



Article

Chronic Perfluorooctanoic Acid Exposure in Young Adult Zebrafish Affects Scale and Internal Skeleton Bone Metabolism, as Well as Offspring Bone Development

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Abstract

Perfluorooctanoic acid (PFOA) is one of the most extensively investigated per- and polyfluoroalkyl substances (PFAS), a class of highly fluorinated organic compounds widely used in consumer products. While assessing the long-term health effects of chronic PFOA exposure in humans remains challenging, simple animal models like zebrafish serve as essential tools to identify potential toxicological risks. The aim of this study was to investigate the chronic effects of PFOA on bone metabolism in young adult zebrafish, as well as the potential transgenerational impacts on their offspring. Fish fed a PFOA-enriched diet exhibited a significant decrease in body weight and length compared to the untreated control group. Explanted scales, which accumulated PFOA within the mineralized matrix, showed a reduced surface area, impaired osteoblastic activity, and strong osteoclastic activity localized in the central region of the scale. Furthermore, endoskeletal analysis revealed a series of malformations across multiple sites, including the vertebrae, ribs, fins, operculum, and branchial arches. The viability and general development of embryos derived from PFOA-treated parents showed no significant variations compared to controls, despite accumulating PFOA. Nevertheless, osteogenesis was specifically inhibited in the trunk of these PFOA-exposed embryos. Overall, this study highlights not only early perturbations in bone remodeling but also the transgenerational impact of PFOA on skeletal development in the offspring.

Keywords: zebrafish; bone; PFAS; PFOA



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1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are synthetic, highly fluorinated organic compounds that have been widely used in consumer products for decades. Because of the exceptional strength of their carbon–fluorine bonds, PFAS exhibit high resistance to

thermal, chemical, and biological degradation. Consequently, they are extremely persistent environmental pollutants, often referred to as “forever chemicals” [1]. PFAS have been detected globally in water, soil, and air. Although human exposure can occur through multiple pathways [1], dietary intake represents the primary source [2]. Furthermore, these compounds exhibit long half-lives once accumulated in human tissues [1]. Indeed, global biomonitoring of human blood samples has highlighted the ubiquitous presence of polyfluorinated compounds at ng/mL levels [3].

Perfluorooctanoate (PFOA) is one of the most extensively investigated PFAS. Unlike other persistent organic pollutants, PFOA is highly water-soluble, leading to substantial chronic toxicity in aquatic organisms and in humans via drinking water and fish consumption [4]. Following exposure, PFOA is rapidly absorbed through the lungs, gastrointestinal tract, and skin, and is distributed to the blood, liver, kidneys, and bones, with an elimination half-life of approximately 4 years in humans [5]. Notably, PFOA and other PFAS have been detected in human rib samples collected during autopsies of men with a mean age of 56 years [6]. While assessing the long-term health effects of chronic PFOA exposure in humans remains challenging due to confounding factors, pollutants, and co-morbidities, animal models serve as essential tools to identify potential health risks.

In rodents, PFOA exposure has been associated with weight loss, altered lipid metabolism, and an increased incidence of various tumors [3]. This enhanced carcinogenicity is largely mediated by the activation of the peroxisome proliferator-activated receptor- α (PPAR- α) pathway [3]. By targeting and activating PPARs, PFAS modifies cell differentiation and bone development, ultimately leading to metabolic disorders and skeletal fragility. In particular, PFOA interacts with multiple transcription factors, including PPAR- α , PPAR- γ , and estrogen receptor α (ER α), thereby acting as an endocrine-disrupting chemical (EDC) with systemic adverse effects [7].

PPARs are nuclear receptors critical for lipid metabolism and cell differentiation, and they are expressed in both osteoblasts and osteoclasts. This widespread expression makes bone tissue a major target for PPAR agonists, including PFOA, which acts as a PPAR- α agonist [5], promoting osteoclastogenesis and subsequent bone loss [8]. Other PPAR agonists, such as bezafibrate, also alter bone resorption. Consistently, epidemiological data indicate a negative correlation between bone mineral density and perfluorooctane sulfonic acid (PFOS) in both sexes, whereas this negative association is limited to females for PFOA [5]. PFOA can further impair bone health by competing with calcitriol for binding with the vitamin D receptor (VDR). This competition induces structural alterations and receptor dysregulation, which alters the expression of vitamin D-responsive genes in osteoblasts and, ultimately, reducing the mineralization rate [9]. Overall, both in vivo and in vitro studies have firmly established bone tissue as a primary target site for PFOA accumulation [5].

A substantial body of epidemiological evidence demonstrates that PFAS adversely influence reproductive functions. Specific concern has been raised regarding prenatal exposure, as PFAS can cross the placental barrier and lead to adverse birth outcomes [5,10]. Because they interfere with endocrine processes, particularly during vulnerable developmental windows such as pregnancy and early life, PFAS are classified as EDCs that impair perinatal, neuronal, and metabolic development and compromise reproductive success [11]. For instance, prenatal exposure to PFAS is negatively associated with birth weight, length, and head circumference, with thyroid hormone disruption suspected as a mediating mechanism [10]. Furthermore, infants from mothers living near PFAS-contaminated water sites exhibit increased rates of preterm birth, birth weights below 1 kg, and higher first-year mortality [12].

The zebrafish (*Danio rerio*) represents an excellent model organism for studying skeletal alterations in both adult and embryonic stages [13] and has been widely utilized in PFAS toxicity screening [14]. Moreover, it serves as a valuable model for investigating the maternal–fetal transfer of PFOA, allowing researchers to evaluate transgenerational effects on early embryonic stages, with a specific focus on skeletal development [15].

Therefore, the aim of this study was to investigate the chronic effects of PFOA on bone tissue development and metabolism in young adult zebrafish, as well as the potential transgenerational impacts on their offspring.

2. Results

2.1. PFOA Treatment Affects Young Adult Fish Growth

Four-month-old zebrafish of both sexes were fed a diet containing PFOA for 10 weeks. At the end of the exposure period, PFOA-treated fish (PFOA) exhibited a significant decrease in growth rate (Figure 1A), showing a 35% reduction in body weight (Figure 1B) and a 14% reduction in body length (Figure 1C) compared to the untreated control group (CTL). No sex-dependent differences were detected.

