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# P-gp expression in brown trout erythrocytes: evidence of a detoxification mechanism in fish erythrocytes

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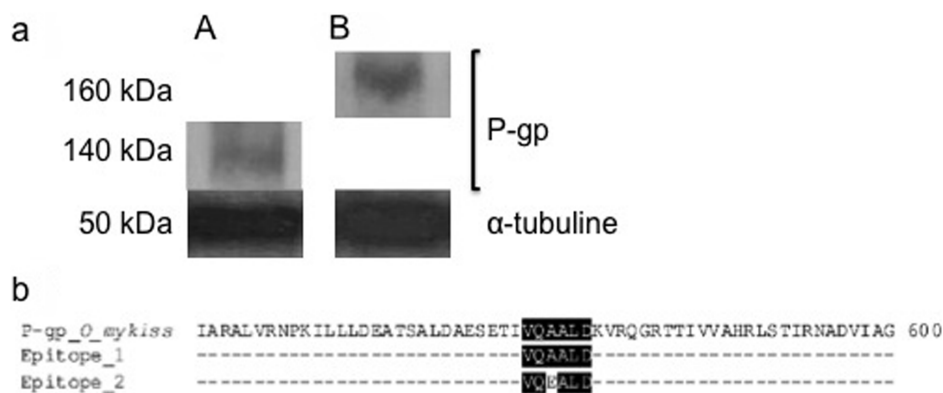
Blood is a site of physiological transport for a great variety of molecules, including xenobiotics. Blood cells in aquatic vertebrates, such as fish, are directly exposed to aquatic pollution. P-gp are ubiquitous “membrane detoxification proteins” implicated in the cellular efflux of various xenobiotics, such as polycyclic aromatic hydrocarbons (PAHs), which may be pollutants. The existence of this P-gp detoxification system inducible by benzo [a] pyrene (BaP), a highly cytotoxic PAH, was investigated in the nucleated erythrocytes of brown trout. Western blot analysis showed the expression of a 140-kDa P-gp in trout erythrocytes. Primary cultures of erythrocytes exposed to increasing concentrations of BaP showed no evidence of cell toxicity. Yet, in the same BaP-treated erythrocytes, P-gp expression increased significantly in a dose-dependent manner. Brown trout P-gp erythrocytes act as membrane defence mechanism against the pollutant, a property that can be exploited for future biomarker development to monitor water quality.

The bloodstream is a hub for the transport and inter-reaction of a variety of physiological molecules (oxygen, nutrients, hormones and waste) and also for the accumulation of xenobiotics (drugs and/or pollutants). Whatever the type of pollution (acute or chronic), the various pollutants are either found directly in the blood or accumulated in organs, tissues and cells before being gradually released into the circulation. Erythrocytes are the major cellular component of blood. In mammals, including man, mature erythrocytes are not nucleated, unlike fish, where they are<sup>1</sup>. Among fish, Salmonids are integrative organisms with regard to environmental conditions and water quality. They are “bioaccumulation” organisms<sup>2</sup>. Brown trout (*Salmo trutta fario*) provide a ubiquitous and highly sensitive index of water quality. Furthermore, the brown trout is not only native to rivers but may also be fish-farmed.

P-gp, the well-known “Multidrug Resistance” (MDR) protein encoded by the MDR1 or ABCB1 gene, have been described as membrane transporters involved in resistance to chemotherapy in cancer<sup>3</sup>. In reality, P-gp belongs to the evolutionarily conserved family of the ATP-binding cassette (ABC) proteins found in practically all living organisms, whether prokaryotic<sup>4</sup> or eukaryotic<sup>3,5–13</sup>. P-gp acts as xenobiotics efflux pump transporting various substrates out of cells. It recognizes various molecules with different structures and properties, including pollutants such as polycyclic aromatic hydrocarbons (PAHs) (e.g. benzo-a-pyrene (BaP)), heavy metals (e.g. cadmium), pesticides (e.g. diazinon, DDT), insecticides (e.g. chlorpyrifos) and various pharmaceutical drugs, such as chemotherapeutic drugs (e.g. Vinca alkaloids, actinomycin D, taxol, antracyclines), calcium channel blockers (e.g. verapamil)<sup>3,5,6,11,12,14–22</sup>. Due to this property, P-gp is involved in the natural defence mechanisms of cells, tissues and organs, and forms an essential part of the innate defence system known as “chemo-immunity”<sup>23</sup>.

