marked multilocus disequilibrium, support the view that the populations of *D. pulex* were not capable of sexual reproduction.

The demonstration that *D. pulex* populations consist of genetically different clones raises significant evolutionary and ecological problems. The nonoccurrence of competitive exclusion suggests that the relative fitnesses of clones are unstable. The situation seems to be one of contemporaneous disequilibrium, in which environmental changes shift genotypic fitnesses before exclusion results. The coexistence of clones needs to be considered in relation to current views on the limits to niche overlap. Studies on vertebrate communities have suggested that there are constraints on the amount of resource overlap allowable between coexisting species (*18*). There is growing evidence that zooplankton communities fail to show such restraints on resource overlap—the clones of *D. pulex* probably use identical food items. This tolerance of overlap does not result from the presence of unlimited resources; the growth of zooplankton populations is normally limited by food shortage. A more fundamental reason for the tolerance of resource overlap can be offered. Stable species or clonal coexistence depends upon equality of relative fitnesses—although this equality may exist only as a long-term average. It is important to recognize that the limiting resource is not, in many cases, the primary determinant of relative fitness. For instance, a *Daphnia* population could be food-limited, but fitness differences among genotypes in the population be caused by differing susceptibility to some physical factor, such as oxygen tension. Only in cases where relative fitnesses are determined primarily by the limiting resource should there be any limit to resource overlap. It seems likely that studies on interactions between coexisting clones will contribute much to our understanding of constraints on species packing.

PAUL D. N. HEBERT
TERI J. CREASE

Department of Biology,
University of Windsor,
Windsor, Ontario N9B 3P4

References and Notes

1. G. E. Hutchinson, *Am. Nat.* **95**, 137 (1961).
2. P. Richerson, R. Armstrong, C. R. Goldman, *Proc. Natl. Acad. Sci. U.S.A.* **67**, 1710 (1970); W. J. Greeney, D. A. Bella, H. C. Curl, *Am. Nat.* **107**, 405 (1973).
3. R. Petersen, *Am. Nat.* **109**, 35 (1975).
4. G. E. Hutchinson, *Ecology* **32**, 571 (1951).
5. P. D. N. Hebert, *Aust. J. Zool.* **25**, 371 (1977); B. J. Manning, W. C. Kerfoot, E. M. Berger, *Evolution* **32**, 365 (1978); D. Frey, in *The Evolution and Ecology of Zooplankton Communities*, W. C. Kerfoot, Ed. (University Press of New England, Hanover, N.H., 1980).
6. P. D. N. Hebert, *J. Anim. Ecol.* **46**, 399 (1977).
7. _____, *Heredity* **33**, 327 (1974); _____ and R. D. Ward, *ibid.* **36**, 331 (1976); P. W. Young, thesis, Cambridge University (1975).
8. J. L. Brooks, *Mem. Conn. Acad. Arts Sci.* **13**, 5 (1957).
9. E. Berger and J. Sutherland, *Heredity* **41**, 13 (1978).
10. Parthenogenetic eggs are produced ameiotically resulting in offspring that are genetic replicates of their parent. These eggs may develop into either males or females; sex is determined by environmental factors.
11. R. G. Stross, *Ecology* **47**, 368 (1966); J. Davison, *J. Gen. Physiol.* **53**, 562 (1969).
12. AMY and GOT were separated on tris-borate gels, while the other enzymes were processed on tris-HCl gels. A tris-glycine electrode buffer was used for both types of gels. Gel buffers and staining procedures were similar to those described [P. D. N. Hebert, thesis, Cambridge University (1972)].
13. Individual clones were maintained in 100-ml beakers containing synthetic pond water made by adding 48 mg of $NaHCO_3$, 38 mg of $CaSO_4 \cdot 2H_2O$, 30 mg of $MgSO_4$, and 0.5 mg of KCl to 1 liter of distilled H_2O. Animals were fed every other day with aquarium-cultured algae (primarily *Scenedesmus*). Mortality during the establishment of clones was usually less than 5 percent.
14. The variation was shown to be heritable by isolating individual females and typing their parthenogenetic progeny. In all cases maternal and offspring phenotypes corresponded. Interpreting the genetic basis of the allozyme variation was complicated because of the absence of sexual reproduction. Variation at the LDH, PGI, and PGM loci was interpreted by comparing *D. pulex* phenotypes with those observed in sexually reproducing species such as *D. magna* and *D. carinata*. Studies on these species have shown that PGI and PGM homozygotes have a single banded phenotype, while PGM heterozygotes have two bands (PGM is a monomer) and PGI heterozygotes have three bands (PGI is a dimer). One *D. pulex* LDH phenotype was similar to that observed in homozygous individuals of sexually reproducing species. The second LDH phenotype had nine bands, spaced in three zones. In each zone the central band stained most strongly. Individuals possessing the phenotype were presumably heterozygous at the LDH locus. If so, the functional LDH molecule is a dimer and all three zones of LDH activity in the usual homozygote are synthesized by a single locus. Amylase phenotypes were not analyzed in *D. magna* or *D. carinata*, but the patterns seen in *D. pulex* are compatible with the hypothesis that AMY homozygotes have a single banded phenotype, while heterozygotes have two.
15. More than three bands may be present in *Daphnia* homozygous at the LDH locus as a result of proteolytic degradation of LDH molecules. Such breakdown was occasionally seen in *D. pulex*, but could be readily distinguished from the heterozygote pattern.
16. In species of *Daphnia* that reproduce by cyclical parthenogenesis unmated ephippial females resorb their eggs and cast empty ephippia.
17. P. D. N. Hebert, *Genetics* **77**, 335 (1974).
18. R. H. MacArthur and R. Levins, *Am. Nat.* **101**, 377 (1967); R. M. May and R. H. MacArthur, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1109 (1972); J. Roughgarden, *Theory of Population Genetics and Evolutionary Ecology* (Macmillan, New York, 1979).
19. We thank Ms. J. Loaring for assistance with the electrophoresis, Drs. R. M'Closkey and M. Weis, and the referees for helpful comments on the manuscript. Supported by an NSERC grant to P.D.N.H.