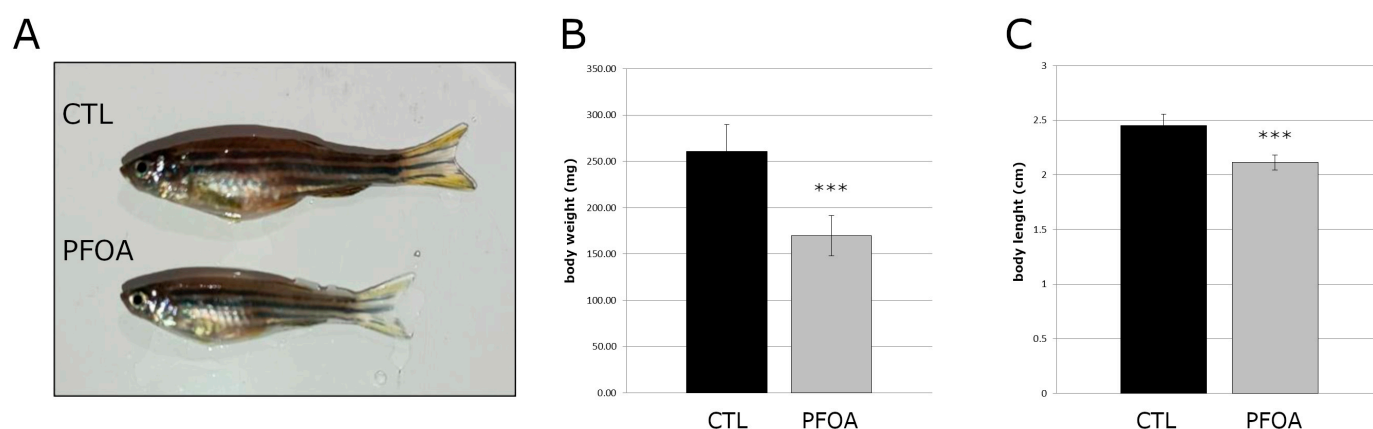


Figure 1. (A) Images of CTL and PFOA fish that show decreased body size. (B) Body weight of treated fish resulted in −35% compared to the CTL (PFOA vs. CTL, $p < 0.001$). (C) Body length of treated fish resulted in −14% compared to the untreated fish (PFOA vs. CTL, $p < 0.001$ (***)).

2.2. PFOA Exposure Modulates Bone Metabolism in Adult Zebrafish Scales

Explanted scales were used to evaluate alterations in bone development in PFOA-treated fish. First, we confirmed that scales from treated fish had accumulated PFOA within the mineralized matrix. Mass spectrometry analysis confirmed the presence of a significant amount of PFOA in the scales of treated fish (Figure 2A).

Whole-scale calcein staining was utilized to identify growth defects or resorbed regions within the mineralized matrix of the scale. A 41% reduction in surface area was observed in scales from PFOA-treated fish (Figure 2B). Detailed analysis of the mineralized matrix revealed fewer circuli in the scales of PFOA-treated fish—a phenomenon underlying the impairment of scale growth—as well as the presence of resorption lacunae localized within the central region of the scale (Figure 2C).

To assess whether the lacunae in the mineralized matrix resulted from osteoclastic resorption, we performed histochemical and biochemical analyses of TRAP enzymatic activity—a well-established marker of osteoclastic activity—in the scales of PFOA-treated fish and untreated controls. Histochemical analysis revealed intense TRAP staining in 50% of the PFOA-treated scales. In contrast, this activity was negligible in untreated controls (Figure 3A). Interestingly, this resorbing activity was localized exclusively within the central

region of the scale (Figure 3B). Biochemical analysis of TRAP activity further confirmed these findings (Figure 3C).

