Conversion of Ectoderm to Mesoderm by Cytoplasmic Extrusion in Leech Embryos
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populations show significant within-population heterogeneity. This may be because the three r-populations became genetically differentiated from each other due to random fixation of alleles during the 188 generations preceding the present experiment. The rK-populations derived from them would be similarly genetically heterogeneous. This between-population variation would affect the measured traits. We can deal with this by comparing each replicate rK-population to the r-population from which it was derived. These comparisons yield no significant difference in growth rate at the lowest density between r_1 and rK_1 or r_3 and rK_3 but the difference between rK_2 and r_2 (-0.82) is statistically significant.

Our previous work has shown that certain larval characters, such as larval feeding rates and pupation site choice have become differentiated between the r- and K-populations (*9–11*). These behavioral traits contribute to adaptation at high population density: in food-limited environments increased pupation height increases viability (*10*) and increased larval feeding rate increases larval competitive ability (*11*). These characters have also become differentiated between the r- and rK-populations (*12*), confirming that the rK-populations are adapting to these crowded environments.

The results of the experiments reported here confirm earlier observations of fitness trade-offs arising from density-dependent natural selection (*6*). These experiments also show that these trade-offs can arise simply from the process of *Drosophila* adapting to crowded population conditions. The r-populations has spent nearly 200 generations in the low-density conditions. The possibility that during the course of the current experiment (generation 201 to 225 in the r-populations) there were further major phenotypic changes in the r- or $r \times r$-populations is minimal. Consequently, the changes now observed between the r- and rK-populations and between the $r \times r$- and $r \times rK$-populations may be safely attributed to changes in the high-density populations as they adapt for 25 generations to these novel environments.

As pointed out above, the primary characters responsible for the differences in population growth rate seem to be larval attributes. These larval differences have presumably developed in response to the high larval densities. Although there are differences in the timing of adult reproduction in the r- and K-populations (*13*), the likelihood that these time differences influence the evolution of the larval characters is minimal because they should have no effect on larval densities.

Much of the current theory in life history evolution assumes that trade-offs in fitness components are important determinants that constrain the direction of evolution (*14*). For instance, in *Drosophila* populations that experience a wide range of population densities through time, neither the r phenotype or the K phenotype described here would be most fit at all times. There are few well-documented examples of such trade-offs during evolution. Rose (*15*) has shown that natural selection for increased longevity in *D. melanogaster* has been accompanied by a decline in early fecundity of females. The possibility exists that natural selection may act further to offset the maladaptive features of trade-offs (*16*). Our populations of *Drosophila* represent one of the few instances in which trade-offs have been repeatedly demonstrated to affect the outcome of natural selection. Understanding the genetic and physiological underpinnings of these trade-offs becomes an important goal for future research in evolutionary ecology.

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Conversion of Ectoderm to Mesoderm by Cytoplasmic Extrusion in Leech Embryos

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The role of cytoplasmic domains in the determination of the fates of ectodermal and mesodermal cells has been investigated in leech embryos. When yolk-deficient cytoplasm (teloplasm) was extruded from the animal pole of the zygote, the ectodermal precursor blastomere was converted to a mesodermal fate. This change of fate can be prevented by replacement of the extruded animal teloplasm with teloplasm from the vegetal pole. The fate of the mesodermal precursor blastomere was unaffected by teloplasm extrusion or rearrangement. These results demonstrate that ectodermal and mesodermal determination of fate involves a binary decision dependent on the position of teloplasm along the animal-vegetal axis.

THE SPECIFICATION OF ECTODERMAL and mesodermal fate occurs early in the development of most animals and is achieved by a variety of mechanisms, including the segregation of localized determinants during cleavage (*1*). Like many invertebrates, glossiphoniid leeches develop in a highly determinate manner, meaning that early cell divisions are stereotyped and give rise to unique cells with predictable fates (*2–4*). The separation of segmental ectodermal and mesodermal lineages occurs with the cleavage of a single cell, when blastomere D′ gives rise to cells DNOPQ and DM (*5*) at the fourth cleavage (Fig. 1A). DNOPQ, which is situated more toward the animal pole than DM, subsequently produces four bilateral pairs of ectodermal stem cells (the N, O/P, O/P, and Q ectoteloblasts) and 13 micromeres, whereas DM, which is situated more toward the vegetal pole than DNOPQ, gives rise to one bilateral pair of mesodermal stem cells (mesoteloblasts) and two micromeres (*6*). Each teloblast undergoes a series of highly unequal divisions to generate a chain (bandlet) of smaller blast cells. Subsequently, the left and right bandlets coalesce along the ventral midline into a sheet of cells called the germinal plate