7 August 1979; revised 23 January 1980

Elastase Digestion of Demembranated Sperm Flagella

Abstract. *The changes in adenosine triphosphate-reactivated motility resulting from digestion of Triton-demembranated sea urchin sperm flagella by elastase are those expected if the elastic interdoublet linkages between flagellar microtubules are particularly sensitive to digestion by elastase and take part in regulating the amplitude of flagellar bending.*

The nine microtubular doublets in the periphery of flagellar axonemes are held together at 96-nm intervals by "interdoublet linkages" (*1*), which appear to consist, at least in part, of a protein that has been named "nexin" (*2*). These interdoublet linkages must be highly extensible to accommodate the sliding between microtubules that occurs during flagellar bending. Extension to between two to four times the rest length appears to be required during normal bending. Even greater extension of the interdoublet linkages has been seen in electron micrographs of partially disintegrated sperm flagella (*3*).

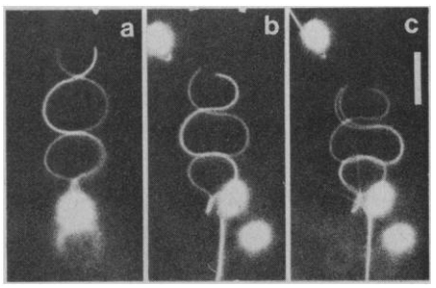

Fig. 1. Multiple-flash dark-field photomicrographs of a motile, demembranated sea urchin spermatozoon during exposure to elastase. Photographs were taken (a) 47 seconds, (b) 192 seconds, and (c) 266 seconds after mixing with elastase; this flagellum disintegrated at 306 seconds. Each photograph was taken with three flashes, with the flash frequency adjusted to twice the beat frequency. Superposition of the first and third images then verifies the accuracy of the adjustment of flash frequency and the recorded beat frequency (scale bar, 10 μm).

Active sliding can occur over distances much greater than those normally encountered during flagellar bending if sperm flagella are first digested with trypsin (*4*). Trypsin digestion causes damage to both the interdoublet linkages and the radial spokes, which can be detected by electron microscopy (*5*). The interdoublet linkages are more likely to be the structures that restrict the range of sliding since *Chlamydomonas* mutants lacking radial spokes still require trypsin digestion before active sliding disintegration can occur (*6*).

