including warming in northwestern North America and Greenland, increased drought in the western United States, and increased precipitation in the Canadian Arctic (*19*). We can also eliminate the known effects of forest harvest on $\delta^{15}N$ values (*20*), because there is no indication of land-use change in all but one of these watersheds. Finally, although we cannot fully exclude an increased influx of isotopically depleted N from vegetated catchments as temperature increased after the Little Ice Age (*21*) and as glaciers receded (*22*), the broad geographic distribution of our sites suggests that such mechanisms are unlikely to produce the consistent pattern observed. We contend that coherent historical changes in sedimentary $\delta^{15}N$ are explained most parsimoniously by elevated deposition of Nr from atmospheric and, ultimately, human sources.

Although the effects of Nr deposition on watersheds have been studied extensively in more-populated regions of the northeastern United States and Europe, the effects of low-level chronic Nr deposition have not been given the same attention. Our results show that the sources of N to ecosystems throughout the Northern Hemisphere have changed substantially over the past century; however, the biogeochemical and ecological effects of this widespread disturbance are essentially unexplored. A growing body of literature, primarily from arctic and alpine regions, has identified substantial changes in algal communities over a similar 100- to 150-year period, including accelerated turnover in species composition, enhancement of planktonic communities, and greater abundance of nitrophilous taxa (*7, 15, 23*). These ecological shifts have been previously attributed to climate warming and attendant limnological effects, including prolonged lake stratification and an expansion of algal habitat. Our results point to an important alternate and potentially synergistic mechanism that further explains processes underlying the accelerating pace of global ecosystem change.

Two implications emerge from these findings. First, although most of the detectable change in lake N geochemistry from anthropogenic Nr has happened in recent decades with widespread industrial N fixation, it began with fossil fuel use and industrialization near the beginning of the 20th century. Second, despite relatively low current rates of atmospheric Nr deposition in remote areas (*4, 24*), increases since preindustrial times are large enough to be imprinted coherently in the lake sediment record and thus are likely to permeate the biosphere at the hemispheric scale.

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Supporting Online Material

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Database S1

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High Relatedness Is Necessary and Sufficient to Maintain Multicellularity in *Dictyostelium*

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Most complex multicellular organisms develop clonally from a single cell. This should limit conflicts between cell lineages that could threaten the extensive cooperation of cells within multicellular bodies. Cellular composition can be manipulated in the social amoeba *Dictyostelium discoideum*, which allows us to test and confirm the two key predictions of this theory. Experimental evolution at low relatedness favored cheating mutants that could destroy multicellular development. However, under high relatedness, the forces of mutation and within-individual selection are too small for these destructive cheaters to spread, as shown by a mutation accumulation experiment. Thus, we conclude that the single-cell bottleneck is a powerful stabilizer of cellular cooperation in multicellular organisms.

Many of the major transitions in evolution have resulted from groups of lower-level units cooperating so extensively that they merged into a higher-level unit (*1–7*). When the units belong to the same species, such as cells in multicellular organisms and individuals in eusocial insect colonies, high relatedness is essential (*2–7*). High relatedness reduces conflicts among units and can select for some to sacrifice themselves for others, and these factors yield the reproductive division of labor seen in both kinds of systems (germ-soma and worker-reproductive). High relatedness is nearly universal in these entities; eusocial insects likely evolved only from monogamous families [relatedness (r) = 1/2] (*8*), and multicellular organisms are typically clonal cell groups (r = 1) (*5*). But little direct evidence supports the hypothesis that conflict reduction via clonality is either necessary or sufficient to maintain multicellularity in any eukaryotic system.

Theoretical models show that a combination of sufficiently high mutation rates plus within-individual selection could favor the spread of cheaters in clonal systems (*9–11*). It has been suggested that key steps in metazoan development

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might reflect a history of such within-organism selection; cell movements might be the relics of an ancient jockeying for position in the germ line, and induction events could evolve as manipulations of other cell lineages to perform somatic functions (*1*). However, the crucial mutation rates to cheaters have not been measured, partly because in most systems, a mutation that destroys subsequent development is lethal and cannot be studied for cheating properties. We tested both the necessity and sufficiency of relatedness in multicellularity by exploiting the social amoeba *Dictyostelium discoideum*, which has a unicellular stage that allows development-destroying mutations to be propagated and characterized (*12*, *13*).