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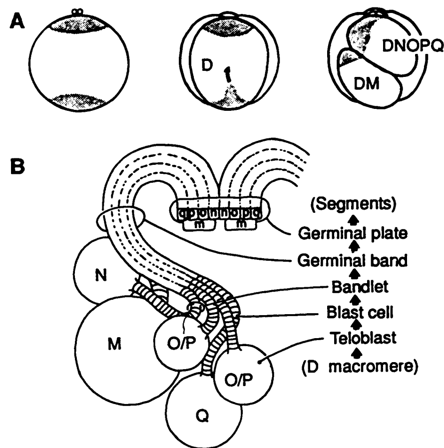

Fig. 1. Schematic summary of glossiphoniid leech development. (**A**) (**Left**) Zygote. After two polar bodies (small circles) are extruded at the animal pole (top), teloplasm (shaded regions) forms as two pools at the animal and vegetal poles. (**Center**) Four-cell embryo. Cell D, the parent of cell D′, inherits both pools of teloplasm. Most of the vegetal teloplasm then migrates to the animal pole and mixes with the animal teloplasm between the second and fourth rounds of cleavage (arrow). (**Right**) Twelve-cell embryo. Two of the seven micromeres at the animal pole are shown. DNOPQ and DM each inherit a mixture of animal and vegetal teloplasm. (**B**) Stage 8. Shown are the five teloblasts on the left side of the embryo and their bandlets of blast cells, which coalesce to form the left germinal band. The left and right germinal bands then join at the future ventral midline (top of figure) to form the germinal plate.

Table 1. Fate of the DAn and DVg blastomeres in control and experimental embryos assessed at early stage 8. Numbers of ETB or MTB derived from these blastomeres are indicated. The micromeres derived from these cells were not included in this analysis. (ETB, ectoteloblasts and bandlets; MTB, mesoteloblasts and bandlets; *n*, number of embryos.)

Group (*n*)	Fate of DAn blastomere			Fate of DVg blastomere		No. of unhealthy embryos
	ETB (*n*)	MTB (*n*)	Other (*n*)	MTB (*n*)	Other (*n*)	
Control (72)	8 (69)			2 (69)		3
Vg^x (34)	8 (30) 8 (3)* 7 (1)			2 (33) 1 (1)		
An^x (43)	8–9 (6) 5–6 (3)	3–6 (5) 2 (18) 1 (4)	(2)†	2 (37)	(1)†	5
Centrifuged An^x (27)	9–11 (3) 8 (14) 6–7 (3)	2 (4)	(1)‡	2 (25)		2

*These embryos also had disorganized germinal bands. †Fate was one large cell without bandlets. ‡Fate was >20 cells without bandlets.

(Fig. 1B). The blast cells derived from DNOPQ ultimately generate segmental epidermis, neurons, and glia, whereas those derived from DM give rise to segmental muscle, nephridia, a few neurons, and contractile fibers of the embryonic integument (*3, 7, 8*).

After zygote deposition and approximately 2 hours before first cleavage, two large pools of yolk-deficient cytoplasm [teloplasm (*2*)] arise at the animal and vegetal poles (Figs. 1A and 2A). Teloplasm, which is enriched in mitochondria, endoplasmic reticulum, and polyadenylated RNA (*9*), segregates to blastomere D′ during the first three rounds of cleavage and then to DNOPQ and DM. Teloplasm is thought to induce the formation of teloblasts in the D′ lineage, because redistribution of teloplasm to other blastomeres by centrifugation (*10*) or by reorientation of cleavage planes (*11*) results in a correlated redistribution of teloblast-forming potential. Before the fourth cleavage, most of the vegetal teloplasm moves toward the animal pole of cell D′. As a result, DNOPQ and DM each inherit a mixture of animal and vegetal teloplasm (*12*) (Fig. 1A), and this led to the proposal that the two pools of teloplasm are equipotent with respect to ectodermal and mesodermal fate determination.