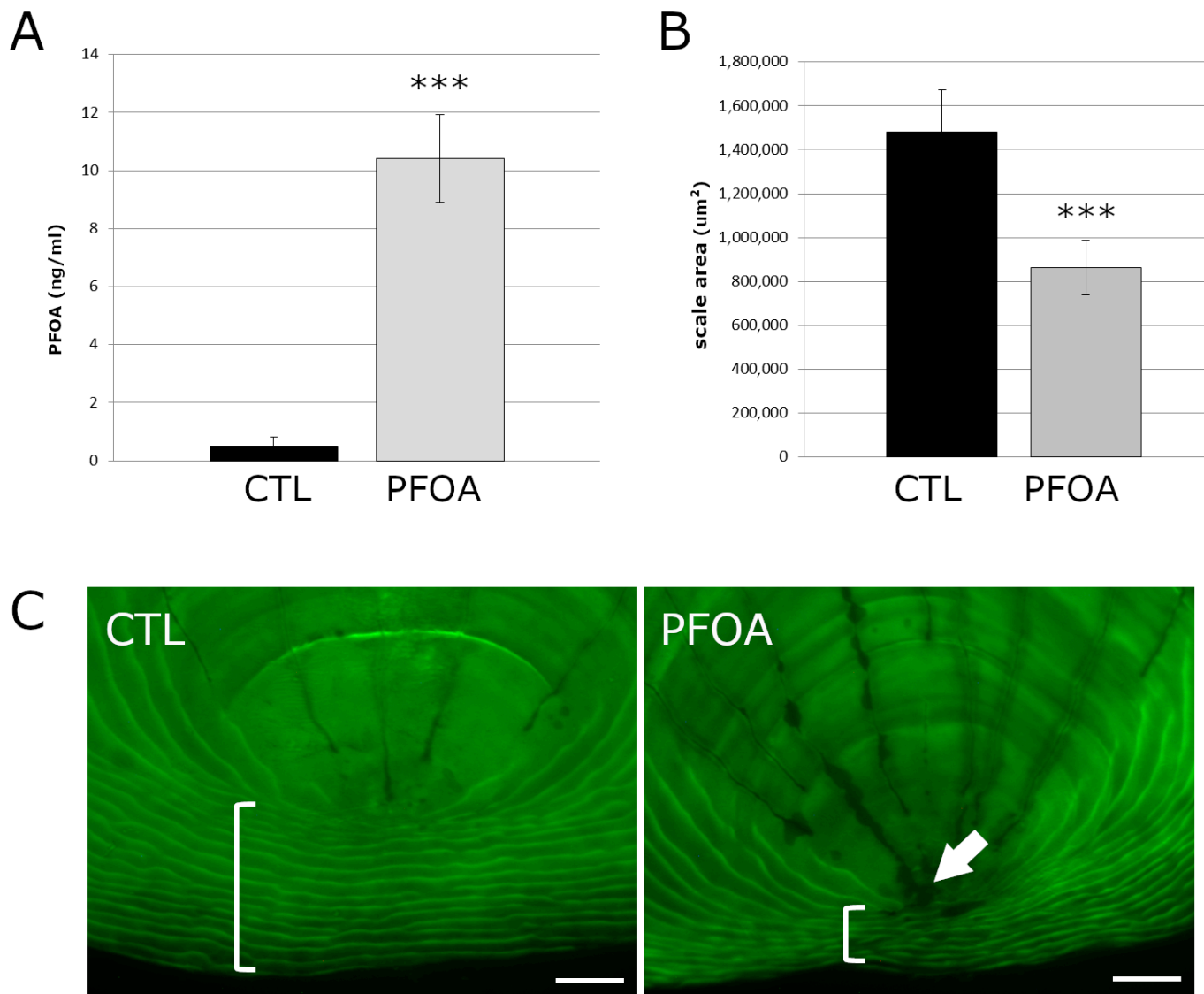


Figure 2. Scale analysis from PFOA fish and controls (CTL). (A) Mass spectrometry analysis confirmed PFOA accumulation in scales from treated fish compared to controls (PFOA vs. CTL, $p < 0.001$ ***). (B) Measurement of the total scale area (in μm^2) revealed a 41% reduction in the PFOA group (PFOA vs. CTL, $p < 0.001$ ***). (C) Representative images of calcein staining of the scale from PFOA and CTL. Resorption sites are visible at the center of PFOA-treated scale (white arrows), accompanied by a reduced number of the growth circuli (white brackets) compared to controls (scale bar: 0.1 mm).

In addition, osteoblastic behavior on scales was evaluated using an ALP activity assay. PFOA-treated scales exhibited impaired ALP activity across all samples tested, as demonstrated by histochemical staining (Figure 4A). A 46% reduction in enzymatic activity was subsequently quantified in the PFOA-treated group via biochemical assay (Figure 4B).

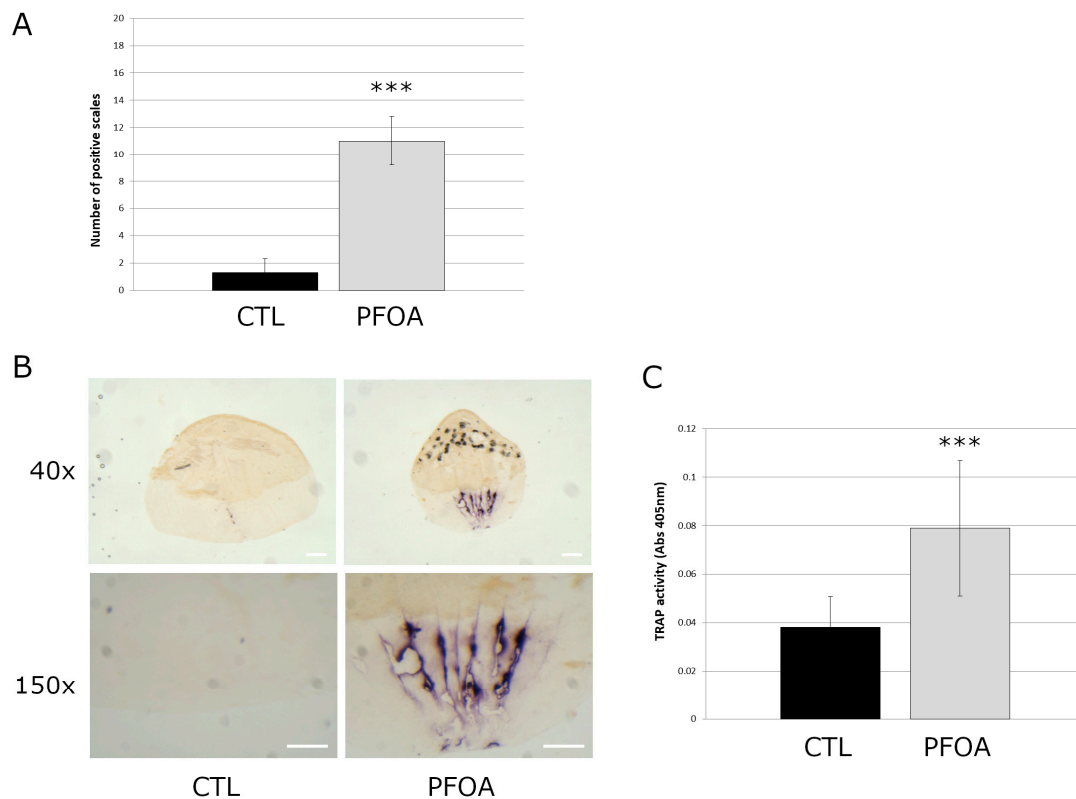


Figure 3. Histochemical TRAP assay in scales from CTL and PFOA fish. (A) TRAP activity is detected in 50% of scales from PFOA-treated fish compared to untreated controls. (PFOA vs. CTL, $p < 0.001$ (***) (B) In TRAP-positive scales, active osteoclasts are exclusively localized in the central region of scales from PFOA-treated fish, as indicated by purple staining (magnification: 40 $\times$, upper panel; 150 $\times$, lower panel) (scale bar: 0.1 mm). (C) Biochemical TRAP assay confirms increased resorption activity in PFOA-treated scales (PFOA vs. CTL, $p < 0.001$ (***)).

