of shortening. These
361 parameters are important indicators for congenital heart diseases as well as generalized
362 cardiovascular diseases. Neonates with congenital heart disease often have altered heart rate,
363 stroke volume, ejection fraction and fraction of shortening, which are diagnostic tools as well as
364 indicators for treatments (Tsai et al. 2008, Beaufort-Krol et al. 2007 and Lutin et al. 1999). In our
365 study, we found significant alterations on these functional parameters: heart rate was slightly
366 decreased at 0.5 mg/kg and increased by over 50% relative to vehicle control in the 1 and 2
367 mg/kg groups. Stroke volume changes were in the opposite direction: relative to vehicle controls,
368 it was slightly higher at 0.5 mg/kg and lower at the two higher doses. Overall, cardiac output was
369 either roughly the same as the vehicle control at all dose groups except for the 1 mg/kg group,
370 which was elevated by 49.9% (data not shown). Meanwhile, ejection fraction and fraction
371 shortening were elevated in all dosed groups. These are all suggestive of disrupted heart
372 development.
373 Myofibril consists of the whole contractility machinery in the myocardium: myosin,
374 actin, topomyosin, troponin, etc. Its rate of ATP hydrolysis is an estimation of cardiac sacromere
375 function (Lionne et al. 2003). Myofibril ATPase activity is an indirect indicator of cardiac
376 function, which can be altered when cardiotoxicity occurs (Cappelli et al. 1989). Calcium is an
377 important regulator for myofibril ATPase activity; cardiaovascular diseases such as
378 cardiomyopathy can alter calcium sensitivity of myofibril ATPase activity (Chang et al. 2005).
379 The cardiac myofibril ATPase assay demonstrated that the calcium-dependent ATPase activity

did not differ across dose groups, but the calcium-independent ATPase activity increased with the dose of PFOA, thus the ratio of calcium-independent ATPase activity to calcium-dependent ATPase activity increased, (Figure 5). This might suggest that the impact of developmental PFOA exposure on cardiac function is primary, directly affecting contractility machinery.

Our primary hypothesis was that PFOA affected cardiac function directly. From our observations, developmental exposure to PFOA in an avian model leads to alteration of heart morphology and function, which are effects similar to what has been observed in human congenital heart disease as well as generalized cardiovascular diseases. The similarities suggest that PFOA could directly target heart development. However, the observation of left ventricle structure via echocardiography showed structural changes similar to an early stage hypertensive heart; as a result, we cannot eliminate the possibility that PFOA's effect on the heart is secondary to hemodynamic change. Future studies may include assessments of blood pressure. In addition, evaluating adult animals exposed to PFOA during development may provide more detailed information about the developmental basis of adult cardiovascular disease.

In humans from the U.S., serum PFOA concentrations as high as 5,100 ng/mL have been reported for occupational exposures (Olsen et al. 2007) and as high as 88 ng/mL have been reported in non-occupationally exposed humans (Olsen et al. 2003). The difference in blood concentration between humans with high occupational concentrations and our lowest dose group (1230.8 ng/mL in the 5 mg/kg group) is 0.24, which suggests that if developing humans are exposed to occupational concentrations of PFOA, a risk of heart anomalies may exist. For humans with high background concentrations (88 ng/mL), the difference between our lowest dose group is 14. This value suggests that developmental cardiotoxicity may be an endpoint of concern for highly exposed (non-occupational) human populations, especially when combined

403 with other risk factors. In a review of epidemiological studies evaluating the health effects of
404 PFOA, Steenland et al. (2010) examined data on cardiovascular disease of workers who were
405 exposed to PFOA. One study that they examined indicated a positive trend between serum PFOA
406 and mortality associated with cardiovascular disease whereas another study indicated no positive
407 trend. Steenland et al. (2010) concluded that data are currently insufficient for inferring
408 associations between PFOA exposure and cardiovascular disease. However, the workers in the
409 studies examined by Steenland et al. (2010) were likely adults during their exposure period. A
410 lack of effects observed in studies of occupationally-exposed adults cannot be taken to mean that
411 developing humans will respond similarly, especially when the increased sensitivity of
412 developing systems relative to adult systems is considered.

