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**Competing interests statement**

The authors declare competing financial interests: details accompany the paper on Nature's website (<http://www.nature.com/nature>).

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**Pfiesteria shumwayae kills fish by micropredation not exotoxin secretion**

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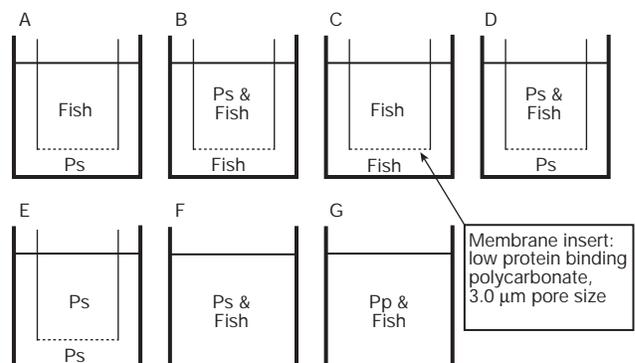
*Pfiesteria piscicida* and *P. shumwayae* reportedly secrete potent exotoxins thought to cause fish lesion events, acute fish kills and human disease in mid-Atlantic USA estuaries<sup>1–7</sup>. However, *Pfiesteria* toxins have never been isolated or characterized<sup>8</sup>. We investigated mechanisms by which *P. shumwayae* kills fish using three different approaches. Here we show that larval fish bioassays conducted in tissue culture plates fitted with polycarbonate membrane inserts exhibited mortality (100%) only in treatments where fish and dinospores were in physical contact. No mortalities occurred in treatments where the membrane prevented contact between dinospores and fish. Using differen-

tial centrifugation and filtration of water from a fish-killing culture, we produced 'dinoflagellate', 'bacteria' and 'cell-free' fractions. Larval fish bioassays of these fractions resulted in mortalities (60–100% in less than 24 h) only in fractions containing live dinospores ('whole water', 'dinoflagellate'), with no mortalities in 'cell-free' or 'bacteria'-enriched fractions. Video-micrography and electron microscopy show dinospores swarming toward and attaching to skin, actively feeding, and rapidly denuding fish of epidermis. We show here that our cultures of actively fish-killing *P. shumwayae* do not secrete potent exotoxins; rather, fish mortality results from micropredatory feeding.

Massive fish kills in mid-Atlantic USA estuaries involving several million Atlantic menhaden, *Brevoortia tyrannus*, have been attributed to dinoflagellates of the toxic *Pfiesteria* complex (TPC)<sup>9</sup>. Potent ichthyotoxins secreted during *Pfiesteria* blooms are thought to be responsible for mortality as well as for deeply penetrating, so-called 'Pfiesteria-specific' skin ulcers in these fish<sup>1,5,9</sup>. However, earlier investigations attributed the menhaden ulcers to fungal infections<sup>10,11</sup>, and *Aphanomyces invadans*, a highly pathogenic oomycete<sup>12</sup>, is now considered the aetiologic agent<sup>13,14</sup>. We recently demonstrated that *A. invadans* is a primary pathogen, able to elicit menhaden ulcer disease in the absence of *Pfiesteria* species or other environmental stressors<sup>15</sup>. Thus, the role of *Pfiesteria* species in menhaden lesion events is now questioned<sup>13–16</sup>.

In contrast to the oomycete-induced ulcers of wild menhaden, laboratory exposure of fishes to an unidentified *Pfiesteria* species elicited rapid, widespread epidermal erosion, osmoregulatory dysfunction and death, with potent exotoxins assumed responsible<sup>4</sup>. However, direct attachment of *P. shumwayae* dinospores to skin, gills, olfactory organs, the oral mucosa and the lateral line canal, associated with extensive tissue damage, has been observed<sup>16</sup>. A direct physical association with these fish tissues had not to our knowledge been previously reported, and this suggested an alternative mechanism of pathogenesis for *P. shumwayae*. To better understand this association and to clarify the consequences of dinospore attachment, we conducted laboratory challenges using a sensitive larval fish bioassay.