In the *D. discoideum* life cycle (*14*–*16*), the unicellular stage consists of asexually reproducing haploid amoebae. When they run out of bacterial prey, they sense starvation, and thousands of cells aggregate together to form a multicellular fruiting body. Roughly 80% of the cells form a sorus of hardy fertile spores; the remaining 20% die to build a sterile stalk that holds these spores aloft and helps with their dispersal. When multiple genetically distinct clones aggregate together, one clone may cheat by forming disproportionately more spores, while forcing others to form more than their share of the sterile stalk (*12*, *16*, *17*). Facultative cheaters retain the ability to cooperate and form normal fruiting bodies on their own (*18*). We focused here on obligate cheaters, which cannot fruit on their own, so their spread may lead to the demise of cooperative development.

We ran two experimental evolution studies with the laboratory strain AX4: a low-relatedness experiment and a mutation-accumulation experiment (Fig. 1A) (*19*). In the first, we artificially constructed low-relatedness conditions and allowed evolution to proceed. Beginning with a clonal isolate of AX4, we transferred 10^6 spores to new nutrient plates with bacteria and allowed them to produce multicellular fruiting bodies. We did this for 31 generations of fruiting body formation (roughly 290 cell divisions) for each of 24 lines. Although each line starts out with maximal genealogical relatedness, the relatedness that matters for selection on social mutants depends on how well mixed they are with wild type (*20*). Tests using labeled and unlabeled clones under the same conditions generated randomly mixed fruiting bodies (*19*) (fig. S1).

Instead of doing an exactly parallel experiment under clonal conditions, which would yield results only for the particular number of cell generations and selection coefficients in the experiment, we estimated the mutation rate to obligate cheaters, which allows us to understand selection on clonal life cycles for any desired number of cell generations or selection coefficients. In our mutation accumulation study, we used a single clonal isolate to initiate 90 experimental lines (*19*). We put each through 70 single-cell bottlenecks, amounting to 1000 cell generations, by transferring one random isolated colony of cells from one nutrient plate with bacteria to another (Fig. 1A). In mutation accumulation studies, drift is maximized and selection is minimized by bottlenecking (*21*). Here, direct selection in multicellular fruiting bodies is completely eliminated because transfers were done before that stage occurred. Ten control lines went through the same treatment but without the single-cell bottlenecks.

For both experimental evolution experiments, we assessed the cheating ability of the evolved lines in social competition with their ancestors. We prepared 1:1 experimental mixes of the ancestor with each line with one of them fluorescently labeled. We also prepared labeled and unlabeled control mixes of each ancestor. We