To test this hypothesis directly, we selectively removed newly formed animal or vegetal teloplasm from zygotes of *Helobdella robusta* (*13*) approximately 1 hour before first cleavage. This was done by immobilization of zygotes in a suction chamber and piercing of the vitelline and cell membranes over the target teloplasm with a glass micropipette (tip diameter, 10 μm). Embryos were then inverted so that the opening of the suction chamber covered the wounded end of the cell, and the target teloplasm, along with some yolky cytoplasm, was removed by gentle suction. Embryos with no visible residual teloplasm at the operated pole were either allowed to develop under normal culture conditions (*14*) or were fixed and sectioned to confirm the success of the removal procedure (*15*) (Fig. 2). Approximately 60% of the embryos that were left to develop underwent the first four rounds of cleavage on schedule, with normal geometry and with normal segregation of the remaining pool of teloplasm. After fourth cleavage, experimental embryos resembled control embryos in that they had three macromeres, the normal complement of micromeres at the animal pole, and two cells derived from the obliquely equatorial cleavage of D′. These two cells were analogous by shape and position to DNOPQ and DM but will be referred to as DAn and DVg, respectively, to avoid confusion regarding their fates. Either DAn or DVg was injected with the lineage tracer rhodamine dextran amine (RDA) (*16*) at this time. Embryos that cleaved abnormally during the first four cleavages were discarded and were not considered further.

The majority (30/34) of embryos from which vegetal teloplasm was extruded (Vg^x embryos) developed normally for at least 60 hours [to early stage 8 (*17*)], at which time they were processed for epifluorescence microscopy (*18*) (Table 1). At this stage, ectodermal and mesodermal cell lineages can be distinguished by the number and position of their teloblasts and bandlets (*2*) (Fig. 3), the timing and

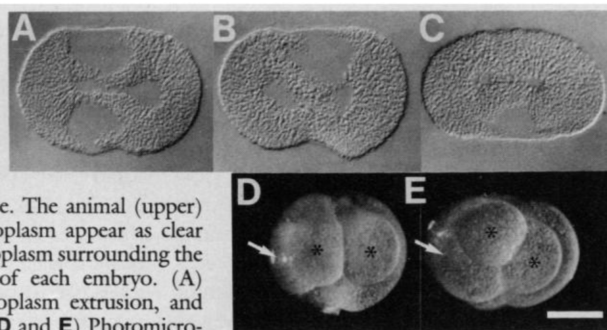

Fig. 2. Teloplasm extrusion efficacy and early cleavages in operated embryos. (**A** through **C**) Meridional sections parallel to the spindle axis through one-cell embryos fixed (*15*) just before first cleavage and viewed with Nomarski optics. Yolky cytoplasm has a granular appearance. The animal (upper) and vegetal (lower) pools of teloplasm appear as clear yolk-deficient regions, as does cytoplasm surrounding the dividing nucleus in the center of each embryo. (A) Control embryo, (B) vegetal teloplasm extrusion, and (C) animal teloplasm extrusion. (**D** and **E**) Photomicrographs of operated embryos after the cleavage of DAn. (D) Vg^x embryo in which DAn has cleaved with a normal animal-vegetal orientation to yield two NOPQ daughter cells (asterisks). The arrow points to the left M teloblast and is orthogonal to the plane of cleavage of DVg. (E) An^x embryo in which DAn has cleaved at an oblique DM-like orientation to yield two mesoteloblasts (asterisks). As in (D), the arrow points to the left M teloblast and is orthogonal to the plane of cleavage of DVg. Scale bar is 100 μm for (A) through (C) and 110 μm for (D) and (E).