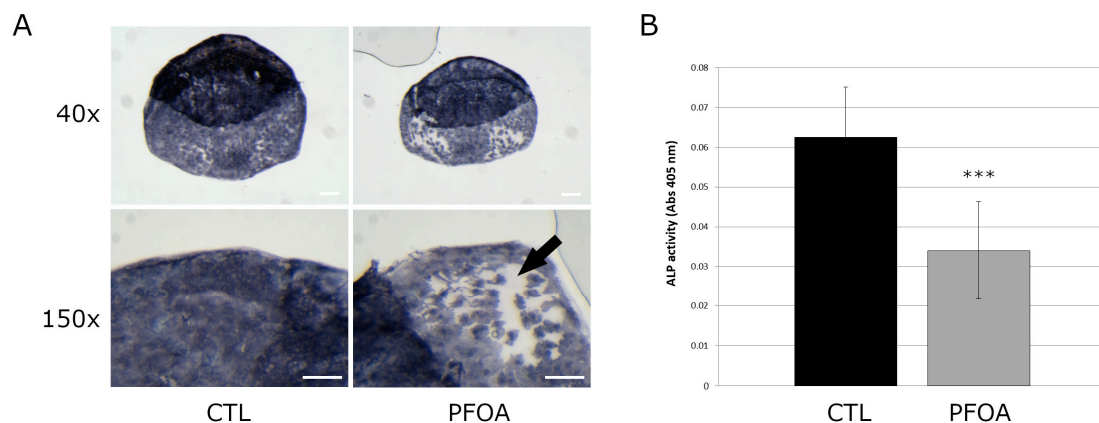


Figure 4. Assessment of ALP activity in CTL and PFOA fish scales. (A) Histochemical staining of ALP activity appears in blue, revealing white areas that indicate an absence of activity (black arrow) in all scales from PFOA fish (magnification: 40 $\times$, upper panel; 150 $\times$, lower panel) (scale bar: 0.1 mm). (B) These findings are confirmed by the biochemical ALP assay, which quantified a 46% reduction in enzymatic activity in PFOA scales compared to controls (PFOA vs. CTL, $p < 0.001$ (***)).

2.3. PFOA Exposure Perturbs Endoskeletal Growth and Remodeling

The analysis of the internal skeleton has been performed using Alizarin Red S fluorescent staining and highlights a series of alterations in the bone structures at different sites.

All vertebrae from PFOA-treated fish exhibited impaired growth and altered morphology compared to untreated controls. Specifically, major defects were detected in the neural channel as well as in both hemal and neural arches (Figure 5). Bone structures surrounding the neural channel appeared resorbed, whereas the arches were shorter and displayed heterogeneous thickness. These alterations worsened caudally, with the last vertebrae exhibiting the most severe phenotype.

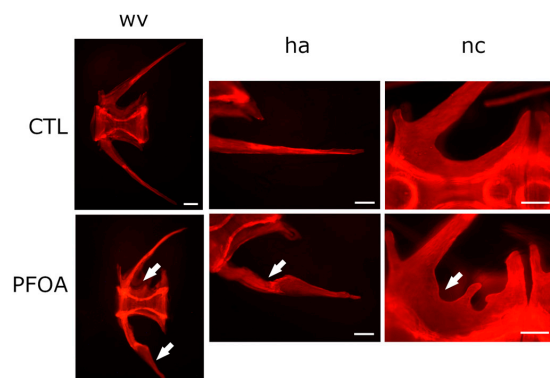


Figure 5. Alizarin Red S fluorescence imaging of explanted caudal vertebrae from PFOA and CTL. Structural impairments are highlighted by white arrows in the whole vertebra (wv, 30 $\times$). Higher magnification reveals alterations in the growth and morphology of the hemal arch (ha, 60 $\times$) and neural channel (nc, 168 $\times$) in PFOA samples (scale bar: 0.1 mm).

PFOA treatment also induced morphological alterations in the fin radials, particularly in the dorsal (Figure 6) and anal fins. These alterations affected both the size and shape of the apical region of the radials and led to the formation of anomalous bifurcations at the distal ends of the neural arches of the corresponding vertebrae.

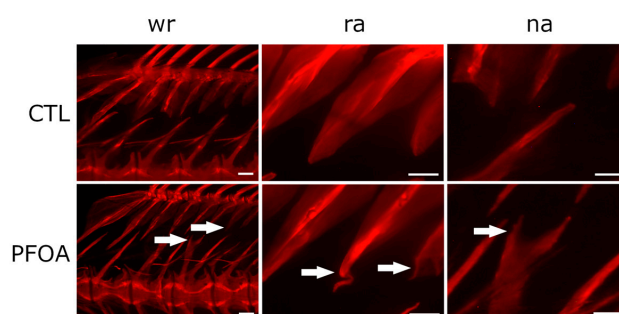


Figure 6. Alizarin Red S fluorescence imaging of radials of the dorsal fin from PFOA-treated fish and controls (CTL). Structural impairments of radials and corresponding neural arches are highlighted by white arrows (wr = whole radials, 20 $\times$). Higher magnification reveals alterations in size and morphology of the radials (ra, 120 $\times$) and neural arches (na, 120 $\times$) in PFOA samples (scale bar: 0.1 mm).

Bone alterations in PFOA-treated fish were also observed within the operculum (Figure 7), characterized by size reduction, and altered morphology along its margins and in the articular site. Still, within the cranial region, the pharyngeal arches displayed developmental defects and structural discontinuities (Figure 7).

Severe morphological abnormalities and altered thickness were observed in the ribs of the PFOA-exposed group (Figure 8A). To determine whether these skeletal alterations were associated with osteoclastic remodeling, isolated ribs were subjected to histochemical TRAP staining. Elevated resorption activity was prominently detected in PFOA samples along both the proximal and distal segments (Figure 8B), with an increase of 21% and 30%, respectively, if compared to controls (Figure 8C).