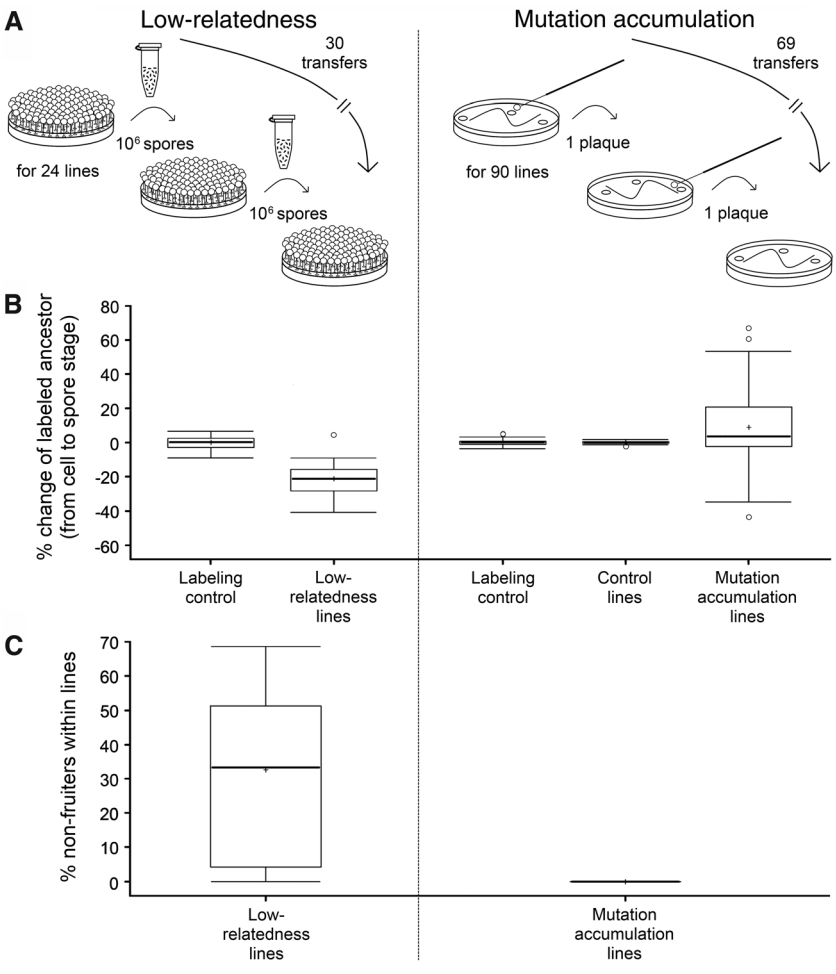

Fig. 1. Cheating in the low-relatedness and mutation accumulation studies. (**A**) Basic setup for both experiments. (**B**) Cheating differences among low-relatedness lines, mutation accumulation lines, and controls. (**C**) Percentage of clones isolated from the low-relatedness and mutation accumulation lines that could not fruit.

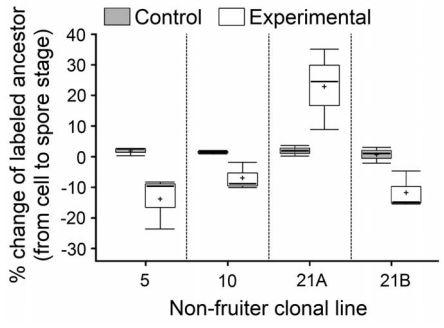

Fig. 2. Three of four nonfruiting clonal isolates significantly cheated their ancestor relative to labeling controls.

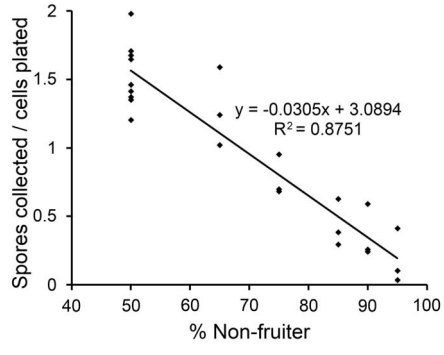

Fig. 3. High percentages of cheaters in a mix decrease total spore production of the entire mix as measured by the number of spores produced per cell plated.

plated these mixtures of starved cells onto nutrient-free substrates, which allowed them to form fruiting bodies, and we collected the resulting spores. Then, we calculated the percent change of the ancestor in each experimental and control mix as: 100 × [(% ancestor in spores) – (% ancestor in cells)]/(% ancestor in cells).

As predicted, the low-relatedness lines significantly cheated their ancestor compared with labeling controls ($P < 0.05$, $n = 24$, 20, Games-Howell post hoc test) (Fig. 1B). The mutation accumulation lines were slightly cheated by their ancestor on average compared with both labeling controls and control lines ($P < 0.05$, $n = 90$, 84, 10, respectively, Games-Howell post hoc test) (Fig. 1B), although, as expected from the accumulation of random mutations, the variance among individual lines was large. More striking was the nature of the cheating in the low-relatedness lines. Of individual clones isolated from these lines, an average of 31.23 ± 24.53% (SD) could not form fruiting bodies on their own, and presumably they spread by cheating in chimeras with others lacking the mutation (Fig. 1C). We tested four nonfruiting clonal isolates from three low-relatedness lines and found that three out of four of these nonfruiters were strong cheaters (Fig. 2) {nonfruiter 5 [$t(2) = 3.801$, $P = 0.031$], nonfruiter 10 [$t(2) = 3.196$, $P = 0.043$], and nonfruiter 21B [$t(2) = 5.091$, $P = 0.018$]}; nonfruiter 21A showed a trend in the opposite direction [$t(2) = –2.59$, $P = 0.061$]. In 1:1 mixtures with their ancestor, they did not reduce total spore production (fig. S4). However, as the percentage of nonfruiters increased above 50%, which occurred in many of the experimental lines (Fig. 1C and fig. S3), the number of spores produced decreased markedly (Fig. 3) (regression $y = –0.0305x + 3.0894$, $R^2 = 0.8751$, $P < 0.0001$). Low relatedness had favored obligate cheaters that greatly lower multicellular cooperation.

