

## Uptake and Depuration of Four Per/Polyfluoroalkyl Substances (PFASs) in Northern Leopard Frog *Rana pipiens* Tadpoles

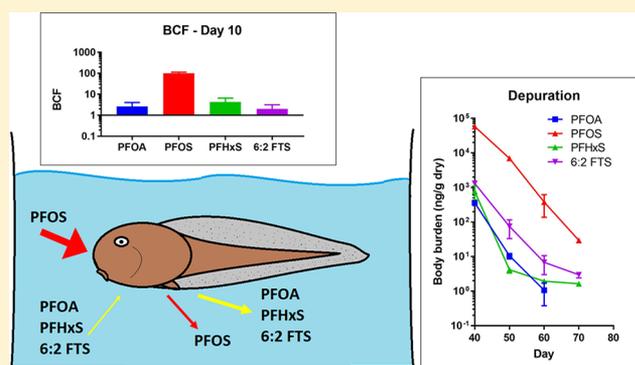
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### S Supporting Information

**ABSTRACT:** Per/polyfluoroalkyl substances (PFASs) are persistent organic contaminants that are ubiquitous in surface waters. To date, their effects on aquatic systems, especially amphibians, are poorly understood. We examined the uptake and depuration of perfluorooctanesulfonate (PFOS), perfluorohexanesulfonate (PFHxS), perfluorooctanoic acid (PFOA), and 6:2 fluorotelomer sulfonate (6:2 FTS) in northern leopard frog (*Rana pipiens*) tadpoles. Whole-body concentrations were examined every 10 d during constant aqueous exposure to targeted concentrations of 10, 100, and 1000  $\mu\text{g/L}$  for 40 d and for 30 d during depuration. Effects of PFAS exposure on length and development were also examined. Rapid uptake led to steady state concentrations by 10 d for most exposures. PFOS accumulated to the highest levels with whole-body bioconcentration factor (BCF) values at 40 d ranging from 19.6 to 119.3. The remaining PFASs were not found to bioconcentrate (BCF < 1.0 at 40 d). Furthermore, some BCF values decreased during the exposure phase, suggesting dilution due to growth and/or changes in toxicokinetics over ontogeny. During depuration, half-lives ranged from 1.2 to 3.3 d for all compounds. All PFASs tended to induce developmental delays, though statistical significance was only seen for PFOS and PFHxS. These sublethal effects observed at environmentally relevant concentrations are concerning and merit further study.



## INTRODUCTION

Per/polyfluoroalkyl substances (PFASs) have been used in a variety of industries and commercial products, along with use as a component of hydrocarbon fire-fighting foams, which has led to their global distribution.<sup>1–3</sup> Contamination of both surface waters and groundwater with PFASs has been well documented, with perfluorooctanesulfonate (PFOS) concentrations reaching 121 ng/L in Lake Ontario,<sup>4</sup> 226 ng/L in the Mississippi River,<sup>5</sup> and 120  $\mu\text{g/L}$  in a well from the impacted Federal site of Wurtsmith Air Force Base.<sup>6</sup> The same well at Wurtsmith had many other PFASs at high concentrations including perfluorooctanoic acid (PFOA, 220  $\mu\text{g/L}$ ), perfluorohexanesulfonate (PFHxS, 260  $\mu\text{g/L}$ ), and 6:2 fluorotelomer sulfonate (6:2 FTS, 46  $\mu\text{g/L}$ ). Despite the prevalence of PFASs, accumulation and toxicity are poorly understood for some of these compounds.

The structure and chemistry of PFASs make them highly resistant to degradation, resulting in extensive potential for bioaccumulation and toxicity.<sup>7,8</sup> While bioconcentration and biomagnification of PFOS has been shown across multiple trophic levels,<sup>9</sup> these studies are missing for many other PFASs. Additionally, toxicity within aquatic systems is poorly understood. A few studies undertaken with model fish species, including zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*), describe toxicity, uptake, and bioaccumulation.<sup>10,11</sup> In zebrafish, toxicity is positively correlated with chain length as well as functional group, with sulfonates more toxic than carboxylates.<sup>11</sup>

