moved. Through a 1-cm² window, right wing limb buds were removed and replaced by right wing limb buds from donor embryos; normal orientation was maintained. Cutting was done with fine glass needles. Grafted limbs were held in place by fine glass tacks and allowed to heal 2 to 3 hours under a warming lamp. Tacks were removed; 2.5 μg of amphotericin B and 50 units each of penicillin and streptomycin (Gibco, Grand Island, N.Y.) in Tyrode's solution were added per egg; the eggs were sealed with Parafilm and incubated on their sides to hatching. Approximately 10 percent of the operated embryos hatched. In the embryos which did not hatch the morphology of the transplanted limbs was normal.

6. G. Filogamo and G. Gabella, *Arch. Biol.* **78**, 9 (1967); L. Roncali, *Monit. Zool. Ital.* **4**, 81 (1970).
7. S. L. McMurty, L. M. Julian, V. S. Asmundson, *Arch. Pathol.* **94**, 217 (1972). Homozygous dystrophic embryos were from line 413, derived recently from line 304, maintained by the Department of Avian Sciences, University of California, Davis. Normal embryos were from a commercial White Leghorn flock, Donsing Hatcheries, Rio Linda, California.
8. T. A. Linkhart, G. W. Yee, B. W. Wilson, P. S. Nieberg, in preparation.
9. E. Cosmos and J. Butler, in *Research in Muscle Development and the Muscle Spindle*, B. Q. Banker, R. J. Przybylski, J. P. Van der Meulen, M. Victor, Eds. (Excerpta Medica, Amsterdam, 1972), p. 149.
10. V. Askanas, S. A. Shafiq, A. T. Milhorat, *Arch. Neurol.* **24**, 259 (1971); J. H. Peacock and P. G. Nelson, *J. Neurol. Neurosurg. Psychiatry* **36**, 389 (1973).
11. J. B. Harris and M. W. Marshall, *Exp. Neurol.* **41**, 331 (1973); W. B. Douglas and E. Cosmos, in *Exploratory Concepts in Muscle* (11), A. T. Milhorat, Ed. (Proceedings of the International Conference, Carefree, Arizona, 15 to 19 October 1973, Excerpta Medica, Amsterdam, in press).
12. C. R. Ashmore and L. Doerr, *Exp. Neurol.* **30**, 431 (1971); E. X. Albuquerque and J. E. Warnick, *Science* **172**, 1260 (1971); J. Jedrzejczyk, J. Wieckowski, T. Rymaszewska, E. A. Barnard, *ibid.* **180**, 406 (1973).
13. S. A. Cohen and G. D. Fischbach, *Science* **181**, 76 (1973); D. B. Drachman, *Ann. N.Y. Acad. Sci.* **228**, 160 (1974); E. X. Albuquerque, J. E. Warnick, F. M. Sansone, R. Onur, *ibid.*, p. 224.
14. Supported in part by PHS grant NS 01957 and by a grant from the Muscular Dystrophy Associations of America, Inc. T.A.L. is an NSF predoctoral trainee in physiology. We thank Dr. U. K. Abbott for her help and encouragement in development of the transplantation technique, Ms. P. S. Nieberg and Mr. F. Lantz for technical assistance, and Mr. J. Schenkel for his initial work in the study.

7 August 1974

Nitrogen Fixation in Marine Shipworms

Abstract. *Nitrogen fixation is associated with four shipworm species. A bacterium capable of fixing nitrogen under anaerobic conditions and of liquefying cellulose in culture has been isolated from the gut of one species. High fixation rates (up to 1.5 micrograms of nitrogen per milligram dry weight per hour), which resulted in a doubling of cellular nitrogen in as little as 1.4 days, was associated with* Teredora malleolus *from the Sargasso Sea. Three species from coastal waters were assayed, and of these juveniles showed the highest fixation rates. Nitrogen fixation activity appeared to be inversely related to the ability of shipworms to obtain combined-nitrogen compounds in their diet. It could be a significant source of nitrogen for shipworms and perhaps other oceanic organisms that ingest terrestrial plant material.*

Wood is notoriously low in combined-N compounds, and this presents a nutritional problem to any organism feeding on it. Woody plant tissue contains only 0.03 to 0.10 percent N, and the C : N ratio of most wood species is about 300 to 500 (*1*). In contrast, the C : N ratio of phytoplankton, the major food of herbivores in the sea, is about 5 to 10 (*2*).