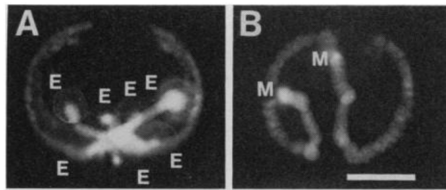

Fig. 3. Vg^x embryos form normal ectodermal and mesodermal lineages. Dorsal view of Vg^x embryos at early stage 8 in whole-mount under rhodamine epifluorescence (*18*). Anterior is up. (**A**) Embryo in which DAn was injected with RDA and gave rise to a normal DNOPQ lineage consisting of eight ectoteloblasts (seven are visible and marked E) and bandlets. (**B**) Embryo in which DVg was injected with RDA and gave rise to two normal mesoteloblasts (M) and bandlets. Scale bar is 100 μm.

orientation of blast cell divisions (*4*) (Fig. 4, A and D), and the mesodermal contribution of contractile fibers to the embryonic integument (*7*). Vg^x embryos were normal by all these criteria and the RDA labeling patterns confirmed that DAn and DVg had produced the DNOPQ and DM lineages, respectively (Fig. 3). Because Vg^x embryos were indistinguishable from control embryos in all aspects examined, we concluded that vegetal teloplasm is not required for normal development up to stage 8 (*19*).

In marked contrast to the results for Vg^x embryos, in embryos from which animal teloplasm was extruded (An^x embryos), ectodermal teloblasts and bandlets were usually lacking after 60 hours of development (Table 1). In the majority (27/43) of these embryos, DAn gave rise to one or more mesodermal teloblasts and bandlets, as judged by the following criteria: (i) the first division of the blast cells in each bandlet was transverse rather than longitudinal and occurred at a distance of 10 to 12 blast cells rather than 20 to 30 blast cells from the parent teloblast (*4*); (ii) the second blast cell divisions were oriented 90° relative to the first and occurred at distances of 16 to 18 blast cells from the parent teloblast; (iii) anterior (older) portions of each bandlet formed hemisomites (*20*); (iv) scattered cells were typically present outside the bandlets, corresponding to putative contractile fibers of the provisional integument (*7*); and (v) the bandlets were deep rather than superficial (Fig. 4).

The number of mesoteloblasts produced by DAn varied from one to six; however, in most cases, two were produced, as in the normal DM lineage. The fate of DVg was not affected by animal teloplasm extrusion. In all but one embryo, DVg generated two mesoteloblasts and bandlets (Fig. 2E). In general, mesodermal bandlets derived from DAn and DVg were disorganized; however, the anterior portions of bandlets from sibling teloblasts were usually joined as in normal mesoderm (*21*). In other An^x embryos, DAn either failed to cleave (2/43 embryos) or produced a DNOPQ-like lineage consisting of five to nine ectoteloblasts and bandlets (9/43 embryos) (*22*). In no case did DAn or DVg generate a mixture of ectodermal and mesodermal teloblasts.

We examined An^x embryos for early differences between the DNOPQ and DM lineages to determine the stage at which DAn began to deviate from the normal DNOPQ fate. During normal development, DNOPQ produces three micromeres at specific locations near the animal pole and then cleaves meridionally, whereas DM produces two micromeres and then cleaves obliquely (Fig. 2D) (*6*). In four out of five An^x embryos, DAn generated only two micromeres and then cleaved obliquely, much like a normal cell DM (Fig. 2E). It therefore appears that DAn adopts a mesodermal identity within one to three cell cycles of its birth. The fact that it does so in the presence of a normal complement of other embryonic cells (specifically, DVg and the A-, B-, and C-derived micromeres and macromeres) makes it unlikely that intercellular signals determine ectodermal fate.