If the elastic resistance of these inter-

0036-8075/80/0321-1365$00.50/0

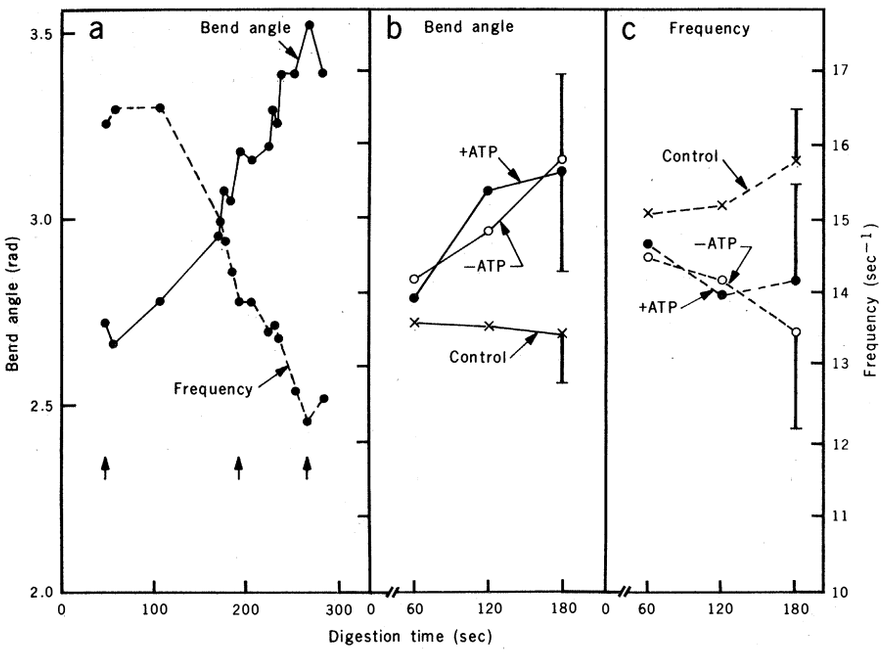

Fig. 2. (a) Measurements of bend angle and beat frequency during elastase digestion of the flagellum shown in Fig. 1. The data corresponding to the three photographs in Fig. 1 are indicated by the arrows at the bottom of the figure. (b and c) Measurements of bend angle and beat frequency of samples of flagella after elastase digestion for 60, 120, or 180 seconds. Means of the results obtained from three experiments with different sperm samples are shown; each point represents measurements of between 39 and 67 flagella. Mean values of the standard deviations are shown for the data at 180-second digestion times; other standard deviations were comparable. In each experiment, elastase digestion was carried out both in the absence and presence of $MgATP^{2-}$, as indicated; the control preparations contained $MgATP^{2-}$, but elastase was omitted.

doublet linkages opposes sliding during normal flagellar bending, a gradual proteolysis of the interdoublet linkages should be revealed by a gradual increase in the amplitude of bending. However, during digestion by trypsin, demembranated sea urchin sperm flagella show various changes in motility, but do not show a gradually increasing amplitude of bending (*7*).

The extensibility of the interdoublet linkages suggests that nexin may be chemically similar to other elastic proteins, such as elastin and resilin, and may be particularly sensitive to digestion by elastase. Elastase might therefore be expected to cause a more specific disruption of the interdoublet linkages than is obtained with trypsin.

Changes in motility resulting from the digestion of demembranated sea urchin sperm flagella by elastase are illustrated in Figs. 1 and 2. Spermatozoa from the sea urchin, *Lytechinus pictus*, were demembranated with a solution containing Triton X-100 (*7*) and then diluted 1:100 with reactivation solution (*8*) containing $MgATP^{2-}$, elastase (1.5 μg ml^{-1}) (from hog pancreas; Calbiochem, 324689), and soybean trypsin inhibitor (2.0 μg ml^{-1}) (Sigma, T9003). This trypsin inhibitor does not inhibit elastase (*9*) and is included to eliminate the effect of any contaminating trypsin-like enzyme. Motility of the reactivated spermatozoa was then monitored by dark-field light microscopy, and photographs of a selected spermatozoon were taken at intervals during the course of digestion, with the use of stroboscopic illumination at a frequency that was adjusted to twice the beat frequency. A printed record of the flash frequency for each photograph then provided precise information about the beat frequency of the flagellum, and measurements on the photographs provided information about the bend angle, which is a convenient measure of the amplitude of the movement (*7*, *10*). Examples of photographs and data for one sperm flagellum are shown in Figs. 1 and 2a. This spermatozoon was initially attached to the microscope slide by its head; about midway through the elastase digestion period, the sperm flagellum typically became detached from the head, and in this case the basal end of the flagellum then became attached to the microscope slide so that its movement could continue to be recorded. The results show a gradual increase in bend angle, and a gradual decrease in beat frequency, during the period of digestion by elastase. After 306 seconds, the flagellum stopped beating and disintegrated by sliding of microtubular doublets, just as in the case of digestion by trypsin. Disintegration was often preceded by a few seconds of very erratic beating. Data obtained from control spermatozoa in similar types of experiments have been presented (*7*, *10*).