Table 1. Nitrogen fixation rates, dry weights, and lengths of nine individuals of *Teredora malleolus* collected from a 30-m-long pine log in the Sargasso Sea, September 1973. Three of the shipworms assayed (17, 18, and 38 mm long) had no measurable N_2 fixation. On later inspection it was noted that these worms had been damaged on extraction from the log.

Dry weight (mg)	Length (mm)	N_2 fixation (μg mg^{-1} hour^{-1})
11.2	12	1.56
11.4	9	0.57
21.7	15	1.51
58.9	23	0.21
151.3	29	0.0003

Shipworms are bivalve mollusks (family Teredinidae), the adults of which are obligatory wood borers and cellulose metabolizers (*3*). They have a functional gill (ctenidium) capable of filtering plankton for food (*4*); however, shipworms have been raised to sexual maturity on wood in filtered seawater with no plankton available for feeding (*5*). Since cellulose constitutes a major portion of the shipworm diet, and juvenile shipworms can grow extremely rapidly [from 0.2 mm to 6 cm in length in 1 month (*6*)], how they obtain combined-N compounds sufficient for protein metabolism is puzzling. We present data showing that N_2 fixation is associated with shipworms and suggest that this process may contribute to the nitrogen metabolism of these organisms.

Nitrogen fixation was demonstrated in juveniles and adults of four shipworm species (Fig. 1, Table 1). The acetylene reduction technique (*7*) was used to assay for N_2 fixation (*8*). Three species, *Psiloteredo megotara, Lyrodus pedicellatus*, and *Teredo navalis*, were obtained from wood held in a running seawater system in Woods Hole, Massachusetts. The fourth species, *Teredora malleolus*, typically found inhabiting floating wood in offshore waters, was obtained from a pine log found floating at 32°18′N, 60°21′W in the Sargasso Sea in September 1973.

For the three coastal species, N_2 fixation was inversely related to shipworm dry weight (Fig. 1). All individuals weighing less than 2.0 mg and 65 percent of those weighing more than 10 mg had measurable N_2 fixation rates. The relation between shipworm body weight and rate of N_2 fixation fits an inverse hyperbolic curve. On a logarithmic plot (Fig. 1) the data exhibit a statistically significant linear relation ($P < .05$) with a correlation coefficient of .76. Juvenile shipworms had the highest N_2 fixation rates: one *L. pedicellatus* juvenile fixed up to 81 ng of N_2 per milligram dry weight per hour; and three *L. pedicellatus* and one *T. navalis* juveniles, each less than 600 μg (dry weight), averaged 31 ng mg^{-1} hour^{-1}. The only larvae tested, those of *L. pedicellatus*, had no associated N_2 fixation.

Adult *Teredora malleolus* from the Sargasso Sea had N_2 fixation rates of up to 1.5 μg mg^{-1} hour^{-1}, exceeding the fixation rates of coastal shipworms by a factor of almost 20 (Table 1). These data suggest an N_2 fixing system which assumes greater importance when there is a dietary deficiency of combined N, for example, in areas of low phytoplankton density like the Sargasso Sea (*9*), or when the animals

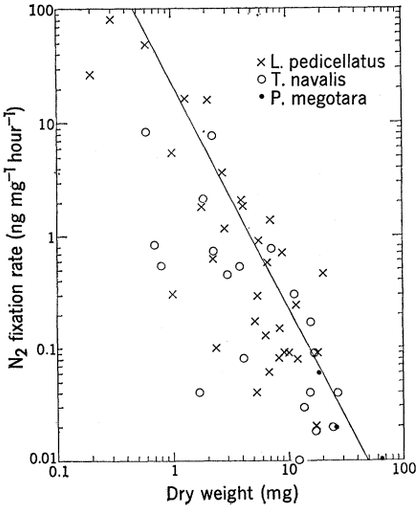

Fig. 1. Rate of nitrogen fixation associated with three coastal shipworm species.

are young and the small gill appears inefficient for suspension feeding.