Our previous research in both laboratory and field has focused on the development of environmental biomarkers for PAH pollution based on MDR1/P-gp expression, using the freshwater ciliated protozoan *Tetrahymena pyriformis* for water pollution and the vinegar fly *Drosophila melanogaster* for air pollution. In these two models, P-gp expression was clearly induced in a dose-dependent manner, after exposure to different concentrations of various classes of PAHs<sup>5,6,11,12</sup>.

In the present work, we aimed to analyze P-gp expression in the erythrocytes of brown trout and their response to BaP, a PAH model, in order to develop an aquatic biomarker.



**Figure 1 | P-gp expression in brown trout erythrocytes.** (a). Western blot analysis of P-gp in brown trout: P-gp protein was analyzed in total protein extract from (A) a dry pellet of brown trout erythrocytes or (B) liver. P-gp weighed 140 kDa in A and 160 kDa in B. The monoclonal anti-tubulin antibody DM1A labelled a tubulin protein presented a molecular weight of about 50 kDa, used as internal control. The samples derive from the same experiment and gels were processed in parallel. The full-length blot and Coomassie blue gel are presented in Supplementary Figures 1a and 1b. (b). Sequence alignment of *Oncorhynchus mykiss* P-gp with the two different epitopes (VQAALD and VQEALD) recognized by C219 antibody. The alignment was performed using ClustalW. Conserved residues and similar residues are shaded in black and grey, respectively.

## Results

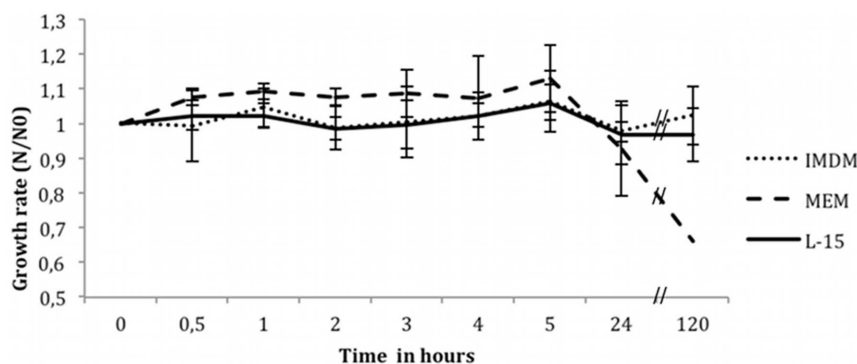
**Expression of P-gp in brown trout erythrocytes.** The expression of P-gp in erythrocytes was analyzed from a dry pellet of brown trout erythrocytes by Western blotting (Fig. 1, and Supplementary Fig. 1a, and b). The results showed that the monoclonal anti-P-gp antibody C219 clearly recognized a protein having a molecular weight (MW) of 140 kDa (Fig. 1a). As expected, in the brown trout liver extract used as the positive control, a P-gp of 160 kDa was labelled<sup>23</sup>. A second band of about 75 kDa was also labelled by C219 antibody in trout erythrocytes. Moreover, the sequence alignment of rainbow trout P-gp available in Genbank with the “VQEALD” and “VQAALD” epitopes was recognized by C219 antibody<sup>24</sup> was then checked *via* CLUSTALW. The results confirmed that the VQAALD epitope, recognized by C219 monoclonal antibody, was present in trout P-gp (Fig. 1b). The expression of P-gp in the brown trout erythrocytes quantified under these conditions was  $0.84 \pm 0.05$  a.u.