Although qualitatively similar changes in motility are typical of all of the spermotozoa in these experiments, there is considerable variability in the extent and rate of appearance of the changes. Data from larger samples of spermatozoa were obtained by a slightly different procedure. A suspension of demembranated spermatozoa was diluted 1:10 with reactivation solution containing elastase and trypsin inhibitor, as above, and incubated for a measured interval. The suspension was then diluted 1:10 again, with reactivation solution containing $MgATP^{2-}$ and chicken ovoinhibitor (2.0 μg ml^{-1}) (Sigma, T1886), a trypsin inhibitor that is also an effective inhibitor of elastase (*9*). Measurements (not given) showed no changes with time in the frequency and bend angle of individual flagella in the final reactivation solution containing ovoinhibitor although, after the longer digestion periods, the flagella eventually disintegrated. These suspensions were sampled by photographing 15 to 20 flagella that were attached to the microscope slide either by the sperm head or the basal end of the flagellum (Fig. 2, b and c). This procedure also revealed a gradual increase in bend angle as the period of incubation with elastase was increased prior to the addition of inhibitor. In control experiments in which the elastase was omitted, the bend angle was essentially constant. With this procedure, spermatozoa can be digested by elastase in reactivation solution either with or without ATP (Fig. 2b). The results show that the effects of digestion by elastase do not require the presence of ATP (and, therefore, of motility) during the digestion period.

In both types of experiments, the increase in bend angle is largely compensated by a decrease in beat frequency. Therefore, the average velocity of sliding between flagellar microtubules, which is proportional to the product of bend angle and beat frequency, is nearly constant. Since the sliding velocity may be the most direct measure of the active process responsible for generating sliding between the flagellar microtubles, elastase digestion probably does not significantly affect the active sliding process during the time frame of these measurements.

The effect of elastase digestion on motility is not the same as the effect of trypsin digestion. Trypsin digestion caused,

initially, a gradual increase in beat frequency without a change in bend angle, corresponding to a gradual increase in sliding velocity (*7*). During the later stages of digestion by trypsin, there were often larger increases in frequency, accompanied by decreases in bend angle.

The effect of digestion by elastase is exactly that expected if the interdoublet linkages are involved in regulating the amplitude of flagellar bending and if these elastic interdoublet linkages are particularly sensitive to digestion by elastase. Elastase appears to have a much more specific effect than trypsin, and may be a better choice for future studies of sliding disintegration following enzymatic degradation of flagella and cilia.

C. J. BROKAW

Division of Biology,
California Institute of Technology,
Pasadena 91125

References and Notes

1. F. D. Warner, in *Cilia and Flagella*, M. A. Sleigh, Ed. (Academic Press, London, 1974), pp. 11-37; K. Summers, *Biochim. Biophys. Acta* **416**, 153 (1975).
2. R. E. Stephens, *Biol. Bull.* **139**, 438 (1970).
3. G. E. Olson and R. W. Linck, *J. Ultrastruct. Res.* **61**, 21 (1977).
4. K. E. Summers and I. R. Gibbons, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 3092 (1971).
5. ———, *J. Cell Biol.* **58**, 618 (1973).
6. G. B. Witman *et al.*, *ibid.* **76**, 729 (1978).
7. C. J. Brokaw and T. F. Simonick, *ibid.* **75**, 650 (1977).
8. The reactivation solutions for these experiments contained 0.15*M* KCl, 20 m*M* tris buffer, 2.0 m*M* EGTA, 2 m*M* dithiothreitol, 2 percent polyethylene glycol, and $MgSO_4$, adenosine triphosphate (ATP), and $CaCl_2$ to give 0.20 m*M* $MgATP^{2-}$, 1.0 m*M* Mg^{2+}, and 10^{-9}*M* Ca^{2+}, at *p*H 8.2. All incubations and measurements were made at 18°C.
9. J. Bieth, in *Frontiers of Matrix Biology*, J. Bieth, G. M. Collinlapinet, L. Robert, Eds. (Karger, Basel, 1978), vol. 6, pp. 1-82.
10. C. J. Brokaw, *J. Exp. Biol.* **71**, 229 (1977).
11. ——— and I. R. Gibbons, in *Swimming and Flying in Nature*, T.Y.-T. Wu *et al.*, Eds. (Plenum, New York, 1975), pp. 89-126.
12. I thank Dr. G. B. Witman for suggesting an examination of the effects of elastase and T. F. Simonick for assistance with these experiments. Supported in part by NIH grant GM 18711.