We exposed larval sheepshead minnows, *Cyprinodon variegatus*, to *Pfiesteria* spp. in six-well tissue culture plates containing polycarbonate membrane inserts (Millicell). This created two compartments within each well ('in', inside insert; 'out', outside insert), allowing separation of fish from dinospores across a permeable membrane (Fig. 1). Mortalities occurred only in treatments where fish and *P. shumwayae* dinospores were in direct physical contact (Fig. 2a: B in, D in, F). Fish physically separated from dinospores (A in, B out versus in) did not die, even if they resided within the same well as dying fish in contact with dinospores (B in versus out). Fish in negative controls (C) and fish exposed to a non-pathogenic strain



**Figure 1** Experimental design for the membrane insert study using larval *Cyprinodon variegatus* exposed to *Pfiesteria shumwayae* (Ps) and *P. piscicida* (Pp).

## letters to nature

of *P. piscicida* dinospores (G) exhibited no mortalities. When fish and *P. shumwayae* dinospores were in physical contact, mortalities began within several hours of assay initiation, reached 7.4–25.9% by 24 h and 92–100% by 48 h (Fig. 2a). Dinospore densities increased significantly after 24 h in treatments where they were in contact with fish (B in, D in, F), but remained near initial levels in treatments where they were separated from fish (A out, D out) (Fig. 2b). Reactive ammonia levels (Fig. 2c) remained below 0.25 mg l<sup>-1</sup> and dissolved oxygen concentrations (Fig. 2d) remained above 6.5 mg l<sup>-1</sup> in all treatments.

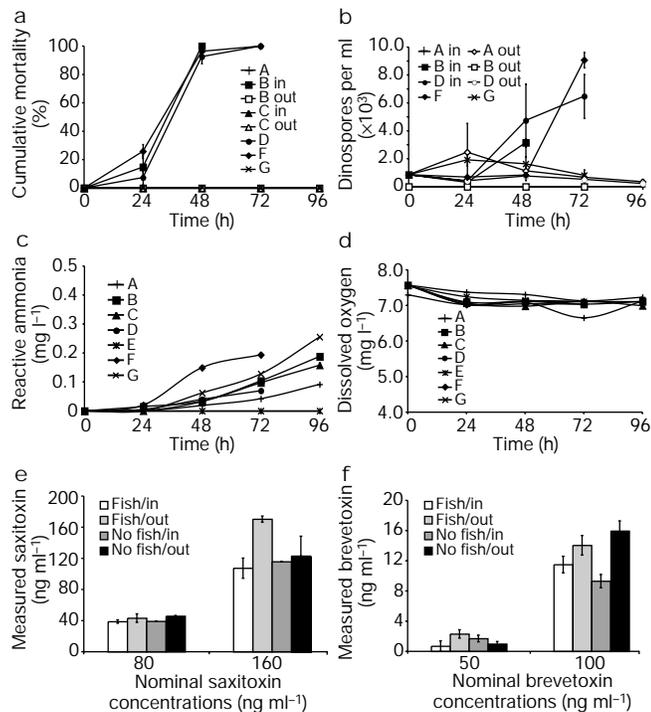
To test permeability of the polycarbonate membrane to representative lipophilic and hydrophilic algal toxins, we exposed larval fish, sequestered in clean water within membrane inserts, to a range of brevetoxin (PbTx-2) and saxitoxin (STx) concentrations placed outside the insert. Additional replicates were run without fish. After a 24-h period of exposure and equilibration, we measured toxin concentrations inside and outside the inserts. Both toxins readily diffused across the polycarbonate membrane, and rapidly equilibrated, in the presence and absence of fish (Fig. 2e, f). Fish exposed to 100 ng ml<sup>-1</sup> (nominal) FbTx-2 exhibited 100% mortality within 24 h. No mortalities occurred at lower PbTx-2 doses, in STx exposures, or in controls.

We conducted fractionation studies to investigate exotoxin secretion further and to evaluate potential contributions of con-