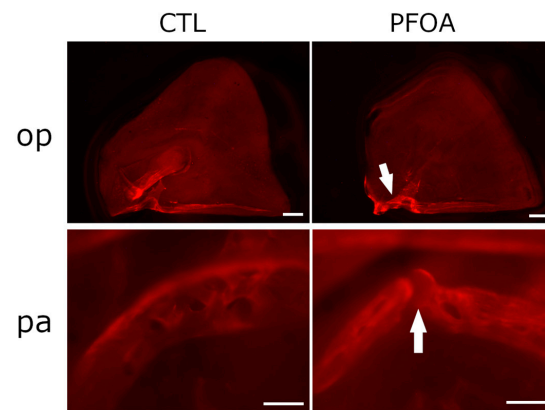


Figure 7. Alizarin Red S fluorescence microscopy of the operculum (op; upper panel) and pharyngeal arch (pa; lower panel) in CTL and PFOA-treated fish. In the operculum, the PFOA group displays structural and morphological abnormalities, specifically within the joint area (white arrow) (scale bar: 0.1 mm). In pharyngeal arches, an altered morphology is evident in the PFOA group, with matrix fragmentation indicated by the white arrow (scale bar: 0.1 mm).

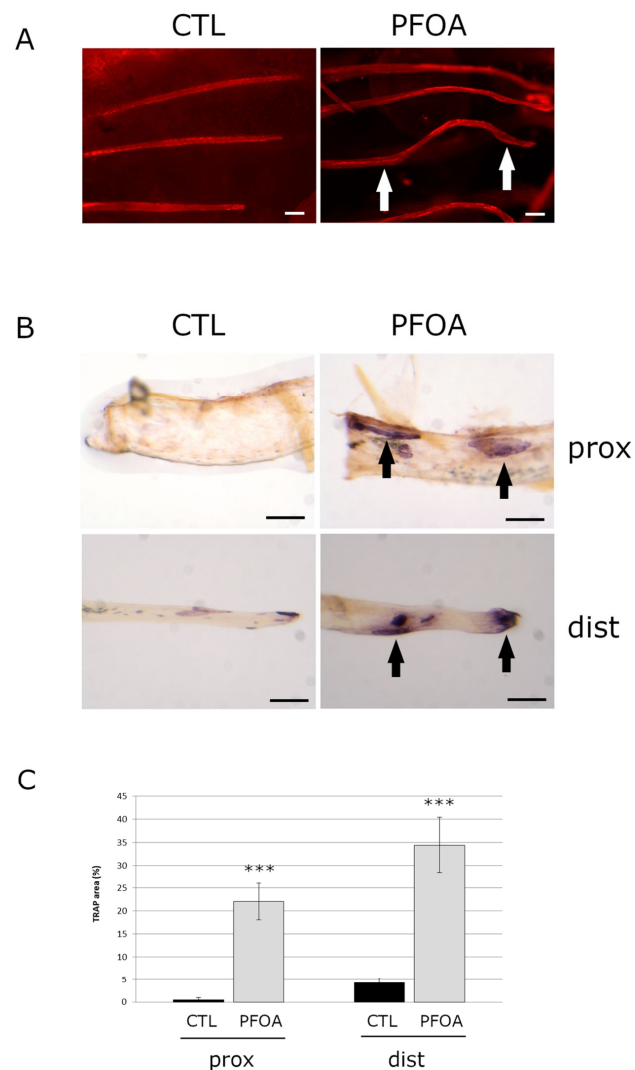


Figure 8. Analysis of ribs from CTL and PFOA zebrafish. (A) Alizarin Red S fluorescence revealed altered morphology in the ribs of the PFOA-exposed group. (B) Histochemical TRAP analysis shows, in purple, high resorption activity in both the proximal (prox) and distal (dist) regions of the rib in PFOA-treated fish (scale bar: 0.1 mm). (C) Quantification of TRAP staining area in both the proximal (prox) and distal (dist) regions (PFOA vs. CTL, $p < 0.001$ (***)).

2.4. Embryos from Adult PFOA-Treated Fish Show Impaired Osteogenesis in the Trunk

After 10 weeks, both CTL and PFOA fish were mated to evaluate the maternal transfer capability of PFOA to the progeny and its influence on vitality and skeletal development. The viability of embryos derived from PFOA-treated parents showed no significant variations compared to the controls (Figure 9A). To confirm that the fertilized eggs and the embryos had accumulated PFOA from the treated parents, a quantitative mass spectrometry analysis was performed. Both fertilized eggs (Figure 9B) and embryos (Figure 9C) produced by treated parents contained great amounts of PFOA compared to controls from untreated parents.