These obligate cheaters are similar to the laboratory-created obligate cheater *fbxA*$^-$ (*12*, *22*) in that they can increase under low relatedness to the point where they drastically lower population spore production. Our results show that such mutations occur naturally and can increase to levels that strongly decrease cooperation in the population in only a few generations. This agrees with bacterial studies (*23*, *24*) but shows that such cheaters are a threat to multicellular development in a eukaryote.

Obligate cheaters are absent or very rare in the field, where relatedness is high (around 0.9 or higher) (*22*), owing to some combination of dispersal structure and some ability to exclude foreign clones from fruiting bodies (*25*, *26*). Our mutation accumulation experiment shows that obligate cheaters do not arise often enough to threaten clonal development. In contrast to the low-relatedness lines, although some mutation accumulation lines cheated, there were no nonfruiters (Fig. 1C) in 90,000 mutational opportunities (90 lines times 1000 generations). The Poisson 95% upper bound number of mutations is 3.69, yielding an upper-bound mutation rate of 4.10×10^{-5} per cell per generation. We used this estimate to predict the maximum spread of such mutants when there are single-cell bottlenecks:

$$\Delta q = \frac{q(1-q)S}{1+qS} + (1-q)F \qquad (1)$$

Here, the first term is the standard between-organism selection equation for haploids, with frequency q of cheaters and selection coefficient S against them at the multicellular level. The second term is the fraction of normal fruiter clones at the single-cell bottleneck stage $(1 – q)$ times the fraction F of their descendent spores that become cheaters via mutation plus within-individual selection favoring cheaters. In the next steps, we assumed that mixed fruiting bodies suffer no fitness cost and that there is no reverse mutation to noncheaters. These assumptions make it easier for cheaters to spread. Let D be the number of rounds of clonal cell division from initial spore to fruiting body. Then, F is the product of nonfruiters that arise by mutation (μD) and the selective advantage of each, $(1 + s)/(1 + \mu Ds)$, where s is the within-individual selection coefficient favoring cheaters and $1 + \mu Ds$ is mean within-individual fitness. This assumes that each cheater cell gets a single opportunity to cheat; we do not consider cell-division rate mutants (*9*–*11*), as there is little cell division during the *D. discoideum* multicellular stage (*27*).

With F defined this way, the equilibrium frequency of cheaters $\{\Delta q = 0; q = –F/[S(1 + F)]\}$ is a function of rounds of cell division D (Fig. 4). The solid line represents obligate cheaters ($S = –1$; on their own, they produce no spores and die) that obtain the maximal cheating advantage ($s = 0.25$, if we assume 100% become spores in mixtures compared with the normal 80%). *D. discoideum* fruiting bodies have 1000 to 100,0000 cells, which require about 10 to 17 rounds of division. In this range, nonfruiters have little opportunity to increase (Fig. 4). If we allow for mortality by letting $D = 20$, the frequency of nonfruiters still remains below 0.001. Even with 60 divisions (which, for reference, could produce a mass equal to a great blue whale), it is still only about 0.003. Clearly the combination of mutation to nonfruiters plus cheating selection poses little threat to *D. discoideum* multicellularity under clonal development. Indeed, removing cheating selection entirely ($s = 0$) shows that the small threat of losing the ability to fruit is primarily a conventional issue of mutational load (Fig. 4, dotted line) and is only modestly raised by cheating. We have assumed that cells cannot survive starvation without fruiting, but even if we allow 50% of such cells to survive ($S = –0.5$), the equilibrium frequencies of cheaters double and remain small. Only if multicellular-level costs are small (e.g., $S = –0.1$) can cheaters spread somewhat more easily (Fig. 4, dashed line), but these are not cheaters that destroy multicellularity.