413 As PFOA is present in wild birds, it is also important to consider the implications of our
414 findings on environmental health. The range of PFOA concentrations reported in wild birds is
415 approximately 0.06 to 2 ng/mL (Martin et al. 2004; Bossi et al. 2005; Falandysz et al. 2007).
416 Generally, levels of PFOA in wild birds are an order of magnitude lower than perfluorooctane
417 sulfonate (PFOS), another PFAA of environmental and human health concern. We observed
418 functional effects in all exposure groups and morphological effects in the two highest dose
419 groups. The average PFOA serum concentration that we observed in our lowest dose group
420 (1230.8 ng/mL) is 2 x $10^4$ to approximately 600 times higher than reported concentrations in
421 wild birds. At current PFOA exposure levels, our study suggests that wild birds are not at risk of
422 developing heart anomalies.

423

424 **Future directions**

425 We are in the process of evaluating potential mechanisms behind the effects that we described.
426 PFOA is a known PPARα agonist; agonism of PPARα may regulate expression of proteins
427 involved in fatty acid transport, catabolism, and energy homeostasis (Peters et al. 2005). Yang
428 (2010) reported that PFOA exposure could lead to increased PPARα mRNA levels and increased
429 inflammatory cytokine (IL-1B, IL-6 and TNF-alpha) levels in livers of male Japanese medaka
430 (*Oryzias latipes*). Another study by Sun et al. (2008) found an association between PPARα
431 expression and TNF-alpha levels. Furthermore, IL-1B was found to be able to modulate TGF-
432 beta signaling via IL-6 activation (Luo et al. 2009) and increased TNF-alpha could suppress the
433 TGF-beta receptor population (Yamane et al. 2003). These studies have indicated the possibility
434 that PFOA acts through PPARα, which then influences inflammatory cytokines such as IL-1B,
435 IL-6 and TNF-alpha. We hypothesize that disrupted heart development could occur through
436 TNF-alpha mediated suppression of TGF-beta super family signal transduction. We are
437 evaluating the plausibility of this hypothesis with our current research. In addition, because
438 serum/plasma concentrations of PFOS are higher in wild birds than are PFOA concentrations, we
439 plan to repeat the ultrasound studies with chickens exposed to PFOS.
440

