species: they were observed in a particular strain which unfortunately was lost.

1 M.C. Santa-Cruz is a member of the Cytology and Histology Department of the University Autónoma, Madrid.
2 H. Bauer, Z. Zellforsch. mikrosk. Anat. *23*, 280 (1935).
3 W. Beermann, Chromosoma, Berl. *41*, 297 (1973).
4 K. Hägele, in: Handbook of Genetics, vol. 3, p.269. Ed. C.R. King. Northwestern University, 1975.
5 H.G. Keyl, Chromosoma *8*, 739 (1957).
6 M.C. Santa-Cruz, A. Villanueva and J.L. Díez, submitted for publication (1978).

Oxygen concentration of the cultural fluid and variations in the mating reactivity of the marine ciliate *Euplotes crassus*[1]

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Summary. Mating reactivity of the ciliate *Euplotes crassus* improved after the amount of oxygen in the cell culture fluid was increased. It was supposed that mating reactivity of *E. crassus* may be influenced in nature by variations of the oxygen concentration in the environmental sea water.

Sexually mature ciliates can mate when appropriate cell encounters occur under suitable physical and chemical conditions. Furthermore, because of the various ecological niches that ciliates exploit, special conditions of the extracellular factors are often required by a given species to initiate conjugation.

We observed that, analogously to what is described in Tetrahymena[3], mating of 2 stocks of *E. crassus* was greatly increased when cells of starving cultures were transferred from test-tubes to depression-slides or to petri plates. We tested what appeared to be one of the most likely hypotheses, that is whether the amount of oxygen dissolved in the cultural fluid was appreciably varied after cell transfer and whether such a variation could affect the cell mating reactivity.

Materials and methods. The 2 stocks we dealt with (H_2 and H_7) which belong to complementary mating-types extremely reactive with each other[4], derived from specimens collected from the Tuscan coast. Stocks were cultured at 22–23 °C in Erd-Schreiber medium after having been inoculated with the green alga *Dunaliella salina* and maintained under a rhythm of 12-h light and 12-h dark. Intraclonal conjugation with various degrees of intensity occasionally occurred in the unmixed cultures for different time periods. The 2 conditions (a clonal age of about 500 consecutive fissions and a heterozygous constitution of the mt locus) which, according to Heckmann[5], are required for selfer pair formation in *E. crassus*, were satisfied in both stocks. The first selfer pair formation was observed more than 1 year after stocks were cultured in the laboratory. This means an age of more than 300 fissions, calculating that cells performed on average 1 fission/day under our routine culturing conditions. The heterozygous constitution was known after a genetic analysis of the F_1-clones derived from heterotypic pairs obtained in the mixture between stocks H_2 and H_7: a nonparental mating-type appeared in $\frac{1}{4}$ of the progeny. (In a species such as *E. crassus* with a multipolar mating-type system controlled by one-locus multiple alleles with serial dominance relationship[6], this finding implies that both parents are heterozygous.)

Experimental procedure started by preparing several 30-ml test-tubes each containing 1 ml of a cell culture plus 25 ml of food. In the presence of an optimal quantity of food, the maximum fission rate (about 2 fissions/day) was obtained for 3–4 days and a density of 1.0 to 1.5×10^3 cells/ml was finally reached. Then cells entered the stationary phase and arrested in G_1-phase; dividers and macronuclear DNA replicating cells (cytologically detectable by the presence of replication bands in the macronucleus[7]) were found only exceptionally. It was only under starvation that selfer pairs formed. Then 2 types of protocols were followed. According to the first, we checked the amount of oxygen in the 5-day starved test-tube cultures. 3 cultures with similar low oxygen levels were chosen for each of the 2 stocks. While 1 test-tube culture was left as control (see below), 2 were fractioned into 2 series of 10 experimental subcultures by transferring 2.5 ml cell culture volumes with a Pasteur pipet to 3-ml 1-depression slides. The bubbling that occurs on cell culture transfer was deliberately carried on in each subculture for at least 10 sec. One of the 2 series was then transferred from the 1-depression slides to a 50-ml beaker where the oxygen sensor was dipped in order to monitor how much the oxygen level had risen by bubbling in the subcultures. The other series was maintained in the 1-depression slides covered with coverslips sealed tight to depressions by means of silicone grease. At 1-h intervals, subcultures were examined after being killed with a few drops of formalin. As controls, cells in a 2.5-ml volume were taken and immediately killed from the 3rd test-tube culture, which in the meantime had been kept sealed with a sheet of parafilm. According to the 2nd protocol, starting again from test-tube cultures with low oxygen levels, we prepared an experimental 10-subculture series of the stock H_2, that was graded according to different oxygen levels. Each subculture consisted of a volume of 15 ml in a 50-ml beaker. Initial oxygen levels, except for 1 subculture kept as control, were increased to different levels by bubbling pure oxygen for a few sec. After having monitored the oxygen levels, each subculture was divided into two 8-ml test-tubes