Control of conflicts is essential in every transition to a higher level of selection (*1*, *2*). In social insect colonies, where relatedness is high but less than one, added control is provided by mechanisms like behavioral policing (*28*). With clonal development in *D. discoideum,* such additional mechanisms do not seem to be needed; high relatedness is both necessary and sufficient to control conflicts that destroy multicellular cooperation in the multicellular stage. Although cell division–rate mutants remain an important avenue for future research, the control of cheating demonstrated here provides a model for why

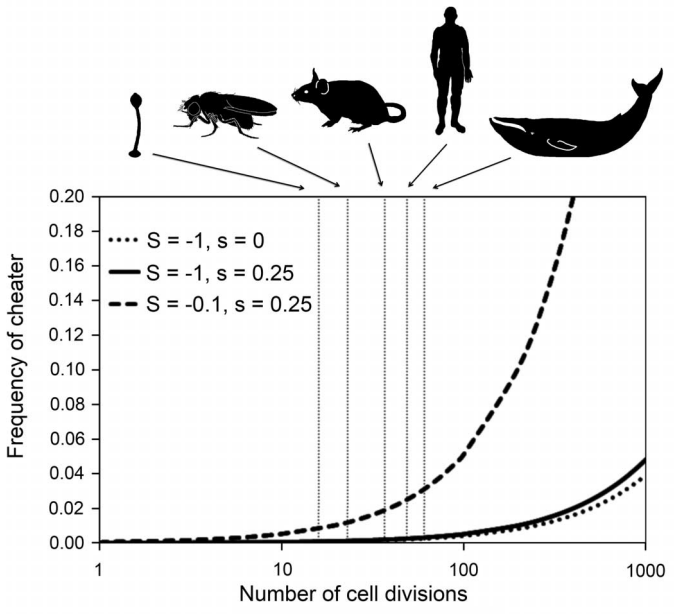

Fig. 4. Cheaters that could eliminate cooperation at low relatedness cannot spread much if single-cell bottlenecks occur at least every 100 generations. Plot of the equilibrial frequency of cheaters under clonal development with maximal estimated mutation rate (4.1×10^{-5}), as a function of the number of cell divisions from the founding cell. S is the selection coefficient against clonal cheater groups, and s is the selective advantage of cheaters within mixed groups. The silhouettes indicate the approximate number of *Dictyostelium* cell divisions that would be needed to produce the mass of a large *Dictyostelium* fruiting body, a *Drosophila* (1 mg), a mouse (25 g), a human (100 kg), and a great blue whale (350,000 kg) on the basis of an estimated 11-μm cell diameter (*29*).

single bottlenecks are so common in other organisms, like metazoan animals, where the experimental manipulations we used are currently difficult or impossible.

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Supporting Online Material

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The Tricarboxylic Acid Cycle in Cyanobacteria

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It is generally accepted that cyanobacteria have an incomplete tricarboxylic acid (TCA) cycle because they lack 2-oxoglutarate dehydrogenase and thus cannot convert 2-oxoglutarate to succinyl–coenzyme A (CoA). Genes encoding a novel 2-oxoglutarate decarboxylase and succinic semialdehyde dehydrogenase were identified in the cyanobacterium *Synechococcus* sp. PCC 7002. Together, these two enzymes convert 2-oxoglutarate to succinate and thus functionally replace 2-oxoglutarate dehydrogenase and succinyl-CoA synthetase. These genes are present in all cyanobacterial genomes except those of *Prochlorococcus* and marine *Synechococcus* species. Closely related genes occur in the genomes of some methanogens and other anaerobic bacteria, which are also thought to have incomplete TCA cycles.