441 **Conclusions**

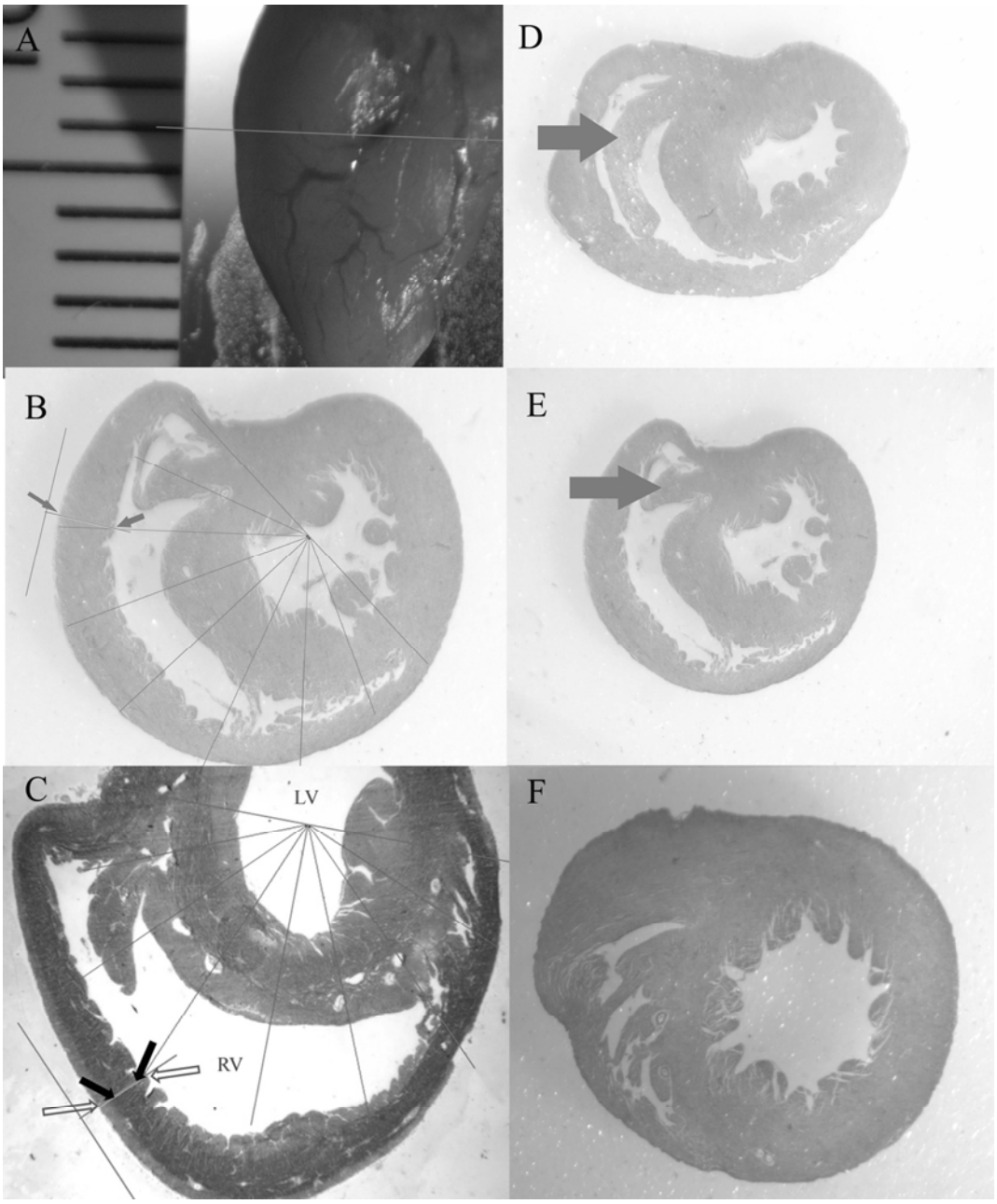
442 This study investigated morphological and functional changes in developing chicken hearts
443 following PFOA exposure. A thinner myosin dense layer in the right ventricular wall was
444 observed and a thickened left ventricular wall and increased left ventricular mass was observed
445 by cardiac ultrasound. Alteration in cardiac function was also observed by ultrasound; the
446 decreased stroke volume along with increased heart rate indicates early compensation for cardiac
447 dysfunction. A cardiac myofibril ATPase assay detected an increase in the calcium-independent

448 ATPase to calcium-dependent ATPase ratio. Our findings suggest that at the doses we

449 administered, PFOA disrupts avian heart development.

450

451 **Figures (note that these will be in separate, high quality files for journal submission)**




452

453 **Figure 1.** Morphological measurement methods.

A: After 24h fixation, hearts were cut at approximately 60% of the length of the heart from the ventricular apex under dissection microscope. Ruler shows the total length (mm). Transverse line shows the position of actual cut.

B: Position of measurements for right ventricular wall thickness in H&E staining. Seven measurements were made on each heart (indicated by radiating lines). The angle between each measurement line was 22.5°. Arrows show the measurement points for one of the seven measurements.

C: Position of measurements for whole right ventricular wall thickness and myosin dense layer thickness in immunohistochemistry. RV: right ventricular. LV: left ventricular. Between open arrows: total right ventricular wall thickness. Between closed arrows: myosin dense layer thickness.

D-F: Anatomical marker for relatively constant position on heart sections. D shows the cut at about 70% length of heart from apex. Arrow shows the marker, which is too long. E shows the cut at 60% length from apex. Arrow shows the marker, which is the size used across all sections. F shows the cut at 50% length from apex; the marker is gone.