**Development of primary culture of brown trout erythrocytes.** For the development of primary culture of erythrocytes, three culture media were used: *i.e.* IMDM, MEM and L-15, supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 1% fungizone and 1% L-glutamine, under different temperature conditions (11°C, 15°C, 18°C or 21°C) with or without shaking. One million cells per mL were seeded in each medium, and the rate of cell growth was assessed over time. For the three culture media tested, the best temperature and stirring conditions for maintaining trout erythrocytes in primary culture were 21°C and 45 rpm (data not shown). These conditions were thus used to track erythrocyte growth rates in

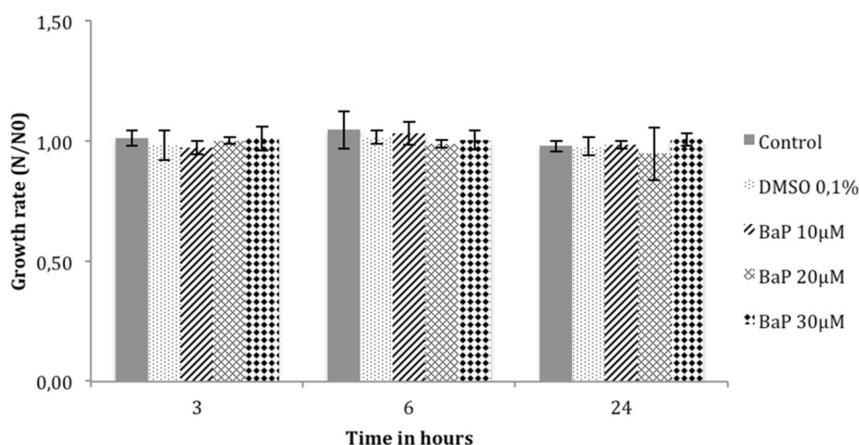
the three tested media in both the short term (24 h) and longer term (5 days). The results presented in Figure 2 show that in all three media, cell growth rates remained relatively stable over 5 h, *i.e.* the short term, with a yield close to 1. In the longer term (1 to 5 days), the growth rate remained close to 1 in L-15 and IMDM media, but fell slightly in the MEM medium. These results suggest that L-15 and IMDM media may be used for the primary culture of brown trout erythrocytes. However, a comparison of cell morphology and cell membrane shape in primary cultures of L-15 and IMDM media showed that erythrocytes in the L-15 medium were less damaged and kept their cell membranes intact (data not shown). Thus, the L-15 medium was chosen for further experiments.

**BaP toxicity analysis on trout erythrocytes.** The toxicity of 10, 20 and 30  $\mu$ M BaP on cultured trout erythrocytes was then studied, by cells counts over time using a Z-2 cell and particle counter. The rate of cell numbers was expressed as  $N/N_0$ , where N and  $N_0$  are cell numbers at time T and  $T_0$  (beginning of experiment or after addition of DMSO or BaP). The results showed that in the presence of BaP at 10, 20 and 30  $\mu$ M, the number of cells remained stable over time without signs of BaP toxicity (Fig. 3).

**P-gp expression in trout erythrocytes after treatment with benzo[a]pyrene.** Using Western blot analysis, the expression levels of P-gp in primary culture were measured for 3, 6 and 24 h in the presence of increasing concentrations of BaP (10, 20 and 30  $\mu$ M) (Fig. 4). Control cultures (untreated cells and in 0.1% DMSO) were performed in parallel. P-gp expression levels in untreated cells and in



**Figure 2 | Growth of brown trout erythrocytes in primary culture over time.** Cells counts were followed over time using a Beckman Coulter Z-2 Counter. N: number of cells at time T.  $N_0$ : number of cells at time  $T_0$  (beginning of experiment).



**Figure 3 | BaP toxicity analysis on brown trout erythrocytes.** Erythrocytes in primary culture were treated with 10, 20 and 30  $\mu\text{M}$  of BaP for 3, 6 and 24 h. Cell growth was analyzed by cell counting on the Beckman Coulter Z-2 Counter. N: number of cells at time T. N0: number of cells at time T0 (beginning of experiment).

0.1% DMSO remained stable over 24 h ( $0.16 \pm 0.01$  and  $0.19 \pm 0.06$  a.u. at 24 h, respectively). There was no significant difference between controls. 0.1% DMSO had no effect on P-gp expression. The addition of 10  $\mu\text{M}$  BaP to the cell cultures did not change the expression rate of P-gp for 6 h, which remained close to the control values ( $0.12 \pm 0.02$  a.u.). In these cells, P-gp rate only increased after 24 h of BaP exposure ( $0.41 \pm 0.05$  a.u.). Conversely, in the presence of both 20 and 30  $\mu\text{M}$  BaP, P-gp expression was clearly induced during the experiment. Indeed, in the presence of 20  $\mu\text{M}$  BaP, P-gp expression increased steadily over time to reach  $0.44 \pm 0.1$ ,  $0.55 \pm 0.1$  and  $0.70 \pm 0.1$  a.u. after 3, 6 and 24 h, respectively. In the presence of 30  $\mu\text{M}$  BaP, P-gp remained high, at  $0.65 \pm 0.01$ ,  $0.42 \pm 0.08$  and  $0.51 \pm 0.01$  a.u. after 3, 6 and 24 h, respectively.