It appears that N_2 fixation could be a significant supplementary source of combined-N compounds for some shipworms. For shipworms from the Sargasso Sea, the time required to double cellular N (T_{DN}) (*10*) through N_2 fixation alone can be as short as 1.4 days. The T_{DN} for the three smallest *L. pedicellatus* (average weight 370 μg) assayed averaged 32 days. Carbon and nitrogen contents of these shipworms were measured with a Perkin-Elmer 240 elemental analyzer, and for 52 shipworms the average C : N ratio was 4.29 (range 3.52 to 9.33), and N constituted an average of 5.74 percent (range 3.78 to 8.27) of shipworm dry weight. The five smallest shipworms assayed had an average N content of 6.08 percent (range 4.60 to 8.21), whereas the five largest averaged 5.67 percent (range 5.16 to 6.37). This suggests that the juvenile shipworms had an adequate supply of combined-N compounds.

A slightly curved, rod-shaped (0.3 by 1.5 μm) bacterium capable of fixing N_2 was isolated from the cecum of *L. pedicellatus*. The isolate is gram-negative and appears to be of the family Spirillaceae. No N_2 fixing bacteria were obtained from the gill or the gland of Deshays (*11, 12*). The N_2 fixing isolate will grow either anaerobically in Bray dishes or Pankhurst tubes (*13*) or aerobically in petri dishes. It will only reduce acetylene under anaerobic conditions. The isolate is capable of liquefying cellulose (Sigma MN-300).

Nitrogen fixation occurs in another wood ingester, the termite (*14*). However, this is apparently the first report showing a potentially significant contribution of combined N to the nutrition of an animal (*15*). This discovery explains in part how shipworms are able to grow on a low-nitrogen diet. In the deep sea there are other cellulose-ingesting mollusks (subfamily Xylophagainae), as well as echinoderms and crustaceans that eat plant material of terrestrial origin, and wood entering the deep sea is rapidly decomposed (*16*). Nitrogen fixation could be an important source of nitrogen for these and possibly other deep-sea organisms.

EDWARD J. CARPENTER
Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543
JOHN L. CULLINEY
Museum of Comparative Zoology, Harvard University, Cambridge, Massachusetts 02138