17 August 1979; revised 19 December 1979

α-Lactalbumin–Casein Induction in Virgin Mouse Mammary Explants: Dose-Dependent Differential Action of Cortisol

Abstract. *The interplay of insulin, cortisol, and prolactin induces synthesis of casein and α-lactalbumin in cultured mammary explants from mature virgin mice. A striking difference has been found between the optimal concentrations of cortisol required for maximal induction of the two milk proteins in vitro: 3×10^{-8} molar for α-lactalbumin and 3×10^{-6} molar for casein. Moreover, 10^{-7} to 10^{-5} molar cortisol caused progressive inhibition of α-lactalbumin accumulation. Such differential actions of cortisol may partly account for the asynchronous synthesis of the two proteins during pregnancy.*

Casein and α-lactalbumin are the major milk proteins synthesized by lactating mammary gland. Casein is a group of phosphoproteins that comprise more than 50 percent of milk protein (*1*); α-lactalbumin is a whey protein that can modify the substrate specificity of galactosyltransferase to include glucose, thus enhancing the synthesis of lactose (*2*).

Juergens *et al.* (*3*) showed that when mammary explants from mice in midpregnancy are cultured in a chemically defined medium containing insulin, cortisol, and prolactin, each at a concentration of 5 μg/ml, there is marked stimulation of the incorporation of inorganic phosphate into the Ca^{2+}-rennin-precipitable proteins, a large portion attributable to casein. Subsequently, Turkington *et al.* (*4*), using the same culture conditions, reported that the triple hormone combination stimulates mammary explants to make α-lactalbumin, which was assayed by the lactose synthetase system. These initial findings have been confirmed by others and these methods of measuring casein synthesis and α-lactalbumin have been widely employed (*5*-*9*).

During our investigation of the role of cortisol in the development of mammary gland in vitro, it became evident that the previous assay procedures for casein and α-lactalbumin were not satisfactory from the standpoint of accurate quantitative measurement. We have therefore used an immunochemical procedure to determine casein synthesis and a modified quantitative assay method to measure the amount of α-lactalbumin, utilizing the lactose synthetase system with pure mouse α-lactalbumin as a standard. With these methods, we have examined the dose-response relation between cortisol and the induction of either casein or α-lactalbumin. To our knowledge, this is the first time such comparative studies have been done in a systematic way. The data presented in this report reveal a striking difference between the concentrations of cortisol needed for maximal induction of casein synthesis and α-lactalbumin accumulation.

Mammary explants from 3- to 4-month-old virgin female C3H/HeN mice were cultured in a chemically defined synthetic culture medium, medium 199, as described previously (*10*). Casein synthesis was determined by incubating explants in the presence of a ^{3}H-labeled amino acid mixture (10 μCi/ml) for 4 days under the indicated conditions and subsequently immunoprecipitating isotopically labeled casein with mouse casein antibody prepared in rabbits (*11*). The amount of α-lactalbumin in mammary explants was determined as the activity of α-lactalbumin in the lactose synthetase reaction, using the modified method of Fitzgerald *et al.* (*12*) with pure mouse α-lactalbumin (*13*) as a standard.

Figure 1 shows the effect of varying the concentration of cortisol on the accumulation of α-lactalbumin, casein, and total protein synthesized in mammary explants from mature virgin mice in culture. During a 4-day culture period, accumulation of casein was low in the culture system with insulin or insulin plus prolactin. However, addition of increasing amounts of cortisol to the medium containing insulin and prolactin effected dose-dependent increases in casein. Maximal accumulation was effected by about 3×10^{-6}*M* cortisol. Addition of cortisol alone or cortisol combined with insulin or prolactin did not cause an increase in casein synthesis.

A different dose-response relation was observed in the accumulation of α-lactalbumin. The level of α-lactalbumin was low in the culture system with insulin, but was elevated 2.5-fold by the addition of prolactin with insulin. Addition of up to 3.0×10^{-8}*M* cortisol with insulin and prolactin caused further increases in α-lactalbumin, and the amount of the milk protein was twice that in the culture with insulin and prolactin. However, at higher concentrations ($> 10^{-7}$*M*), cortisol had an inhibitory effect. Addition of 3×10^{-6}*M* cortisol, an optimal concentration for casein synthesis, reduced the tissue content of α-lactalbumin more than 5-fold in the insulin-prolactin system.

The stimulatory effect of cortisol on total protein synthesis is not apparent up to 3×10^{-8}*M* but is manifested at higher concentrations. This can be explained as follows. About 50 percent of the total protein synthesized in maximally stimulated tissue explants is casein; less than 0.2 percent is α-lactalbumin, as determined by immunochemical analysis with antibody to mouse α-lactalbumin (*13*). In addition, considerable amounts of noncasein proteins are synthesized by mammary explants cultured with insulin alone (*14*), which results in higher baselines for total protein synthesis.

0036-8075/80/0321-1367$00.50/0