Other aquatic taxa, especially amphibians, have been largely overlooked. Amphibians have an important role ecologically, utilizing both aquatic and terrestrial environments, providing an important prey base in each.<sup>12</sup> Amphibians are also frequently used as indicator species for ecosystem health.<sup>13</sup> Importantly for their study in conjunction with PFAS exposure, amphibian metamorphosis is under the control of thyroid hormone,<sup>14</sup> one of the systems most commonly identified as a target of PFAS toxicity.<sup>15,16</sup>

The primary objective of this study was to examine the uptake and depuration kinetics of four PFASs in northern leopard frogs, *Rana pipiens*. We investigated the differences in kinetics based on exposure concentration (10, 100, and 1000  $\mu\text{g/L}$ ) and chemical, with special attention to the time to steady state concentrations. We defined steady state as the time after

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which there is no statistically significant increase in body burden. Also, given the potential influence of PFASs on amphibian metamorphosis, we tested for sublethal effects of exposure on tadpole development and size. We hypothesized that longer chain lengths and the presence of a sulfonate group versus a carboxylate would lead to higher accumulation and increased toxicity as was observed in zebrafish embryos by Ulhaq et al.<sup>11</sup>

## ■ EXPERIMENTAL SECTION

**Study Animals and Husbandry.** Eight northern leopard frog egg masses were collected during early spring (March 2016) from a temporary pond at the Purdue Wildlife Area in West Lafayette, IN, and randomly assigned to outdoor ~100 L wading pools. After hatching, larvae were checked daily for mortality and fed Purina Rabbit Chow *ad libitum*.

**Chemicals and Stock Solutions.** All perfluoroalkyl substances were purchased from Sigma-Aldrich (PFOA 96%, PFOS and PFHxS  $\geq$  98%); 6:2 FTS was purchased from SynQuest Laboratories. For chemical analyses, mass-labeled M8-PFOA, M8-PFOS, M3-PFHxS, and M2-6:2 FTS solutions purchased from Wellington Laboratories were used. Stock solutions consisted of 1 g of chemical dissolved in 1 L (PFOA, PFHxS), 2 L (PFOS), or 5 L (6:2 FTS) of Milli-Q water, then vacuum-filtered (Whatman, ashless, grade 40) before storage in polycarbonate bottles. We adjusted PFOA and 6:2 FTS stock solutions to pH 6.95–7.05 with sodium hydroxide.

**Experimental Design and Procedures.** Treatments consisting of control and exposure to each of the four PFASs at three concentrations (nominally 10, 100, and 1000  $\mu\text{g/L}$ ) were placed in two replicates on adjacent shelves within an environmental chamber. Experimental units consisted of 15 L plastic aquaria filled with 7.5 L of filtered, UV-irradiated well water. Tadpoles ( $n = 35$  per aquarium) were randomly assigned to the experimental units. Prior to addition to aquaria, a subset of animals was examined to confirm development at Gosner stage 26—when hind limb buds start to develop.<sup>17</sup> Tadpoles with visible irregularities in morphology, coloration, or behavior were excluded. Animals were maintained at  $20 \pm 2$  °C with a 12 h/12 h light/dark photoperiod for 10 days to acclimate to indoor conditions and were fed a Tetramin slurry *ad libitum*. Water changes were conducted every 4 d.

Tadpoles were exposed for 40 d using a static renewal approach with all water changed every 4 d. Animals were monitored daily for mortality and abnormalities. A water sample (~5 mL) was taken immediately prior to and after each water change to monitor concentration of test chemicals. Every 10 d, six animals were randomly collected from each aquarium. The animals were euthanized (800 mg/L buffered MS-222), measured (total length at 10 d, snout–vent length otherwise), and staged (Gosner) prior to storage at  $-20$  °C for chemical analyses.

After 40 d, the depuration phase was initiated by removing animals, cleaning each aquarium with a methanol-soaked sponge, and rinsing to remove adsorbed compound. Aquaria were refilled with clean water; animals were returned to the same aquarium and monitored as described above. Water changes were carried out every 4 d with fresh water, and a water sample was taken prior to each water change. Two tadpoles were sampled every 10 d for an additional 30 d.