References and Notes

1. E. B. Cowling and W. Merrill, *Can. J. Bot.* **44**, 1539 (1966).
2. J. D. H. Strickland, *Fish. Res. Board Can. Bull. 122* (1966).
3. L. J. Greenfield and C. E. Lane, *J. Biol. Chem.* **204**, 669 (1953).
4. R. D. Turner, *A Survey and Illustrated Catalogue of the Teredinidae* (Museum of Comparative Zoology, Cambridge, Mass., 1966).
5. K. J. Eckelbarger and D. J. Reish, *Bull. South. Calif. Acad. Sci.* **71**, 48 (1972).
6. J. L. Culliney, unpublished observations of *Teredo navalis* at Beaufort, N. C., June and July 1973; the water temperature was 26° to 28°C.
7. W. D. P. Stewart, G. P. Fitzgerald, R. H. Burris, *Proc. Natl. Acad. Sci. U.S.A.* **58**, 2071 (1967).
8. For the assay, high-purity acetylene gas (1 or 5 cm^3) was injected through a septum into serum bottles (5 or 20 ml, respectively) half-filled with seawater filtered through glass fiber. Blanks with dead shipworms were done with every experiment, and contaminating ethylene in the acetylene was subtracted for each shipworm sample. Shipworms were incubated in the dark at 25°C on ship or 15°C in the laboratory. After incubation for about 6 to 24 hours [shipworms are capable of living anaerobically; see C. E. Lane, R. W. Sims, E. J. Clancy, *Am. J. Physiol.* **183**, 3 (1955)] the worms were killed with 50 percent TCA, the bottles were shook, and 100 μl of the gas phase was withdrawn with a gas-tight syringe. Ethylene production was measured by injection of the gas into a Packard model 417 gas chromatograph with a stainless steel column (1.8 m by 0.3 cm) containing Porapak R and operated at 50°C. To convert moles of ethylene produced to moles of ammonia fixed, the factor 1.5 was used.
9. E. M. Hulburt, *Bull. Mar. Sci.* **14**, 33 (1964); *ibid.* **17**, 330 (1967).
10. $T_{DN} = (\ln 2)/[(N_2$ fixed per day)/(N content)], where nitrogen concentrations are per milligram dry weight.
11. J. D. Popham and M. R. Dickson, *Mar. Biol.* **19**, 338 (1973).
12. Bacteria were initially isolated by aseptically streaking onto Difco Marine Agar in petri dishes. Isolated colonies were transferred to axenic culture and then into modified Hino and Wilson medium [R. J. Seidler, P. E. Aho, P. N. Raju, M. J. Evans, *J. Gen. Microbiol.* **73**, 413 (1972)] with the addition of NaCl (23 g/liter), $MgSO_4$ (5 g/liter), and sucrose (2 percent), but without mannitol. A small piece (0.5 cm^2) of Whatman No. 1 filter paper was added to the medium.
13. N. E. R. Campbell and H. J. Evans, *Can. J. Microbiol.* **15**, 1342 (1969).
14. J. R. Beneman, *Science* **181**, 164 (1973); J. A. Breznak, W. J. Brill, J. W. Mertins, J. C. Coppell, *Nature (Lond.)* **244**, 577 (1973).
15. J. R. Postgate, *J. Appl. Bacteriol.* **37**, 185 (1974); see p. 195.
16. R. D. Turner, *Science* **180**, 1377 (1973).
17. Supported by NSF grant GA 37993 and ONR contract N0014-67-A-0298-0027 with Harvard University. We thank K. L. Smith, Jr., for stimulating advice and Barbara Culliney, Junell Sayles, Charleen Van Raalte, and Charles Price for technical assistance. Charles Remsen and Brian Schroeder advised on microbiological methods, and Ruth Turner kindly confirmed identifications of shipworm species and critically reviewed the manuscript. Contribution No. 3461 from the Woods Hole Oceanographic Institution.

7 October 1974

Pregnancy in Cactus Mice: Effects of Prolonged Copulation

Abstract. *The postejaculatory copulations of cactus mice are demonstrated to function in facilitating neuroendocrine responses necessary for pregnancy. Whereas estrous cycles were altered after just 10 percent of the tests terminated after one ejaculatory series, females became either pregnant or pseudopregnant after 80 percent of tests continued at least to sexual satiety (30 minutes with no copulations).*

While a few mammalian species cease copulating upon attaining their first ejaculation of an episode, most continue and attain multiple ejaculations (*1*). The function of this continued copulatory activity, particularly in species with spontaneous ovulation, is not clear. Copulation is known to function not only in the transfer of sperm from male to female, but also in triggering neuroendocrine reflexes in the female. These neuroendocrine responses, ovulation in some species and the initiation of a functional luteal phase (pseudopregnancy) in others, are as critical to pregnancy as is sperm transfer. Available data on laboratory rats and mice suggest that a single ejaculatory series is sufficient for initiation of the neuroendocrine reflexes producing pregnancy and pseudopregnancy in these species (*2, 3*). In this report, we show that in cactus mice copulation after ejaculation is critical to such responses.

Cactus mice ejaculate after several discrete insertions (intromissions) with intravaginal thrusting (*4*). An "ejaculatory series" is defined as a group of intromissions leading up to and including an ejaculation. A pause of about 7 minutes follows the first ejaculatory series, after which copulation is resumed. However, a second ejaculation rarely is attained (*4*). Indeed, we have never observed more than one ejaculation in a test when the female was in naturally occurring estrus. Ejaculations are readily discriminated from intromissions without ejaculation, using behavioral criteria (*4*). Vaginal smears taken from females receiving many intromissions, but no behaviorally identified ejaculations, reveal an absence of sperm in the female's genital tract. Thus cactus mice are ideal for investigation of stimulatory effects of copulation, since such effects can be separated from possible effects of additional sperm transfer.