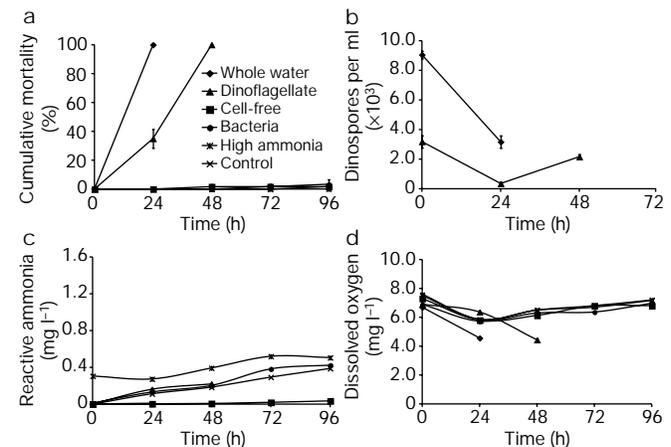
taminating microbial organisms and poor water quality to fish mortality. We centrifuged and filtered water taken from a 38-l bioassay of *P. shumwayae* that actively killed tilapia, *Oreochromis niloticus*. The various fractions were assayed on larval fish. Fish in 'whole water' and in the 'dinoflagellate'-enriched fractions exhibited 100% mortality within 24 and 48 h, respectively (Fig. 3a). Negligible mortality (<3.3%) occurred in the 'cell-free' (putatively 'exotoxin' enriched) and 'bacteria' fractions and controls. Dinospore densities in the whole-water and dinoflagellate fractions ranged from ~350 to ~9,000 cells ml<sup>-1</sup> during exposures (Fig. 3b). Water quality measurements were similar among all treatments. Reactive NH<sub>3</sub> in all treatments remained below 'high' ammonia controls (Fig. 3c). Dissolved oxygen ranged from 7.6 to 4.4 mg l<sup>-1</sup>; the lowest values occurred after fish mortality in the whole-water and dinoflagellate fractions at 24 and 48 h, respectively (Fig. 3d). Similar fractionation studies with larval fish have been repeated six times with identical results (data not shown).

Microscopically, dinospores of *P. shumwayae* placed with fish exhibited rapid chemotaxis, attachment to fish epidermis by a peduncle, and feeding by myzocytosis<sup>17</sup>. Dinospores fed for about 1 min, swelling significantly as the epidermal cell cytoplasm was internalized; they then detached from fish and slowly swam away. Our *P. piscicida* cultures, which have never been pathogenic, did not exhibit attachment and feeding (see Supplementary Information).

Scanning electron microscopy (SEM) of larval fish skin after brief exposure to *P. shumwayae* showed focal epidermal degeneration associated with dinospore feeding. Myzocytosis caused contraction and sloughing of epidermal cells and cellular debris, rapidly creating shallow epidermal erosions seen only in association with numerous adhering dinospores (Fig. 4a). Dinospores attached by the distal end of the peduncle, which exhibited small filopodial extensions. The surface of affected epidermal cells was extensively damaged (Fig. 4b, c). Transmission electron microscopy (TEM) showed the distal peduncular surface of an actively feeding dinospore tightly apposed to a small group of epidermal cells, which, according to the nuclear and cytosolic morphology, were degenerating. Epidermal cell organelles, including mitochondria and rough endoplasmic reticulum, occurred within the peduncle, indicating ingestion and transport toward a large food vacuole within the dinospore epicone (Fig. 4c). Peduncles not yet attached to fish epidermis exhibited small distal



**Figure 2** Results from bioassays using larval *Cyprinodon variegatus* exposed in six-well tissue culture plates fitted with polycarbonate membrane inserts. **a–d**, Mortality, cell counts and water quality from a 96-h bioassay. **e, f**, Measured saxitoxin and brevetoxin concentrations from a 24-h bioassay. **a**, Cumulative fish mortality in treatments containing fish. Survival analysis showed significant differences in mortality rates between fish in contact with dinospores and those separated from contact (Tarone-Ware  $\chi^2 = 226.55$ , d.f. = 7,  $P < 0.001$ ). **b**, Cell count data from selected treatments where dinospores were in direct contact with fish and other selected treatments. **c**, Reactive ammonia concentrations for all treatments (inside and outside water samples pooled). **d**, Dissolved oxygen concentrations for all treatments (inside and outside water samples pooled). **e**, Measured saxitoxin concentrations inside and outside polycarbonate membrane inserts after 24 h, in the presence and absence of fish. **f**, Measured brevetoxin concentrations inside and outside polycarbonate membrane inserts after 24 h, in the presence and absence of fish.



**Figure 3** Fractionation assay with larval *Fundulus heteroclitus* using a *Pfiesteria shumwayae* culture that actively killed tilapia in a 38-l assay. **a**, Cumulative fish mortality during a 96-h fractionation study. Survival analysis showed significantly different mortality rates between fish exposed to fractions containing dinoflagellates and those exposed to other fractions (Tarone-Ware  $\chi^2 = 302.03$ , d.f. = 5,  $P < 0.001$ ). **b**, Dinospore cell count in the 'whole water' and 'dinoflagellate' fractions. **c**, Reactive ammonia concentrations for all treatments over the 96-h exposure. **d**, Dissolved oxygen concentrations for all treatments over the 96-h exposure.

peduncular filopodia and numerous electron-dense, rod-shaped granules (Fig. 4, inset). Both structures were absent in peduncles that had attached to epidermal cells.