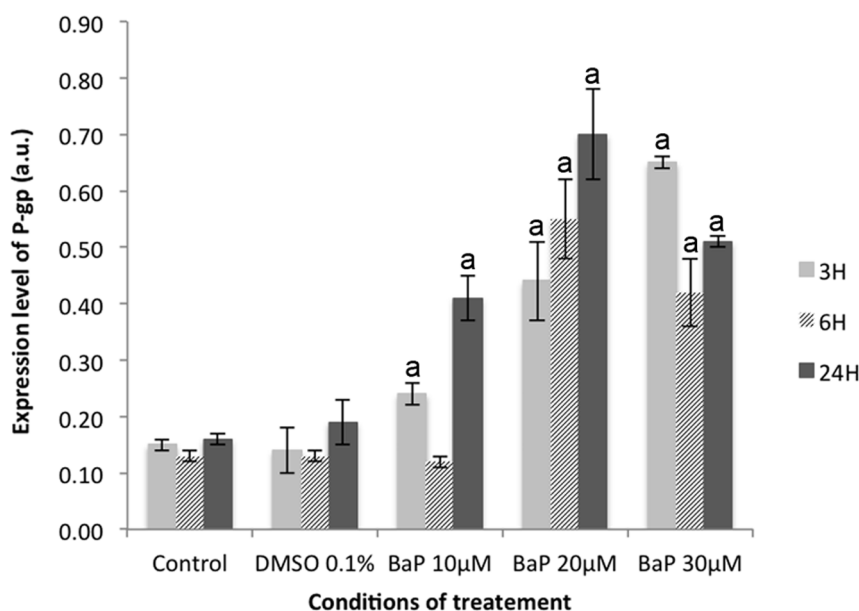
## Discussion

Blood delivers necessary substances such as nutrients and oxygen to cells and transports metabolic waste. Blood is also a site of passage and distribution of all xenobiotics (drugs and/or pollutants) which

enter the organism. In aquatic vertebrates, such as fish, blood cells are directly exposed to the contamination. Therefore, they provide an excellent tool for water pollution detection.

In cells, P-gp is a ubiquitous membrane detoxification protein involved in the defence mechanisms against xenobiotics. It recognizes a large class of xenobiotics, from different drugs<sup>3</sup> to various pollutants, such as PAHs<sup>5,6,11,12,25</sup>. The latter are highly ubiquitous environmental contaminants and include toxic compounds with cytotoxic, genotoxic and carcinogenic properties<sup>26–28</sup>. It has already been demonstrated that P-gp expression may be induced after exposure to PAHs in different cells models and/or organisms, suggesting that P-gp may be used as a potential biomarker of environmental pollution<sup>5,11,12,25,29</sup>.

In this context, our work focused on P-gp expression in erythrocytes using the *Salmonidae* brown trout as a model since it can live in most rivers. Firstly, P-gp expression was studied directly in dry pellets of cells using Western blot analysis. The monoclonal antibody anti-P-gp C219 clearly marked a protein of 140 kDa. As



**Figure 4 | P-gp expression in brown trout erythrocytes in the presence of BaP.** Brown trout erythrocytes were exposed to 10, 20 and 30  $\mu\text{M}$  of BaP. After 3 h, 6 h and 24 h of treatment, the cells were recovered to perform protein extraction. Following extraction, Western blotting was performed and protein expression quantified using Quantity One software (Biorad®). Statistical analysis was performed by Student's t-test. "a" indicate a significant difference ( $p < 0.05$ ) in P-gp expression between BaP treated samples and controls.