**Analytical Procedure.** Depending on available wet weight, whole-body burdens were quantified from two or three pooled tadpole samples ( $n = 3$  for first two samples and  $n = 2$  for

remaining samples). Prior to chemical analysis, samples were lyophilized for ~24 h and dry weight measured. After transfer to 1.5 mL microcentrifuge tubes, tadpoles were spiked with 20  $\mu\text{L}$  of an internal standard solution in methanol (about 250 ng/mL of each mass-labeled PFAS). Extraction was performed by adding tetrahydrofuran (THF, 600  $\mu\text{L}$ ) and water (200  $\mu\text{L}$ ) with the aid of glass beads and mechanical shaking (10 min), sonication (30 min), and centrifugation (20 min) following a modified method from Luque et al.<sup>18</sup> The supernatant was transferred to a 1.5 mL glass injection vial, gently blown down with nitrogen, and solvent exchanged to a 500  $\mu\text{L}$  50:50 v/v methanol:water. This extract was vortexed and transferred to 1.5 mL microcentrifuge tubes and centrifuged for 10 min. A fraction of the supernatant (200  $\mu\text{L}$ ) was transferred back to the 1.5 mL glass injection vials and stored at 4 °C until analysis.

Water samples (250  $\mu\text{L}$ ) were directly transferred to a 1.5 mL glass injection vial, and 230  $\mu\text{L}$  of methanol and 20  $\mu\text{L}$  of an internal standard solution in methanol were added (about 250 ng/mL of each mass-labeled PFAS). This sample was then vortexed and stored at 4 °C. At the highest concentration, 25  $\mu\text{L}$  of samples and 225  $\mu\text{L}$  of ultrapure water were transferred for dilution with the usual amount of methanol and internal standard solution. For both water and tissue samples, laboratory blanks and spiked control samples were prepared with each batch of samples.

Analysis was performed using reverse-phase chromatography with two automated liquid chromatography mass spectrometry (LC-MS/MS) systems which were coupled to either an AB Sciex TripleTOF 5600 (animal and some water samples) or an AB Sciex 3000 triple quadrupole MS (remaining water samples). The mobile phase was a gradient of 0.15% acetic acid in water and 20 mM ammonium acetate in methanol. Instrument control was managed via Analyst TF1.7 and Analyst 1.4.2 software for the 5600+ and the 3000, respectively, and all data were processed in Multiquant 3.0.1. Additional details are provided in the [Supporting Information](#) (SI).

**Data and Statistics.** Aquarium means were calculated on days 10, 20, 30, and 40 for each response variable (i.e., dry weight body burdens, bioconcentration factor (BCF), length, and Gosner stage). Body burden data was log-transformed prior to analysis to achieve normality. For accumulation data (body burden and BCF), repeated measures-analysis of variance (rm-ANOVA) assessed the effect of chemical, exposure concentration, and their interaction. For each treatment, we used one-way ANOVA to test for steady state, assessing the effect of time on body burden. Treatments with significant effects of time were further examined by Tukey's means comparisons to clarify when steady state was reached. For morphometric data (length and Gosner stage), we used rm-ANOVA to assess the effect of chemical and concentration individually. In cases of statistical significance, one-way ANOVA examined the effect of chemical or concentration at 40 d. Coupled with Tukey's means comparisons, this test determined which individual treatments had a significant effect. Statistical analyses were carried out in JMP 12, Graphpad Prism 7, and SPSS. Reported BCF values are the ratios between wet weight PFAS concentrations (ng/g) and PFAS water exposure concentrations ( $\mu\text{g/L}$ ). Wet weight PFAS concentrations were calculated by multiplying the dry weight concentration by % dry matter. For water concentrations, we used mean concentrations over the entire exposure period. During depuration, many values for body burden were below the limits of quantification (LOQ), in which case an estimate of half the LOQ factored into curve fitting. Best fit

first-order decay curves determined elimination constants and half-lives. Laboratory contamination nullified the final PFOA-exposed depuration sample; elimination constants were calculated over a shorter period (20 vs 30 d).