We report here an alternate mechanism of pathogenicity for *P. shumwayae*. In contrast to prior studies<sup>18,19</sup>, our *P. shumwayae* cultures, although highly pathogenic to fish, do not produce exotoxins. Rather, they kill fish by myzocytosis, a feeding mechanism previously observed in non-toxic ambush predator dinoflagellates<sup>20,21</sup> and *Pfiesteria* spp.<sup>22,23</sup>, but not generally implicated in fish mortality. Despite almost a decade of research, isolation and characterization of *Pfiesteria* toxins have not been achieved. However, two distinct fractions with biological activity were recently isolated from *P. piscicida* cultures<sup>8</sup>. A lipophilic fraction was dominated by a phthalate ester determined to be a contaminant of artificial sea salts used to produce culture water. A hydrophilic fraction (designated pPfTx) induced a GH<sub>4</sub>C<sub>1</sub> reporter gene assay, caused cell toxicity and killed brine shrimp and fish<sup>8</sup>. However, typical epidermal pathology attributable to *Pfiesteria* exposure<sup>16</sup> was

not produced by these fractions. The pPfTx was also shown to interact with a P2X<sub>7</sub> receptor suggested to mediate GH<sub>4</sub>C<sub>1</sub> cytotoxicity<sup>24</sup>. Further, pPfTx selectively inhibited the *N*-methyl-D-aspartate neurotransmitter receptor, although an association with animal toxicity was not shown<sup>25</sup>.

On the basis of the recently proposed nomenclature for *Pfiesteria* functional types (for example, tox-A, tox-B and non-inducible)<sup>5</sup>, our *P. shumwayae* cultures should be 'non-inducible' and should not kill fish. We maintained these cultures on algae for more than 24 months, presumably too long to maintain fish-killing capacity<sup>5</sup>. However, using standard protocols<sup>5,26</sup> and environmentally relevant cell densities (300–2,500 cells ml<sup>-1</sup>)<sup>27</sup>, our cultures kill fish within hours (tox-A equivalent) and elicit typical pathology previously ascribed to *Pfiesteria* toxigenicity<sup>4,5</sup>. Thus, our findings challenge the utility of what has been described as the 'gold standard' 38-l *Pfiesteria* toxicity bioassay<sup>19,26</sup>, which uses fish neurobehavioural changes, gross pathology and mortality as sole endpoints of 'toxigenicity'. That assay cannot discriminate between truly toxigenic cultures and cultures that kill fish by myzocytosis. Our findings indicate that fish mortality after *P. shumwayae* exposure results from micropredatory feeding on captive fish prey. Certainly, myzocytosis resulting in widespread skin damage and rapid mortality can convincingly be demonstrated *in vitro*. However, what role, if any, *Pfiesteria* spp. have in morbidity and mortality of wild fishes has recently been questioned<sup>13–16,28,29</sup> and remains unclear. Only assays that routinely apply fractionation or membrane isolation protocols will discriminate between disparate killing mechanisms. We therefore recommend that all strains and cultures of *Pfiesteria* be re-evaluated for pathogenicity by these protocols. □

## Methods

### *Pfiesteria* culture

The clonal culture of *Pfiesteria shumwayae* (VIMS-1049) was established from a water sample obtained from the Pamlico River, North Carolina, on 12 November 1999. It has been deposited with the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP number 2089). Dinoflagellate species were definitively identified by SEM plate tabulations<sup>18,30</sup> and by comparing DNA sequence analysis of the small subunit ribosomal RNA gene with GenBank deposited sequences (*P. piscicida* accession number AF149793, *P. shumwayae* AF080098). Culture identities were intermittently re-verified using species-specific polymerase chain reaction primers described in Supplementary Information.

### Membrane insert study

The experimental design illustrated in Fig. 1 prevented dinospores from making physical contact with fish, yet allowed exotoxins, if present, to diffuse across a membrane and affect fish. We used membrane inserts that fit into wells of tissue culture plates (see below). Cultures of *P. shumwayae* (VIMS-1049) and *P. piscicida* (VIMS-P11) were used at a density of ~1,000 cells ml<sup>-1</sup> in sterile-filtered autoclaved (12‰) York River water supplemented with penicillin (2,500 IU l<sup>-1</sup>) and streptomycin (2.5 mg l<sup>-1</sup>).