a P-gp-positive control we used brown trout liver extract with a molecular weight of about 160 kDa. Indeed, the expression of a 165 kDa P-gp has been reported in liver extract and hepatocyte cell cultures of another trout species, *Oncorhynchus mykiss*<sup>23</sup>. Moreover, depending on species and tissue, the apparent molecular mass of P-gp may vary between 130 and 180 kDa, reflecting differences in glycosylation<sup>30</sup>. The monoclonal C219 anti-P-gp recognized a P-gp of 170 kDa in man<sup>3</sup>, and of 140 kDa in *Drosophila melanogaster*<sup>11,12</sup>. In addition, depending on species, other P-gp molecular weights were also cited, such as a 55 kDa P-gp in cattle<sup>31</sup>, a 66 kDa and a 96 kDa P-gp in the freshwater ciliated protozoan *Tetrahymena pyriformis*<sup>5</sup>. These results suggest that a P-gp of 140 kDa was expressed in the nucleated erythrocytes of brown trout. Furthermore, the C219 monoclonal antibody seems to label another protein with a MW of 75 kDa in trout erythrocytes. Previously, the labelling of an 80 kDa band of unknown identity was also reported in trout liver and cultured hepatocytes by the same antibody<sup>10</sup>.

Secondly, for trout P-gp erythrocytes induction study, we developed the best set of conditions for *in vitro* culture, such as type of culture medium, cell density, temperature and stirring. The results showed that it was possible to achieve primary cultures of trout erythrocytes over time. Indeed, as trout erythrocytes are nucleated, it should be possible to maintain them in culture for several days. The best conditions for *in vitro* culture were Leibovitz's medium (L-15) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% fungizone and 1% L-glutamine, at a temperature of 21°C, with stirring at 45 rpm. The development of primary blood cell cultures in the trout model offers further scope for adaptation to other fish models or to species possessing nucleated erythrocytes. This *in vitro* culture model may be used for future cytotoxicity tests on various xenobiotics and/or for other applications linked tied to a fish health model. After starting red blood cell culture *in vitro*, it was necessary to analyze the response of P-gp to PAHs. For this purpose, we chose to test the action of BaP, which is a cytotoxic, genotoxic and carcinogenic model pollutant in the PAHs class. It has been found in air, water and soil<sup>32,33</sup>. Moreover, BaP, like several other PAHs, has been shown to induce P-gp in other biological models of aquatic and atmospheric pollution<sup>5,11,12</sup>. BaP toxicity at 10, 20 and 30 µM was first tested by analyzing red blood cell numbers in primary culture over time. No toxicity was detected at these concentrations, probably due to the detoxification action of P-gp in the erythrocytes. Similar responses have already been detected in the ciliated protozoan model *Tetrahymena pyriformis* and in SL2 *Drosophila melanogaster* cell cultures in the presence of similar concentrations of BaP and other PAHs<sup>5,12,21</sup>.

This hypothesis of detoxifying action by P-gp was then checked by monitoring P-gp expression over time in primary cultures of erythrocytes, after treatment with increased concentration of BaP. Under these conditions, a clear dose-dependent induction of P-gp expression was detected in trout erythrocytes. With 10 µM BaP, P-gp expression increased only after 24 h of treatment with a 2-fold higher ratio than in the controls. The P-gp induction was clearly noted with 20 and 30 µM BaP from 3 h to 24 h, with a mean ratio 3.6-fold higher than controls. These results suggest that P-gp is involved in the mechanisms of cellular defence against BaP pollutant. Similar findings have already been described in other models<sup>5,6,11,12</sup>.

All these results suggest that trout erythrocytes can be used to develop biomarkers to determine water pollution by PAHs. Indeed, such a development offers two lines of interest. First, the blood being a fluid tissue circulating throughout the body, this biomarker may reflect the overall state of fish contamination, with possible applications in the agri-food sector. It may also reflect the state of water contamination and thus have applications in the field of environmental monitoring. Experiments to develop a P-gp biomarker are currently being tested on brown trout in a natural environment.

## Methods

**Fish model and blood sampling.** Brown trout (*Salmo trutta fario*) were obtained from the "Le Moulin de Pagnat" fish farm, Saint-Saturnin, France. For blood sampling, they were anesthetized using a mix of clove oil in 70% alcohol. Blood samples were then taken by intracardiac puncture onto lithium heparinate anticoagulant. 500 µL of blood were then washed twice with 1 mL of PBS by centrifugation at 800 G for 10 minutes and then the supernatant was removed. After centrifugation, red blood cells were concentrated in the lower portion of the tube and white blood cells, in the form of a thin layer, were seen on the surface of the layer of red blood cells. The fraction of white blood cells was removed. To achieve the primary culture, only the fraction of red blood cell pellets was used (Supplementary Fig. 2). Erythrocytes were counted using a Malassez cell counter. The cells were then used either immediately for primary cell culture or stored at -20°C as dry pellets.