## RESULTS AND DISCUSSION

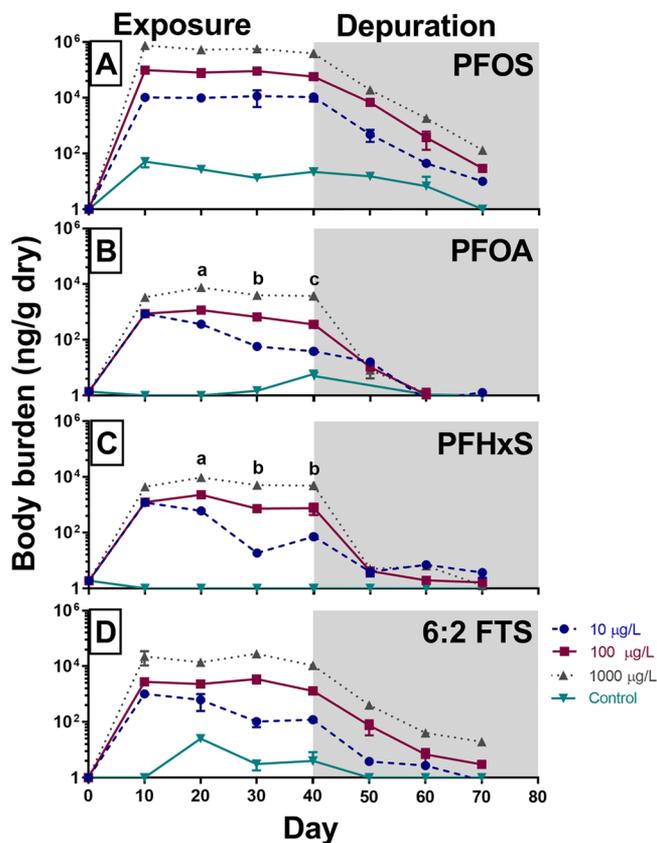
**Water Concentrations.** PFAS water concentrations were nearly constant throughout the exposure period (Figure S1). Average concentrations across the entire exposure period were within 25% of the targeted nominal concentrations for all treatments except for 6:2 FTS which ran ~40% above nominal. Control water samples consistently contained PFAS concentrations below the LOQ of ~0.22  $\mu\text{g/L}$ .

Water samples at the first water change during the depuration period (44 d) showed measurable compounds in only the highest (1,000  $\mu\text{g/L}$ , all compounds) or middle (100  $\mu\text{g/L}$ , PFOS only) exposure concentrations. Measured concentrations were several orders of magnitude below exposure concentrations (0.98–6.9  $\mu\text{g/L}$ ), and PFAS concentrations fell below the LOQ in all treatments after the second water change (48 d).

**Accumulation.** Whole-body burdens were found to be dose and chemical dependent (see Table S5 for statistics). As in trout,<sup>19</sup> the highest body burdens were found following exposure to PFOS (Figure 1A). The remaining chemicals all reached concentrations approximately 2 orders of magnitude below PFOS (Figure 1B, C, D). Steady state concentrations were achieved by 10 d for all compounds and exposures except the highest doses (1000  $\mu\text{g/L}$ ) of PFOA and PFHxS, which peaked at 20 d. Low dose exposures of PFOA and PFHxS resulted in highest body burdens at 10 d with a significant decrease by 30 d. This decline could be attributable to physiological and anatomical changes during development, such as a maturation of organs (e.g., liver or kidney) involved in xenobiotic excretion. Increased hydrophobicity due to chain length and sulfonate group, as previously suggested,<sup>11</sup> may explain the lack of decline seen in PFOS. Further work on tissue distribution and mechanisms of excretion of PFASs by tadpoles is warranted.

**Bioconcentration.** Whole-body BCFs mirrored body burden uptake patterns with maximum values or steady state reached by 10 d. Bioconcentration was greatest for PFOS with whole-body BCFs ranging from 19.6 to 148.7 (Figure 2A). The remaining compounds had maximum BCF values below 10, and all were below one by 30 d (Figure 2B, C, D). This range in BCF is approximately 2 orders of magnitude lower than for an environmentally exposed fish population.<sup>20</sup> Bioconcentration factors for PFOA in adult zebrafish range from 20 to 30,<sup>21</sup> again notably higher than the values found here in tadpoles. These differences may be attributable to the greater permeability of amphibian skin, which permits high flux of water and solutes through the animal.<sup>12</sup> Importantly, these BCF values for PFOS are well below the threshold of 1000 for the USEPA definition of “bioaccumulative”, potentially limiting regulatory action.<sup>22</sup>