### Membrane permeability study

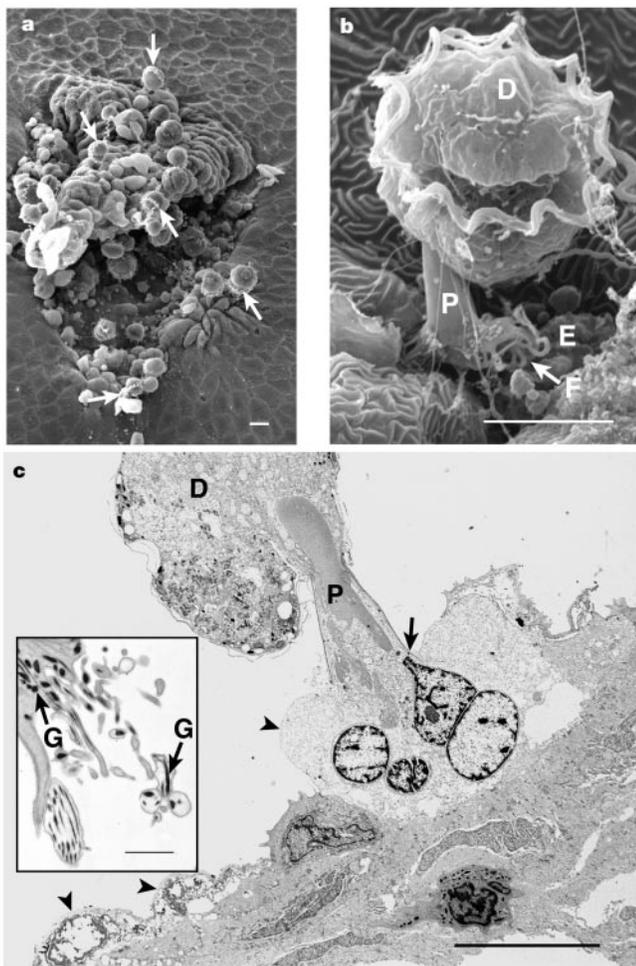
Brevetoxin extracts used in the exposures were produced from a *Karenia brevis* strain isolated from the 1999 bloom at Pensacola Beach, Florida, and maintained at the Environmental Protection Agency (EPA) Laboratory, Gulf Breeze, Florida. Brevetoxin isolation protocols are described in Supplementary Information. Brevetoxin extract (1 vial: 50 µg PbTx-2, 0, 10, 25, 50, 100 ng ml<sup>-1</sup>; STx, 0, 20, 40, 80, 160 ng ml<sup>-1</sup>). A membrane insert (Millicell, polycarbonate, pore size 3 µm) was set into each well and 5 ml of clean filtered ASW (12‰) was immediately added to the insert. Three replicates per dose received five larval fish within the insert, whereas three replicates per dose remained without fish. The assay was run for 24 h in a BSL2 cabinet at room temperature (22 °C) with mortalities recorded at the end of the study. Brevetoxin and saxitoxin analyses are detailed in Supplementary Information.

Saxitoxin (Sigma; 10 µg) was solubilized in 0.5 ml of ASW (12‰) and then dissolved in 62 ml (62.5 ml total volume) of ASW to obtain a high-dose stock solution (160 ng ml<sup>-1</sup>). We distributed 30 ml of this stock to six wells (5 ml well<sup>-1</sup>), and serially diluted 30 ml to obtain the lower concentrations.

The assay was conducted in six-well tissue culture plates (Falcon) over a range of concentrations (PbTx-2, 0, 10, 25, 50, 100 ng ml<sup>-1</sup>; STx, 0, 20, 40, 80, 160 ng ml<sup>-1</sup>). A membrane insert (Millicell, polycarbonate, pore size 3 µm) was set into each well and 5 ml of clean filtered ASW (12‰) was immediately added to the insert. Three replicates per dose received five larval fish within the insert, whereas three replicates per dose remained without fish. The assay was run for 24 h in a BSL2 cabinet at room temperature (22 °C) with mortalities recorded at the end of the study. Brevetoxin and saxitoxin analyses are detailed in Supplementary Information.