From these experiments, we concluded that removal of the animal teloplasm from zygotes causes DAn, the nominal ectodermal precursor, to adopt a mesodermal fate. In contrast, removal of the vegetal teloplasm does not appear to affect embryogenesis. If the two teloplasms are equivalent, as suggested (*10, 12*), then the key determinant of ectodermal and mesodermal fates may be the position of teloplasm along the animal-vegetal axis. In An^x embryos, even though the vegetal teloplasm still migrated toward the animal pole of blastomere D, it rarely appeared to fully replace the extruded animal teloplasm. This observation was confirmed in embryos fixed during the cleavage of cell D′ and then sectioned for assessment of the distribution of teloplasm (*15, 23*). In control embryos ($n = 12$), teloplasm was found in a single pool adjacent to and contacting the animal pole, and approximately 555 ± 103 pl (mean ± SD) of teloplasm was inherited by DAn. Vg^x embryos ($n = 8$) had similar teloplasm distributions, with approximately 428 ± 75 pl of teloplasm partitioned to DAn. In contrast, in An^x embryos ($n = 6$) teloplasm lay more centrally and did not contact the animal pole, and only 180 ± 15 pl of teloplasm was inherited by DAn. To test whether vegetal teloplasm placed at the animal pole could rescue the ectodermal fate in An^x embryos, we embedded 31 An^x embryos, selected randomly from a group of 60, in low–melting temperature agarose at the four-cell stage and centrifuged them with the animal pole oriented centripetally (700*g*, 2 to 4 min). This forced the vegetal teloplasm to the animal pole of blastomere D, the precursor of D′. All of these embryos underwent normal third and fourth cleavages, after which DAn was injected with RDA. Four embryos were fixed during the cleavage of D′ and sectioned for morphometric analysis. The distribution of teloplasm resembled that of uncentrifuged An^x embryos in that only 203 ± 50 pl of teloplasm was inherited by DAn but differed in that teloplasm contacted the animal pole, as in control and Vg^x embryos. Moreover, in 74% (20/27) of the embryos that were left to develop [compared to only 21% (6/29) of uncentrifuged An^x embryos], DAn generated ectodermal teloblasts and bandlets (Table 1 and Fig. 4E). In almost all cases, DVg gave rise to two mesoteloblasts and bandlets. Most of the centrifuged embryos generated an abnormal number of ectoteloblasts or had disorganized bandlets;

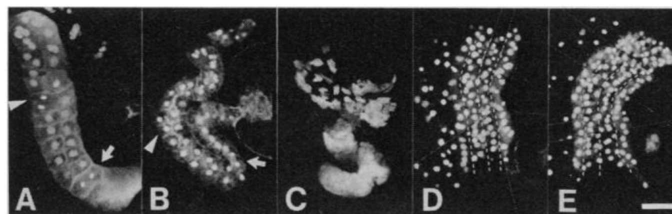

Fig. 4. Conversion of ectoderm to mesoderm and rescue of the ectodermal fate. Stage 8 embryos in which one blastomere was injected with RDA were processed for epifluorescence microscopy (*18*) and optically sectioned at 5-μm intervals on a Bio-Rad (Cambridge, Massachusetts) confocal microscope. Cell nuclei appear as bright spots. Four to eight optical sections were superimposed to generate each image; bandlets appear truncated because cells outside these focal planes are not visible. In each panel, anterior is up. (**A**) Left mesodermal bandlet from a control embryo showing the characteristic orientations of the first (arrow) and second (arrowhead) blast-cell divisions. Primary undivided blast cells are out of focus at the lower right. (**B**) One of two mesodermal bandlets derived from DAn in an An^x embryo showing the mesoderm-like first (arrow) and second (arrowhead) blast-cell divisions. (**C**) Anterior end of a mesodermal bandlet derived from DAn in an An^x embryo showing migratory cells that in normal mesoderm generate the putative contractile fibers of the embryonic integument. (**D**) The right germinal band of a control embryo showing (from right to left) the n, o, p, and q ectodermal bandlets and the characteristic longitudinal orientation of their early blast-cell divisions. Dashed lines separate the bandlets. Extraneous micromere-derived nuclei are present at the left and between bandlets. (**E**) The right germinal band of an An^x embryo in which the ectodermal fate was rescued by centrifugation at the four-cell stage. In this embryo, eight ectoteloblasts were produced by DAn, and four of their bandlets are shown here. As in (D), extraneous micromere-derived nuclei are also present. Scale bar is 25 μm in (A) through (C) and 30 μm in (D) and (E).

nonetheless, well-organized germinal bands formed in about 30% of the cases. Centrifuged control embryos ($n = 5$) developed normally. Thus, vegetal teloplasm can rescue the ectodermal fate when placed at the animal pole.