**Depuration.** Depuration was rapid for all chemicals (Figure 1). Based on whole-body burdens, we calculated first-order elimination constants and half-lives for each treatment group. Half-lives ranged from 1.2 to 3.3 days (Table S4). Due to rapid depuration and a wide sampling interval, half-lives for non-PFOS treatments are based partially on estimated values and should be interpreted carefully. However, these pilot-level

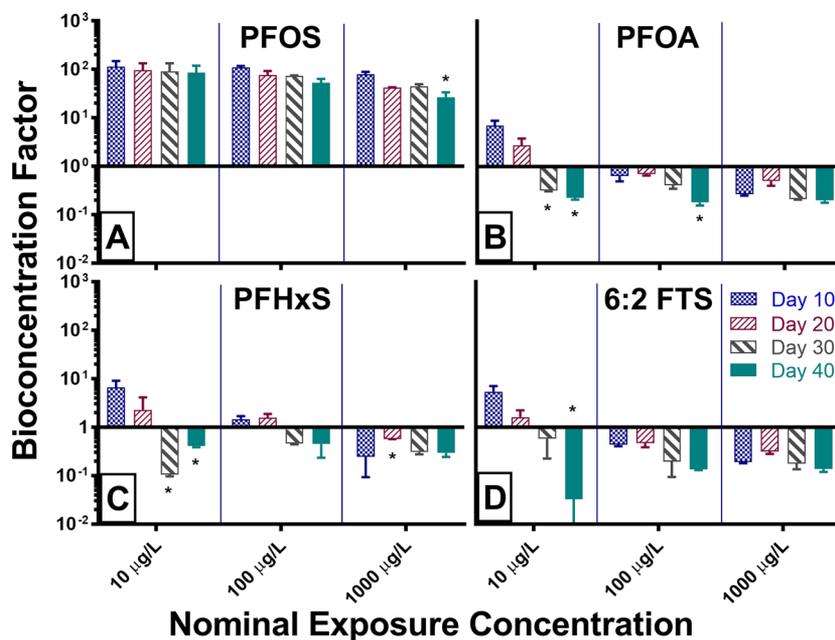


**Figure 1.** Whole-body accumulation and depuration concentrations (ng/g dry weight) of each of the tested compounds. Depuration began at day 40 of the experiment (gray background). Error bars correspond to standard error; some error bars are obscured by symbols. Time points denoted with “a” signify a significant ( $p < 0.05$ ) increase from day 10 body burden in the 1000  $\mu\text{g/L}$  treatment group. Time points with a “b” signify a significant decrease below day 10 body burden in the 10  $\mu\text{g/L}$  treatment group. Time points with a “c” denote a significant decrease relative to day 10 body burden in both the 10 and 100  $\mu\text{g/L}$  treatments. (A) PFOA = perfluorooctanoic acid, (B) PFOS = perfluorooctanesulfonate, (C) PFHxS = perfluorohexanesulfonate, (D) 6:2 FTS = 6:2 fluorotelomer sulfonate.

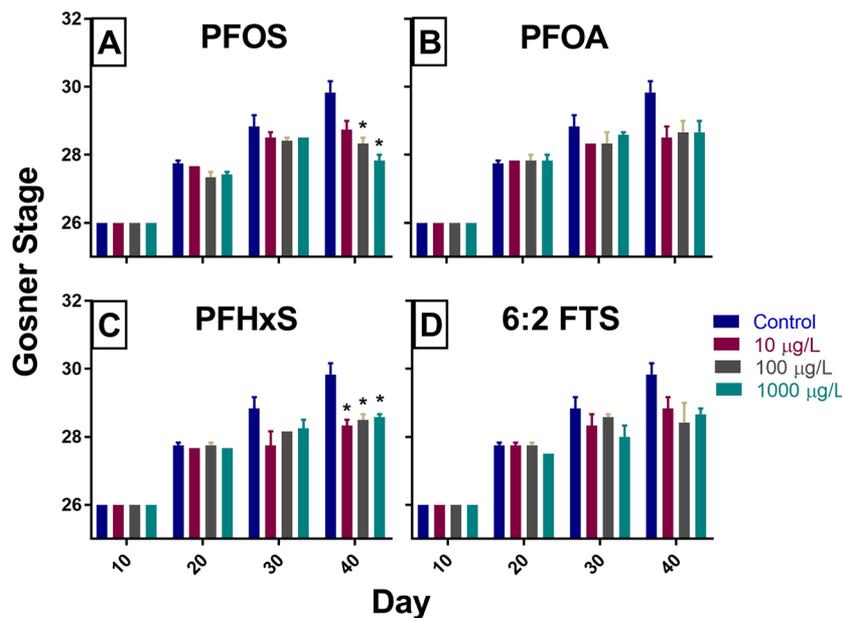
depuration rates and half-lives correspond well with rapid uptake and steady state achieved in 10 d.