### Fractionation study

To identify and select cultures, we obtained materials for fractionation studies from 'standard' 38-l bioassay aquaria<sup>19</sup> that exhibited high mortalities of tilapia (20–30% daily).



**Figure 4** Myzocytosis by *Pfiesteria shumwayae* on the epidermis of larval *Cyprinodon variegatus*. **a**, Scanning electron micrograph (SEM) of early epidermal erosion (<5 min) with numerous attached dinospores (arrows). **b**, SEM of single dinospore (D) attached to and feeding on damaged epidermal cell (E). Note the distal end of peduncle (P) exhibiting filopodial projections (F). **c**, Transmission electron micrograph (TEM) of dinospore (D) attachment (arrow) and feeding on epidermal cells. Note the epidermal cytoplasm and organelles within the dinospore peduncle (P), and the cytoplasmic rarefaction and nuclear degeneration of affected cells (arrowheads). Inset, TEM of distal portion of an unattached peduncle showing filopodial extensions and rod-shaped electron-dense granules (G). Scale bars: **a**, **c**, 10 µm; **b**, 5 µm; inset, 1 µm. Two short video clips of myzocytosis by *P. shumwayae* on larval fish skin (~1 min) can be seen in Supplementary Information.

Protocols for the 38-l bioassays are detailed in Supplementary Information. Water was taken from an aquarium previously amended with an environmental water sample from Slocum Creek, North Carolina, and verified by cell counts, SEM and molecular analyses to contain high densities of *P. shumwayae*.

Water from the actively killing tank was used as a positive ('whole water') control. An enriched 'dinoflagellate' fraction was produced by filtering, rinsing and resuspending 3 l of whole water. Viability of dinoflagellates in this fraction was confirmed microscopically. A 'bacteria' fraction was obtained by centrifuging 3 l of whole water at 8,500 g for 45 min at 10 °C, resuspending the pellet in 12‰ ASW, filtering through a 5-µm filter to remove dinoflagellates and other protozoa, and bringing the filtrate to 3 l using 12‰ ASW. A 'cell-free' fraction was obtained by removing the supernatant from the centrifuged bacterial pellet and filtering through a 5-µm and then a 0.45-µm filter to a volume of 3 l. Filter-sterilized 12‰ ASW was used as a negative control. We also used a 'high ammonia' control consisting of 12‰ ASW with ammonia and pH adjusted to that of the whole water.

## Electron microscopy

Fish killed with trichaine methanesulphonate (MS-222) were fixed in 4% glutaraldehyde with 5% paraformaldehyde in 0.1 M sodium cacodylate buffer at pH 7.2, at room temperature for ~2 h. Samples were washed with three changes of 0.1 M sodium cacodylate buffer, 15–30 min each, and stored overnight at 4 °C in a third change of buffer. They were postfixed with 1% OsO<sub>4</sub> in 0.1 M buffer, pH 7.2, at room temperature for 1 h and then washed with buffer in three changes of 0.1 M cacodylate, pH 7.2, 15–30 min each. The caudal peduncle was cut off the fish with a single-edged razor blade and processed for TEM analysis, and the body of each fish was processed for SEM by standard methods detailed in Supplementary Information.

## Other methods

Larval fish sources, water quality measurements and cell count protocols are detailed in Supplementary Information.

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## Competing interests statement

The authors declare that they have no competing financial interests.

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# Protein phosphatase 1 is a molecular constraint on learning and memory

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Repetition in learning is a prerequisite for the formation of accurate and long-lasting memory. Practice is most effective when widely distributed over time, rather than when closely spaced or massed. But even after efficient learning, most memories dissipate with time unless frequently used<sup>1,2</sup>. The molecular mechanisms of these time-dependent constraints on learning and memory are unknown. Here we show that protein phosphatase 1 (PP1) determines the efficacy of learning and memory by limiting acquisition and favouring memory decline. When PP1 is genetically inhibited during learning, short intervals between training episodes are sufficient for optimal performance. The enhanced learning correlates with increased phosphorylation of cyclic AMP-dependent response element binding (CREB) protein, of Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) and of the GluR1 subunit of the AMPA receptor; it also correlates with CREB-dependent gene expression that, in control mice, occurs only with widely distributed training. Inhibition of PP1 prolongs memory when induced after learning, suggesting that PP1 also promotes forgetting. This property may account for