From these results, we drew three conclusions about the specification of ectoderm and mesoderm in the leech. First, determinants for these fates are not exclusively localized to the animal or vegetal teloplasm, because both ectodermal and mesodermal precursors can arise in embryos having only animal teloplasm (that is, Vg^x embryos) or only vegetal teloplasm (that is, centrifuged An^x embryos). Second, DAn is competent to become either an ectodermal or mesodermal precursor, whereas DVg seems competent to produce only mesoderm. Third, DAn makes a binary decision between ectodermal and mesodermal fates on the basis of the position of teloplasm along the animal-vegetal axis.

These conclusions are consistent with several models for determination of ectodermal and mesodermal fates. In principle, either the relative or absolute volumes of teloplasm inherited by DNOPQ and DM could specify these fates. However, our measurements of these parameters show only slight differences between conditions that induce ectoderm and mesoderm and thus limit the robustness of any such mechanism. We propose instead that factors imparting ectodermal competence are localized to the animal cortex of the early embryo and thus are stable to teloplasm extrusion and are inherited by DNOPQ, and not DM, at fourth cleavage. We further propose that the ectodermal fate requires a short-range interaction between these cortical factors and teloplasm sometime after second cleavage. In the absence of such an interaction (as in An^x embryos), or in the absence of the factors altogether (as in DM), a mesodermal fate is adopted.

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19. The proportion of embryos in which we failed to achieve complete extrusion of the vegetal teloplasm, as assessed by examination of sectioned zygotes fixed shortly after extrusion, was only 13% (6/45 embryos). This is significantly less than the proportion of Vg^x embryos that developed normally (46% or 30/65), even including in the total those embryos that were discarded because of abnormal early cleavages. Therefore, we rejected the possibility that the 30 Vg^x embryos that developed normally did so because they contained residual vegetal teloplasm.
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22. The proportion of embryos in which we failed to achieve complete extrusion of the animal teloplasm was 13% (2/16). By the Fisher-Irwin Exact test, this proportion is not significantly different from the proportion of An^x embryos in which DAn exhibited an ectodermal fate (21%). Thus, it is possible that those embryos that formed ectoteloblasts did so because they contained residual animal teloplasm.
23. We measured teloplasm volumes by viewing serial 14-μm sections mounted on microscope slides using differential interference contrast optics with a video camera. The perimeter of the teloplasm was traced with a digitizing tablet connected to a computer graphics workstation (Polycad 10, Cubicomp Corporation, Berkeley, CA). The perinuclear cytoplasm was included in these measurements because it is continuous with and indistinguishable from teloplasm at this stage of development. The area of the polygon was calculated by the computer program and multiplied by the thickness of the section. Volumes obtained for serial sections were then summed. In sections where the teloplasm was not yet completely partitioned between DAn and DVg, a line was drawn through the teloplasm in alignment with the cleavage furrow.

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The 2.3 Angstrom X-ray Structure of Nitrite Reductase from *Achromobacter cycloclastes*

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The three-dimensional crystal structure of the copper-containing nitrite reductase (NIR) from *Achromobacter cycloclastes* has been determined to 2.3 angstrom (Å) resolution by isomorphous replacement. The monomer has two Greek key β-barrel domains similar to that of plastocyanin and contains two copper sites. The enzyme is a trimer both in the crystal and in solution. The two copper atoms in the monomer comprise one type I copper site (Cu-I; two His, one Cys, and one Met ligands) and one putative type II copper site (Cu-II; three His and one solvent ligands). Although ligated by adjacent amino acids Cu-I and Cu-II are ~12.5 Å apart. Cu-II is bound with nearly perfect tetrahedral geometry by residues not within a single monomer, but from each of two monomers of the trimer. The Cu-II site is at the bottom of a 12 Å deep solvent channel and is the site to which the substrate (NO_2^-) binds, as evidenced by difference density maps of substrate-soaked and native crystals.

Nitrite reductases (NIRs) are part of a denitrification pathway in which nitrate is sequentially reduced to nitrite, nitric oxide, nitrous oxide, and dinitrogen ($NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) (*1*). Denitrification is a process that primarily uses energy, occurs anoxically, and has become increasingly important in eutrophic ecosystems (*2*). Denitrification in soil is an important mechanism for loss of plant nutrients and essentially runs counter to nitrogen fixation, and hence is as important a process as nitrogen fixation and photosynthesis in understanding the nitrogen cycle.

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