Few studies have examined the kinetics of PFASs in amphibians, and only Ankley et al.<sup>23</sup> looked at bioconcentration (PFOS only) in larval amphibians. As observed by Ankley et al.,<sup>23</sup> we found rapid uptake of PFASs. Our sampling regimen, however, did not allow for the detection of the initial, steep alpha uptake curve observed in fish.<sup>24</sup> Likewise, sampling during depuration did not yield values that permit statistical comparison between compounds. Overall, though, the kinetics of PFASs in northern leopard frog tadpoles are faster when compared to adult zebrafish, with steady state reported for PFOA in 20–30 d and half-lives ranging from 10 to 14 d for PFOA to over 40 d for PFOS.<sup>22,25</sup> We suspect that the flux of PFASs through the skin is driving the differences observed; other routes of excretion (biliary, renal, gill clearance) are likely comparable between tadpoles and fish. These values are still much lower than those in mammals, with half-life of PFOA in humans estimated at 3 years.<sup>26</sup>

**Survival, Growth, and Development.** Only sublethal effects were observed for tadpoles, as survival for all treatments was above 90%. Length and development trended below



**Figure 2.** Bioconcentration factor (BCF) over the course of exposure for all compounds tested. Error bars correspond to standard error. Asterisks denote a significant change ( $p < 0.05$ ) from the BCF at day 10. (A) PFOS = perfluorooctanesulfonate. (B) PFOA = perfluorooctanoic acid. (C) PFHxS = perfluorohexanesulfonate. (D) 6:2 FTS = 6:2 fluorotelomer sulfonate.



**Figure 3.** By-chemical average Gosner stage over the course of exposure. Error bars correspond to standard error. Asterisks signify significant difference ( $p < 0.05$ ) in one-way ANOVA from controls on that day. There was no difference in Gosner stage between days 0 and 10. (A) PFOS = perfluorooctanesulfonate. (B) PFOA = perfluorooctanoic acid. (C) PFHxS = perfluorohexanesulfonate. (D) 6:2 FTS = 6:2 fluorotelomer sulfonate.

controls for nearly all exposures (Figure 3, SVL Figure S2). Chemical had a statistically significant effect for both response variables, and exposure concentration also had a significant effect on development (Table S5). This developmental delay was apparent for the higher (100 and 1000  $\mu\text{g/L}$ ) exposures of PFOS and all exposures of PFHxS at day 40 (Figure 3A and C, Table S5). Delayed development (demonstrated as increased time to metamorphosis) has previously been shown only at higher exposure concentrations (3000  $\mu\text{g/L}$ ) of PFOS.<sup>23</sup>

This is the first study to show sublethal effects of PFHxS on amphibians. This was surprising as this C6 chemical was

thought to be less toxic than C8 PFASs.<sup>27</sup> Given that the effects on development were observed at environmentally relevant concentrations (10  $\mu\text{g/L}$ ) and several orders of magnitude below previously reported effects of PFOS,<sup>23</sup> future studies should examine the sublethal effects of PFHxS on other taxa.

These results confirmed our hypotheses regarding chain length and functional group: Significant developmental delay was seen with sulfonates and not the carboxylate, and the longer chain sulfonate was more accumulative. However, the trends seen in this study suggest that effects may be seen with all chemicals studied, regardless of body burden. As such, the

widespread contamination of surface waters with PFASs could pose a significant concern to aquatic communities and ecosystems.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.7b00339.

Additional figures of results and analytical method details. T(PDF)

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### Notes

The authors declare no competing financial interest.

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