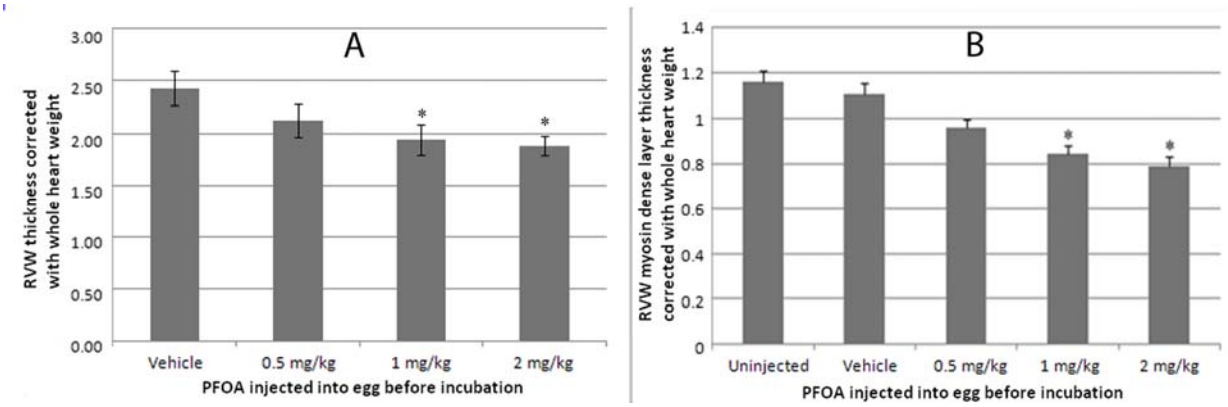


**Figure 2**. Histology data on right ventricular whole thickness and myosin dense layer thickness on D19 chicken embryo hearts. Fertile chicken eggs were injected with 0, 0.5, 1 and 2 mg/kg PFOA per egg and incubated to day 19, hearts were processed as described in the methods section.

A: Right ventricular wall thickness corrected with whole heart weight generated from H&E staining (Uninjected group not shown, N=7-10).

B: Right ventricular wall myosin dense layer thickness generated from immunohistochemistry corrected with whole heart weight (N=11-15).

*: significantly different from vehicle control group ($P<0.05$).