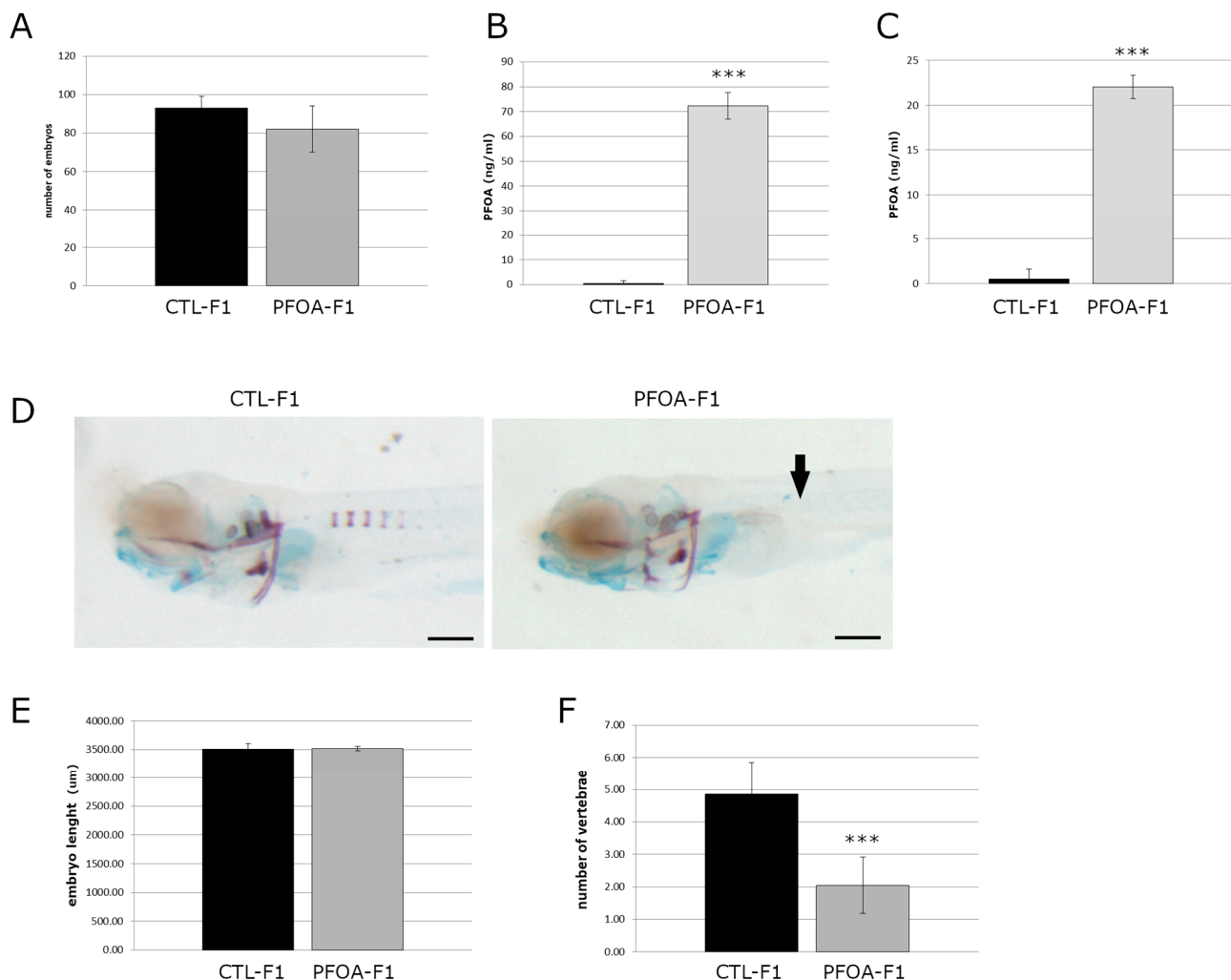


Figure 9. F1 embryo analysis. (A) Viability of embryos (number of embryos) derived from control (CTL-F1) and PFOA-F1 parents showed no significant differences. Quantitative mass spectrometry analysis of PFOA accumulation in (B) fertilized eggs and (C) embryos, displaying a significant increase in the PFOA-F1 group compared to the CTL-F1 group (PFOA-F1 vs. CTL-F1, $p < 0.001$ (***)). (D) Representative images of vital staining for cartilage and bone at 5 dpf; the black arrow indicates a delay/reduction in vertebral mineralization in PFOA-F1 embryos, while cartilaginous structures and cranial bones remain unaltered (scale bar: 200 µm). (E) Total embryo length (µm) indicates that overall development is unaffected between groups. (F) Quantification of the number of mineralized vertebrae, showing a specific 58% inhibition of trunk osteogenesis in PFOA-F1 embryos compared to CTL-F1 controls (PFOA-F1 vs. CTL-F1, $p < 0.001$ (***)). Data are expressed as mean $\pm$ SD.

Skeletal development was evaluated at 5 days post-fertilization (dpf) using vital staining for cartilage and bone, revealing a delay in vertebral mineralization in PFOA-

containing embryos. In contrast, no significant morphological variations were observed in cartilaginous structures or cranial bones (Figure 9D). General development appeared unaltered, as confirmed by the unchanged embryo length (Figure 9E), whereas osteogenesis was specifically inhibited by 58% in the trunk of PFOA-containing embryos compared to controls (Figure 9F).

3. Discussion

Our study highlights that PFOA treatment on young zebrafish induces perturbations in bone remodeling of scales and internal skeleton, but, also, the transgenerational impact of PFOA on skeletal development in PFOA-treated fish offspring. As previously reported by Jantzen et al., embryos of adult fish fed with PFOA were significantly smaller both in weight and length [16]. Our study confirms these findings within a shorter treatment window restricted to young adulthood, a stage where the skeleton is already formed, undergoing maturation, and requiring extensive remodeling. While transient exposure limited to the embryonic stage allows for the recovery of adult body length and weight, it adversely affects reproduction and skeletal development in the subsequent generation [16].

The observed reduction in size does not appear to result from reduced caloric intake or neurological impairment, as no hypophagia or abnormal behaviors were observed in the treated fish. Instead, altered bone growth and remodeling represent the most plausible hypothesis. Indeed, PFOA has been shown to retard skeletal development and induce skeletal malformations in mice [10] as well as in zebrafish embryos [14].

Subsequently, we utilized the fish scale as a model to evaluate alterations in bone metabolism during PFOA exposure. First, we confirmed that scales from treated fish contained PFOA, which had evidently accumulated within their anatomical, predominantly osseous, structures. Indeed, PFAS and PFOA have been shown to readily accumulate in bone tissue and persist over extended periods, as documented in human remains [17] and rodent models [5].

PFOA exposure significantly reduced scale surface area compared to controls, consistent with the overall decrease in fish length and weight, likely reflecting decelerated growth. Typically, fish scale growth occurs via the deposition of concentric rings of mineralized matrix (circuli) mediated by specialized osteoblasts [18].

Analysis of the circuli within the anterior region of PFOA-treated scales showed a marked reduction in both count and thickness relative to controls. Consequently, PFOA exposure appears to downregulate the secretory activity of scale osteoblasts. This finding was validated by measuring osteoblastic alkaline phosphatase (ALP) activity, which was significantly suppressed in the PFOA group. These data align with recent literature showing that PFOA impairs both the osteogenic differentiation of stem cells and the mineralization capacity of mature osteoblasts in vitro [19].

In vivo rodent studies demonstrated that PFOA accelerates osteoblast senescence, resulting in long bone shortening and decreased bone mineral density [10]. Consistently, a delay in osteoblast differentiation and subsequent impairment of operculum formation has been highlighted in zebrafish larvae [20].