**Ethics.** In compliance with the European Directive 2010/63/UE and Decree No. 2013-118 of application of 1 February 2013 on the protection of animals used for scientific purposes ref NOR: AGRG12131951D - ethical approval was not required for this study since fish were anesthetized with an overdose of a natural anesthetic.

**Red blood cell culture development.** The red blood cell culture was achieved using three culture media: Iscove's Modified Dulbecco's Medium (IMDM), Minimum Essential Medium (MEM) and Leibovitz's medium (L-15) (Gibco®, Invitrogen). Erythrocytes were seeded in 25-ml flasks at one million cells per mL in each culture medium, enabling development of optimal culture conditions.

**Cell counts.** Cells in cultures were counted electronically using a Beckman Coulter™ Z-2 cell and particle counter. The results were expressed as N/N0, where N corresponded to cell number at time T and N0, to cell number at T0 (beginning of experiment or after addition of DMSO or BaP).

**BaP solubilization and exposure.** BaP was dissolved in dimethyl sulfoxide (DMSO) as a stock solution at 5 mM. For each experiment, one million cells/mL were seeded in 10 mL of culture medium. The cell cultures were then exposed to concentrations of 10 µM, 20 µM and 30 µM BaP. For each BaP concentration studied, the required volume of BaP was removed from the BaP stock solution. This was then supplemented with the appropriate volume of DMSO, so that the final DMSO concentration remained constant *i.e.* 0.1%, in all analyzed cell cultures. In parallel, two control cultures were carried out: (i) untreated cells and (ii) primary culture of erythrocytes treated with 0.1% DMSO.

**MDR expression studies by Western blot analysis.** For each experiment (red blood cell pellets and primary cell cultures), the erythrocytes were collected by a centrifugation at 800 G for 10 min at 4°C. The cells were then treated with 1 ml of lysis buffer (20 mM tris-HCl, 2 mM EDTA, 2 mM EGTA, 6 µM β-mercaptoethanol, and 0.1% protease inhibitor cocktail, pH 7.5). For protein extraction, this sample was sonicated (5 × 5 s at 4°C) and protein concentrations were determined using a "Coomassie Plus - The Better Bradford Assay" Kit (Thermo Scientific®). For the P-gp positive control, a trout liver extract treated under the same conditions was also used<sup>23</sup>. Western blot analysis was then carried out according to Vaché *et al.*, 2006<sup>12</sup>. Briefly, for each analysis, extracts were diluted to 100 µg·mL<sup>-1</sup> protein with sample buffer (1.25 M tris-HCl, 20% sodium dodecyl sulfate, 20% glycerol, 2 M dithiothreitol, 0.5% bromophenol blue, pH 6.8). For these experiments, the primary monoclonal antibody anti-P-gp C219 (1 : 500, Calbiochem®), the mouse monoclonal antitubulin antibody DM1A (1 : 5000, Sigma®) and the secondary antibody, a horseradish-peroxidase (HRP)-coupled goat anti-mouse (1 : 4000, Promega®) were used. Immune complexes were visualized by chemiluminescence (ECL + Western blotting detection reagent, Amersham®) according to the manufacturer's specifications. The intensities of P-gp and control tubulin bands were analyzed by densitometry using Quantity-One software (BioRad®). For each sample, P-gp expression was determined by calculating the ratio of the density of each MDR protein over the density of the tubulin control. This ratio was expressed in arbitrary units (a.u.).

**Statistical analysis.** Data were expressed as means ± confidence intervals of *n* independent experiments. Each experiment was performed at least in triplicate and then statistically compared using ANOVA and a Student's *t*-test. The comparison was applied on each culture condition and on control groups *versus* BaP-treated groups and *P* < 0.05 was considered statistically significant.

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## Author contributions

E.V. executed the experiments. E.V. and M.B. wrote the main manuscript text. E.V. and I.W. prepared figures 1–4. All authors read and approved the final manuscript.

## Additional information

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