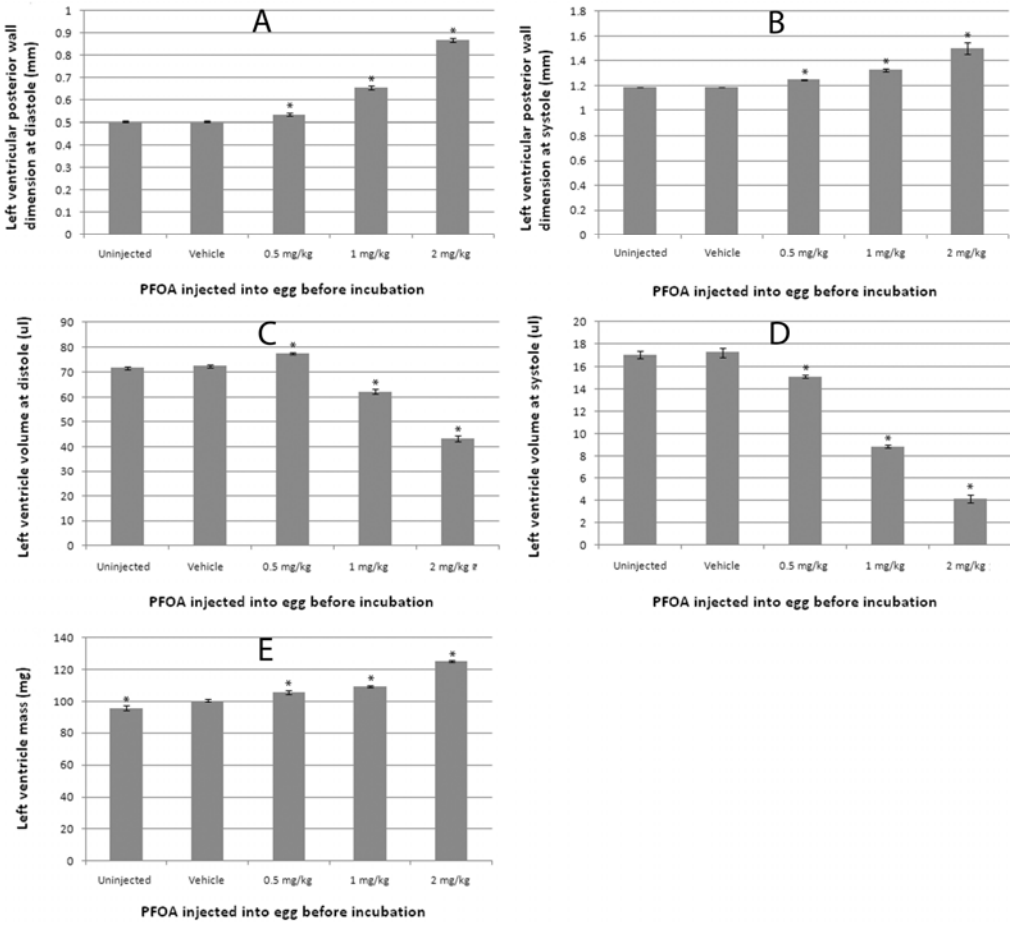


**Figure 3**. Cardiac ultrasound data on structural parameters of one-day-old hatchling chickens (N=9-12). Fertile chicken eggs were injected with 0, 0.5, 1 and 2 mg/kg PFOA per egg and incubated until hatch. By post hatch day 1, hatchling chickens were assessed by echocardiography as described in methods section.

A: Left ventricular posterior wall dimension at diastole of one-day-old hatchling chickens measured by echocardiography.

B: Left ventricular posterior wall dimension at systole of one-day-old hatchling chickens measured by echocardiography.

C: Left ventricle volume at diastole of one-day-old hatchling chickens measured by echocardiography.

D: Left ventricle volume at systole of one-day-old hatchling chickens measured by echocardiography.

E: Left ventricle mass of one-day-old hatchling chickens measured by echocardiography.

*: significantly different from vehicle control group ($P<0.05$).

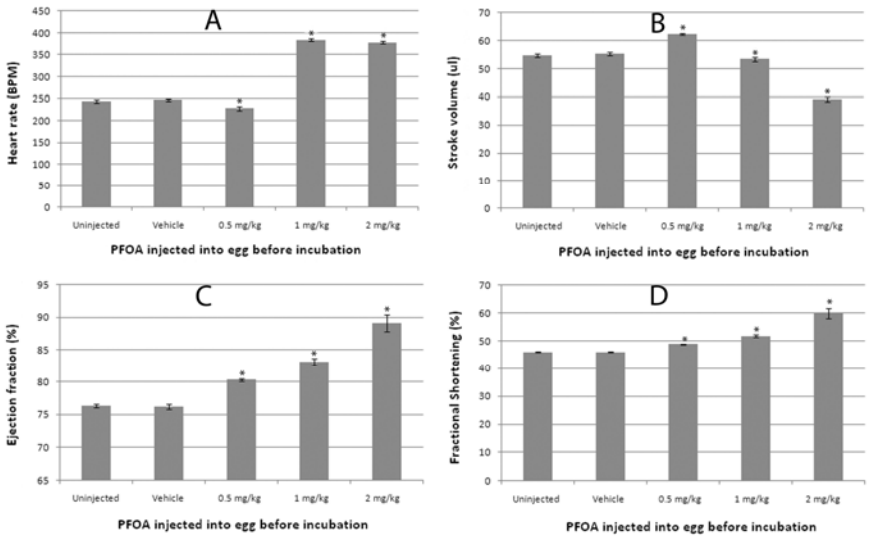


**Figure 4**. Cardiac ultrasound data on functional parameters of one-day-old hatchling chickens (N=9-12). Fertile chicken eggs were injected with 0, 0.5, 1 and 2 mg/kg PFOA per egg and incubated until hatch. By post hatch day 1, hatchling chickens were assessed by echocardiography as described in methods section.

A: Heart rate of one-day-old hatchling chickens measured by echocardiography.

B: Stroke volume of one-day-old hatchling chickens measured by echocardiography.

C: Ejection fraction of one-day-old hatchling chickens measured by echocardiography.