The tricarboxylic acid (TCA) cycle, also known as the Krebs or citric acid cycle, has two functions in bacteria: It oxidizes two-carbon units derived from acetyl–coenzyme A (CoA) producing carbon dioxide and the reduced form of nicotinamide adenine dinucleotide (NADH), which provides the electrons for oxidative phosphorylation, and it provides essential precursor metabolites [e.g., oxaloacetate, 2-oxoglutarate (2-OG), and in some species succinate] that are required for biosynthesis of cellular components. In 1967 two groups reported the failure to detect 2-oxoglutarate dehydrogenase (2-OGDH), which converts 2-oxoglutarate to succinyl-CoA, in various cyanobacteria (*1*, *2*). During the ensuing 44 years, it has become common knowledge that these organisms have an incomplete TCA cycle [see, e.g., comments in (*3*, *4*)]. The absence of 2-OGDH has also frequently been discussed as a contributing factor to explain why most cyanobacteria are obligate photolithoautotrophs (*5*–*7*). Consistent with these initial observations, no fully sequenced cyanobacterial genome encodes the genes for 2-OGDH (*7*). The incomplete TCA cycle has recently been incorporated into metabolic models for *Synechocystis* sp. PCC 6803 [e.g., (*8*–*10*)].

Alternatives to 2-oxoglutarate dehydrogenase are known to participate in the TCA cycles of a few organisms, including those of *Euglena gracilis* mitochondria (*11*) and *Mycobacterium* spp. (*12*). In the latter organism, a thiamine pyrophosphate (TPP)–dependent enzyme, 2-oxoglutarate decarboxylase (2-OGDC), is structurally related to the large subunit of 2-OGDH and produces succinic semialdehyde (SSA). SSA is subsequently oxidized to succinate by succinic semialdehyde dehydrogenase (SSADH). Although cyanobacteria lack homologs of this type of 2-OGDC, *Synechococcus* sp. PCC 7002 and many other cyanobacteria encode homologs of SSADH. This observation, as well as the fact that mutants of *Synechocystis* sp. PCC 6803 lacking succinate dehydrogenase still synthesize succinate (*13*), strongly suggested that cyanobacterial genomes encode a previously unrecognized 2-OGDC. A gene neighborhood analysis revealed that the gene encoding SSADH (SynPCC7002_A2771) occurred as part of an apparent operon comprising two genes in most cyanobacteria, in which one of the genes (SynPCC7002_A2770) encodes an enzyme similar to, but phylogenetically distinct from, acetolactate synthase (IlvB). Because acetolactate synthase is a TPP-dependent enzyme, like 2-OGDH and 2-OGDC (*14*), it seemed possible that these two open reading frames might encode the enzymes replacing 2-OGDH. An examination of transcription data for these genes under many different growth conditions (*15*) strengthened this hypothesis, because the transcript levels of these two genes varied coordinately with those for other genes encoding enzymes of the TCA cycle.

Open reading frames SynPCC7002_A2770 and SynPCC7002_A2771 were separately expressed in *Escherichia coli*, and the resulting soluble proteins were purified (*16*). When the product of SynPCC7002_A2771 was incubated with SSA, succinate was produced and nicotinamide adenine dinucleotide phosphate ($NADP^+$) was reduced (Fig. 1A). When the products from SynPCC7002_A2770 and SynPCC7002_A2771 were incubated with 2-OG, 2-OG was quantitatively converted to succinate (*16*) and $NADP^+$ was reduced; however, 2-OG was consumed but no succinate was produced when $NADP^+$ was omitted (Fig. 1B). When the product of SynPCC7002_A2770 was incubated with 2-OG alone, an aldehyde was produced that could be detected using Schiff's reagent (Fig. 1C). These observations establish that SynPCC7002_A2770 encodes a novel 2-OGDC that produces SSA and SynPCC7002_A2771 encodes SSADH, which jointly replace 2-OGDH and succinyl-CoA synthetase (Fig. 1D).

Peptides from SynPCC7002_A2770 and SynPCC7002_A2771 were detected in the soluble proteome of *Synechococcus* sp. PCC 7002 (*17*). To investigate the function of these two

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High Relatedness Is Necessary and Sufficient to Maintain Multicellularity in *Dictyostelium*

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