Beyond decelerated bone growth, PFOA-exposed scales displayed resorption lacunae affecting the mineralized matrix of the radii within the anterior focus. These resorption events correlated with robust osteoclastic TRAP activity in roughly 50% of the examined scales, while being negligible in the control group. Mechanistically, PFOA has been reported to trigger in vitro osteoclastogenesis via both the suppression of early osteoblast differentiation in co-cultures and the direct activation of osteoclast progenitors [21]. Consistently, further experiments in rodents demonstrate that PFAS accumulate in the bone

marrow and directly stimulate osteoclastogenesis, a mechanism presumably mediated by PPAR γ [8].

These data validate that PFOA stimulates osteoclast activation, while the focal nature of the resorption events points toward an osteoblast-independent mechanism. Consistent with this hypothesis, most osteoclast progenitors are known to compartmentalize at the outer scale margin, a region characterized by differentiating and actively secreting osteoblasts [22], which produce microvesicles that modulate the RANKL/RANK ratio [23]. The focal nature of scale resorption in PFOA-exposed fish suggests that chronic chemical stress triggers a pathway resulting in the long-term, direct activation of osteoclast progenitors within the dermal pocket near the focus. Consistently, prior findings from our laboratory demonstrated that focal scale resorption characterizes the chronic phase of an adult zebrafish inflammatory bowel disease (IBD) model; conversely, marginal resorption, which is mediated by functional osteoblasts, subsided following the acute phase [24].

PFOA-induced skeletal anomalies extend beyond the scale, involving other components such as vertebrae, ribs, neural/hemal spines, and the operculum. These malformations likely result from a combination of growth defects and disrupted remodeling, demonstrated by the emergence of aberrant osteoclastic activity in the ribs of exposed fish. Intriguingly, such internal skeletal alterations in adult zebrafish are characteristically prominent only in aged individuals [25]. Similar phenotypes have been reported in specific genetic models of skeletal disorders, such as the *Chihuahua* zebrafish [26], and in fish subjected to severe thyroid hormone disruptions during growth [27].

The adult zebrafish skeleton undergoes dynamic remodeling to accommodate both physiological and pathological changes. For example, increased mechanical loading through controlled exercise triggers adaptive bone ossification [28]. In a pathological context, prednisolone treatment in adult zebrafish reduces the bone mineral density of the vertebral centra [29]. Intriguingly, these skeletal disruptions manifest at the precise anatomical landmarks identified in our work.

Secondly, our interest was in studying the effects of chronic PFOA exposure in the offspring of these fish. PFOA administered to pregnant mice leads to increased neonatal mortality and reduced growth for the surviving offspring [3]. PFOA also has specific effects on bone tissue of mice offspring, as it has been demonstrated that mice born from pregnant females orally exposed to PFOA throughout the entire pregnancy show increased femoral periosteal area and decreased tibial mineral density. The same study demonstrated that PFOA accumulates in the bone tissue of mice offspring of females treated during pregnancy, both by in utero exposure and lactation, and remains present until old age, with PFOA-induced alterations in bone geometry and mineral density that persist through the entire life of the mice [5]. Another study analyzed the offspring of mice treated with PFOA during pregnancy, highlighting both maternal and fetal toxicity with abnormal growth characterized by general delay of the development, with abnormalities including reduced ossification of different bones, including caudal vertebrae [30]. Our study on PFOA-treated zebrafish offspring confirms the transmissibility of PFOA to offspring and its negative effects on osteogenesis. Despite the significant decrease in vertebral mineralization, we did not detect differences in the growth or survival rate of embryos from PFOA-treated fish.

Given that trunk ossification in zebrafish larvae occurs via intramembranous mechanisms, generated by the osteogenic differentiation of progenitor stem cells, we postulate that embryonically accumulated PFOA predominantly targets osteoblast differentiation within the vertebral centra. Supporting this hypothesis, endochondral ossification during cranial development in PFOA-F1 embryos remains largely unaffected. Notably, the literature confirms that PFOA suppresses essential osteoblastic differentiation markers

in vivo in zebrafish larvae, including *runx2* and *col10a1a* [31], and shifts the fate decision of mesenchymal stem cells toward adipogenesis [32].

4. Materials and Methods

4.1. Ethic Statement

This experimentation was performed in the Zebrafish Laboratory (IRCCS Ospedale Galeazzi Sant'Ambrogio, Milan, Italy) according to Italian and European guidelines on research (EU Directive 2010/63/EU) and following authorization by ASL Varese with Prot. No. 2019/014/DVVS/0078143, Italy. Authorization by ethic committee OPBA-FIIRV n.05/2021 on 15 June 2021.

4.2. Animals

Danio rerio (zebrafish) of AB strain were maintained in a ZEBTEC[®] benchtop system (Tecniplast spa, Buguggiate, Italy) under standard conditions [33]. Both adult fish and embryos were maintained at 28 °C in an E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄) during the treatments.

4.3. Treatments

Four-month-old zebrafish were treated with perfluorooctanoic acid (PFOA, Sigma Aldrich, St. Louis, MO, USA) for 10 weeks. PFOA was dissolved in 95% EtOH to obtain 1 µg/mL solution, then was added to a specific amount of standard fish food (Vipagran, Sera GmbH, Heinsberg, Germany) and left to evaporate for 2 h in order to allow the ethanol to evaporate and to give the fish a PFOA dosage equal to 0.3 mg/kg/day [5]. CTL fish were fed with standard fish food given in the same quantity.

Embryos were obtained from both CTL and PFOA fish 10 weeks after the start of the treatment. Embryos from CTL fish were maintained in E3 medium. All embryos were raised to 5 days post-fertilization (dpf).

4.4. Scale Analysis

Adult fish were euthanized by rapid cooling. Fish bodies were measured for length and weight. Scales were removed from either side of fish bodies using a Dumont[®] stainless steel forceps (Sigma Aldrich, St. Louis, MO, USA) under a light stereomicroscope (Olympus SZX-ZB7, Olympus, Tokyo, Japan) and processed differently depending on the analysis to be performed.

