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 r1 and rK1 or r2 and rK2 but the difference between rK2 and r2 (−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 K-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 rK-populations is minimal. Consequently, the changes now observed between the r- and rK-populations and between the r and rK-populations may be slightly 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.

REFERENCES AND NOTES
17. We thank M. Rose and three referees for useful comments on the manuscript and R. McCleary for statistical advice. Supported in part by a grant from the Department of Energy and by NIH grant BRSK S07 R07006.
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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, glosso-}
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 in 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' 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

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<th>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, ectotelooblasts and bandlets; MTB, mesotelooblasts and bandlets; n, number of embryos.)</th>
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*These embryos also had disorganized germinal bands. †Fate was one large cell without bandlets. ‡Fate was >20 cells without bandlets.
orientation of blast cell divisions (4) (Fig. 4, A and D), and the mesodermal contribution of contractile fibers to the embryonic integument (7). Vg² embryos were normal by all these criteria and the RDA labeling patterns confirmed that DAn and DVg had produced the DNO/PQ and DM lineages, respectively (Fig. 3). Because Vg² embryos were indistinguishable from control embryos in all aspects examined, we concluded that vegetal teloplasms is not required for normal development up to stage 8 (19). In marked contrast to the results for Vg² embryos, in embryos from which animal teloplasms was extruded (An² 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 hemismones (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 mesoteleoblasts produced by DAn varied from one to six; however, in most cases, two were produced, as in the normal DAn lineage. The fate of DVg was not affected by animal teloplasms extrusion. In all but one embryo, DVg generated two mesoteleoblasts 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 An² embryos, DAn either failed to cleave (2/43 embryos) or produced a DNO/PQ-like lineage consisting of five to nine ectoteleoblasts and bandlets (9/43 embryos) (22). In no case did DAn or DVg generate a mixture of ectodermal and mesodermal teloblasts.

We examined An² embryos for early differences between the DNO/PQ and DM lineages to determine the stage at which DAn began to deviate from the normal DNO/PQ fate. During normal development, DNO/PQ 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 An² 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 teloplasms from zygotes causes DAn, the nominal ectodermal precursor, to adopt a mesodermal fate. In contrast, removal of the vegetal teloplasms 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 teloplasms along the animal-vegetal axis. In An² embryos, even though the vegetal teloplasms still migrated toward the animal pole of blastomere D, it rarely appeared to fully replace the extruded animal teloplasms. This observation was confirmed in embryos fixed during the cleavage of cell D' and then sectioned for assessment of the distribution of teloplasms (15, 23). In control embryos (n = 12), teloplasms 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. Vg² embryos (n = 8) had similar teloplasms distributions, with approximately 425 ± 75 pl of teloplasm partitioned to DAn. In contrast, in An² embryos (n = 6) teloplasms 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 teloplasms placed at the animal pole could rescue the ectodermal fate in An² embryos, we embedded 31 An² 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 (700g, 2 to 4 min). This forced the vegetal teloplasms 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 teloplasms resembled that of uncentrifuged An² embryos in that only 203 ± 50 pl of teloplasm was inherited by DAn but differed in that teloplasms contacted the animal pole, as in control and Vg² embryos. Moreover, in 74% (20/27) of the embryos that were left to develop [compared to only 21% (6/29) of uncentrifuged An² embryos], DAn generated ectodermal teloblasts and bandlets (Table 1 and Fig. 4E). In almost all cases, DVg gave rise to two mesoteleoblasts and bandlets. Most of the centrifuged embryos generated an abnormal number of ectoteleoblasts or had disorganized bandlets;
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 trimmer 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 ligand). 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$_3^-$) binds, as evidenced by difference 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$_3^-$ → NO → N$_2$O → 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 important as a process for nitrogen fixation and photosynthesis in understanding the nitrogen cycle.

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**REFERENCES AND NOTES**


5. 2D and 2d by the terminology of K. J. Müller [Z. Wiss. Zool. 142, 425 (1935)].


13. Embryos were obtained from a laboratory colony. *Holobdella robusta* is a species of glossiphoniid leech (M. Shankland, S. T. Bissen, D. A. Weisblat, in preparation) that closely resembles *Holobdella intestinalis*, the object of much previous work on this genus.


15. Sectioned embryos were fixed at 4°C overnight in 4% formaldehyde and 0.1% tri-HCl buffer (pH 7.4), rinsed several times in tri-HCl buffer, dehydrated in an ethanol series, embedded in JB4 embedding resin (Polysciences, Warrenton, PA), and sectioned at 14 µm with a glass knife microtome.


18. Embryos processed for epifluorescence microscopy were fixed for 1 hour at room temperature in 1.0% formaldehyde in 0.08 M sodium cacodylate buffer (pH 7.5), rinsed and manually destained in 0.1 M phosphate-buffed saline (PBS) (pH 7.4), and then incubated at room temperature for approximately 12 hours in each of the following solutions: blocking solution (TBP), consisting of PBS, 2% bovine serum albumin, 1% Triton X-100, and sodium azide (1%); a 1:100 dilution of mouse monoclonal antibody to leech nuclei in TBP; several changes of TBP; a 1:250 dilution of fluorescent-conjugated rabbit secondary antibody to mouse antibodies (ICN Biomedicals, Lisle, IL) in TBP; and several changes of PBS. Embryos were then post-fixed for 1 hour at room temperature in 4% formaldehyde with H3chopher 33258 (5 µg/ml) in 0.1 M tri-HCl buffer, rinsed several times in 0.1 M tri-HCl buffed, dehydrated in an ethanol series, and cleared in methyl salicylate.

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% (69/485) embryos. This is significantly less than the proportion of Vtg 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 Vtg embryos that developed normally did so because they contained residual vegetal teloplasm.


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 embryos in which DAn exhibited an ectodal ectodermal fate (21%). Thus, it is possible that those embryos that formed ectodetrains 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 (Polyquad 10, Cubicon Corporation, Berkeley, CA). The pinacoidal 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.

24. We thank S. Bissen, K. Symes, L. Gizzler, R. Kostenk, C. Wedeen, K. Anderson, and J. Gerhart for reading this manuscript and D. S. Stuart (University of California, Berkeley) for supplying the antibody to leech nuclei. Supported by a postgraduate scholarship from Natural Sciences and Engineering Research Council, Ottawa, Ontario, Canada, to B.H.N. and by NSF grant DCB 87-11262 to D.A.W.

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