D: Fraction shortening of one-day-old hatchling chickens measured by echocardiography.

*: Significantly different from vehicle control group ($P<0.05$).

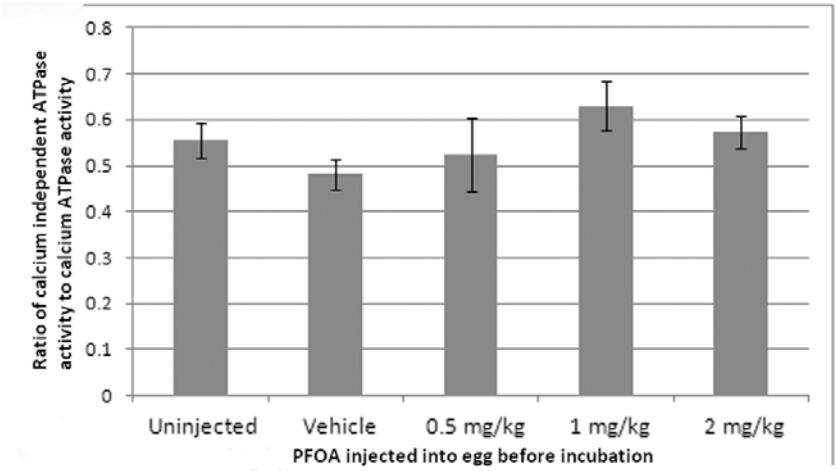



**Figure 5**. Cardiac myofibril ATPase activity data on one-day-old hatchling chicken hearts. After echocardiography assessment was finished, PFOA-exposed one-day-old hatchling chickens were decapitated, hearts were removed for cardiac myofibril ATPase activity assay. For detail, see methods section. Graph shows the ratio of cardiac myofibril calcium independent ATPase activity to calcium ATPase activity. (N=5 for uninjected group, 7-9 for other groups.).

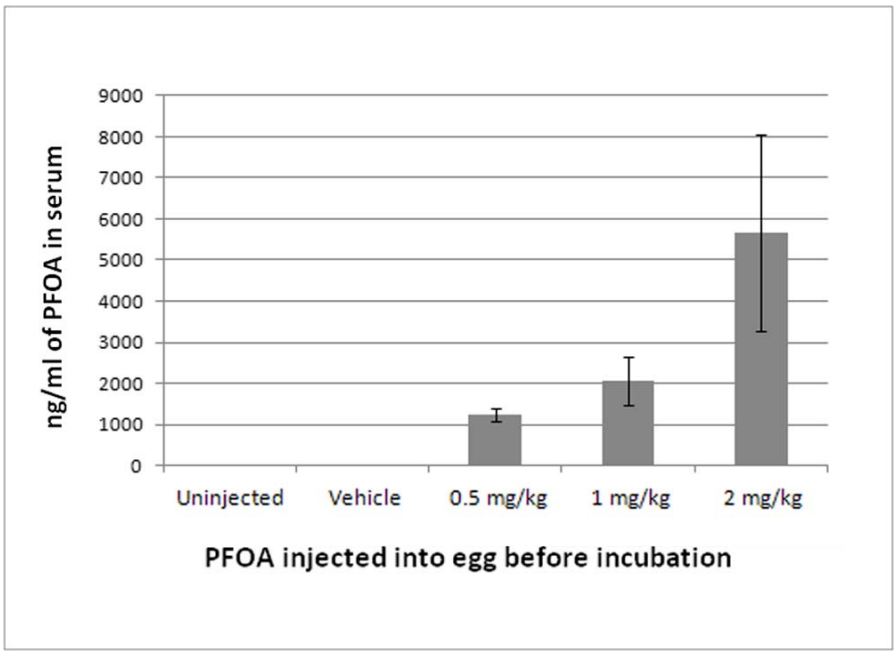



**Figure 6**. Serum PFOA concentrations measured in hatchling chickens. With the method used to quantify PFOA concentration, the limit of quantitation (LOQ) was 50 ng/mL. All samples from uninjected and vehicle groups are below LOQ and are reported as 0 in the graph.

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