For each fish, 20 scales were stained using 0.005% calcein (Bis[*N,N*-bis(carboxymethyl)aminomethyl] fluorescein, Sigma Aldrich, St. Louis, MO, USA) E3 solution [34], repetitively washed and analyzed using a fluorescence microscope (Olympus SZX-ZB7, Olympus, Tokyo, Japan) equipped with a Discovery CH30 camera (TiEsseLab, Milan, Italy).

Scale histological alkaline phosphatase (ALP) activity were analyzed using BCIP[®]/NBT liquid substrate (Sigma Aldrich, St. Louis, MO, USA), and tartrate-resistant acid phosphatase (TRAP) activity was evaluated using Leukocytes acid phosphatase (TRAP) detection kit (Sigma Aldrich, St. Louis, MO, USA); in both cases, the scales were processed according to manufacturer's protocol.

Biochemical ALP analysis was performed according to our method [35] and biochemical TRAP analysis was performed using a test previously described by Persson et al. [36]. Raw data from biochemical analysis were normalized using scale area-based measurements. To perform both these analyses, absorbance was read at $\lambda = 405$ nm using a spectrophotometer (iMark[™] Microplate Reader, Bio-Rad, Hercules, CA, USA).

4.5. Skeletal Analysis

Fish bodies were processed as described by Helland [37] to make them transparent and, through Alizarin Red S staining, to visualize the internal skeleton. Images were acquired using a fluorescence microscope (Olympus SZX-ZB7, Olympus, Tokyo, Japan). Ribs are the only components of the internal skeleton that can be isolated from the fish body and analyzed without using a protocol to make the fish body transparent, so a histological TRAP assay was performed.

4.6. Embryo Histochemical Analysis

CTL and PFOA-F1 were raised to 5 days post-fertilization, then fixed in 3.5% formaldehyde 0.1 M sodium phosphate-buffered solution and processed using a two-color acid-free staining [38] in order to evaluate osteogenesis. The staining uses Alcian blue 8GX (Sigma Aldrich, St. Louis, MO, USA) and Alizarin Red S (ARS, Sigma Aldrich, St. Louis, MO, USA) to stain the cartilage and bone tissue, respectively. Osteogenesis was quantified by counting the number of mineralized vertebral bodies, normalized to the embryo body length (N.V./L). Embryos were analyzed under a light stereomicroscope (Olympus SZX-ZB7, Olympus, Tokyo, Japan) acquiring images using a Discovery CH30 camera (TiEsseLab, Milan, Italy).

4.7. Statistics

Twenty fish were used: 5 male and 5 female fish as CTL, 5 male and 5 female fish treated with PFOA. The entire experiment was repeated 3 times with comparable results, using a total amount of 60 fish. For each analysis performed on scales, 20 scales from each fish were used, for a total of 200 scales analyzed for each histological staining, biochemical assay, or calcein staining. All the fish bodies were used to perform internal skeleton analysis.

Embryos tests were performed on 10 embryos per analyzed point, and the entire experiment was repeated at least 3 times with comparable results.

Data derived from each assay are expressed as mean $\pm$ standard deviation versus control. Statistical significance for p -values was determined using Student's t -test analysis. All significance values were set at less than $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***), and all the experiments were repeated at least three times with comparable results.

4.8. Extraction and LC-MS/MS Analysis of PFOA from Zebrafish Tissues

Zebrafish tissues were collected following sacrifice and immediately dissected. Tissue samples were briefly rinsed with ultrapure water, frozen, and stored at -80 °C until analysis. Before extraction, samples were thawed on ice.

For extraction, 1 mL of methanol containing 0.1% ammonium hydroxide was added to each tissue sample. Samples were homogenized using an ultrasonic horn until complete tissue disruption was achieved. Proteins were precipitated by adding acetonitrile to the collected supernatant at a 1:1 v/v ratio. Samples were vortex-mixed for 1 min and centrifuged at 12,000 g for 10 min. The clarified supernatant was collected, and the extraction was repeated by adding 0.5 mL of 1:1 v/v methanol to the remaining pellet. After vortexing and centrifugation under identical conditions, the supernatants were combined and evaporated to dryness under a gentle stream of nitrogen at 40 °C. The dried residue was reconstituted in 100 μ L of methanol:H₂O (50:50 v/v) containing 2 mM ammonium acetate. Samples were vortex-mixed, centrifuged if necessary to remove residual particulate matter, and transferred to HPLC vials for instrumental analysis. PFOA analysis was performed by HPLC-MS/MS using a Kinetex C18 column from Phenomenex Inc. (Phenomenex Inc., Torrance, CA, USA) The mobile phases consisted of 2 mM ammonium acetate in water as

solvent A and methanol as solvent B. Chromatographic separation was achieved using a gradient starting at 50% B and increasing to 95% B over 2 min, followed by a 4 min hold at 95% B, then re-equilibration to the initial conditions. Mass spectrometric detection was performed in negative electrospray ionization mode. Full-scan acquisition was performed on an Orbitrap mass analyzer (LTQ Orbitrap Velos pro, Thermo Fisher Scientific, Waltham, MA, USA) over the m/z range 400–420 to monitor the accurate mass region corresponding to PFOA. In parallel, selected reaction monitoring was used for targeted confirmation and quantification of PFOA, monitoring the transition from m/z 413 to 369, with m/z 169 used as an additional fragment ion for confirmation.

Blank controls were included throughout the analytical workflow to evaluate potential background contamination. Although PFAS-free certified plasticware was not used, blank samples confirmed the absence of detectable PFOA contamination under the applied analytical conditions. PFOA concentrations were calculated from the instrumental response and normalized to tissue weight.

5. Conclusions

Overall, this study elucidates the detrimental effects of chronic PFOA exposure on the adult zebrafish skeleton. Crucially, it highlights not only immediate disruptions in bone remodeling but also the transgenerational impact of PFOA bioaccumulation in germ cells, resulting in distinct skeletal abnormalities in the F1 generation. Future research will focus on dissecting the precise molecular pathways responsible for these pathologies in both adults and offspring.

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