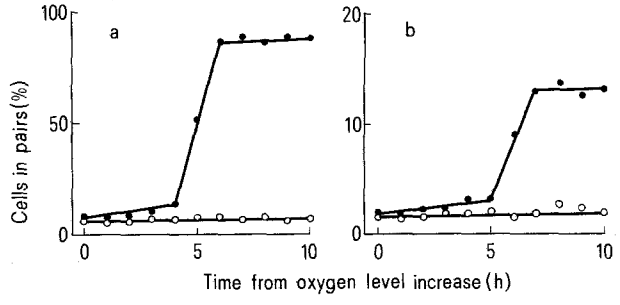

Fig. 1. The effect on the percent of cells in pairs by a sudden increase of the amount of oxygen in the cultural fluid. Oxygen concentration was varied from the initial level of 3.8 in the control (○——○) to 6.4 in the experimental subcultures (●——●) of stock H_7 (plot a); from 4.6 (○——○) to 7.8 (●——●) in the case of stock H_2 (plot b).

which were then sealed with parafilm. The percentage of pairing was examined after 5 h in 1 test-tube, after 10 h in the other test-tube. About 10^3 cells were examined for each point or column reported in the figures. Calculations were made to show percent cells in pairs according to the method used by Bruns and Brussard[8] in Tetrahymena. The data reported in the figures refer to single experiments taken as representative. The oxygen levels were monitored with an oxygen electrode (YSI Model 51 oxygen meter, Yellow Spring Instruments) and all measurements were taken in parts per thousand (‰).

Results. After being transferred from test-tubes, cells revealed an increased readiness to enter conjugation that was particularly evident in the subcultures of stock H_7. Cells slowed down in swimming, grouped and acquired ability in agglutinating with each other. This is a typical cell behavior that precedes the mating union of *E. crassus*[9]. Soon, significantly increased percentages of paired cells were found in the subcultures of stock H_7 ($p < 0.01$) as well as of stock H_2 ($p < 0.02$). Paired cells of stock H_7 from an initial percentage of 6.4 reached a percentage as high as 89.8 (figure 1, a). In the subcultures of stock H_2, percent cells in pairs eventually increased about 7 times (figure 1, b). However, cells of stock H_2 never reached the very high percentages of pairing showed by cells of stock H_7 and they took 1 h longer to reach the maximum of pairing. This difference in the mating reactivity between the 2 stocks is probably due to a constitutional difference of the stocks to undergo selfing, as also indicated by the percentages of pairs initially present in the test-tubes (cf. controls of plots a and b in figure 1). Besides the mechanical stimulus to which cells were undoubtedly submitted in being transferred, we found that the extracellular condition which had become clearly altered was the oxygen level in the cultural fluid. It had become almost $\frac{2}{3}$ higher than the initial value.

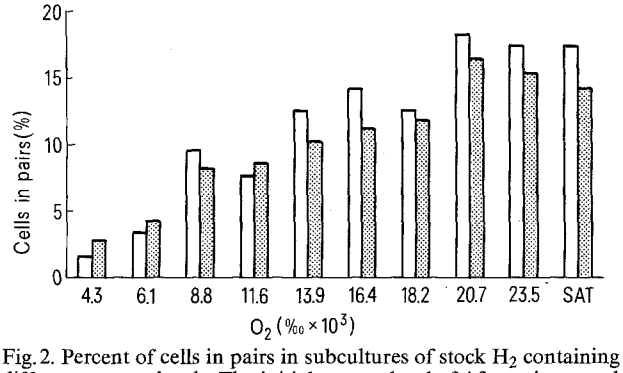

Fig. 2. Percent of cells in pairs in subcultures of stock H_2 containing different oxygen levels. The initial oxygen level of 4.3 was increased in all the subcultures, 1 excepted (control), up to the values reported on abscissa. Saturation = SAT. Each subculture, represented by 2 columns, was examined twice for the percent cells in pairs: the 1st time at 5 h (white column), the 2nd time at 10 h (dotted column) after oxygen addition.

The indication that oxygen could actually function as an external conjugation-stimulating factor, was further indicated by the following experiment. After transfer for test-tubes, cells of the less mating-reactive stock H_2 were kept in beakers to which different amounts of oxygen were added. Percent of cells in pairs increased in a good relation to the amounts of oxygen added to cultural fluid (figure 2). In addition, a reduction of the time lag needed for the cells to enter conjugation was also obtained. At 5 h after oxygen addition, paired cells increased up to around 14%, which had represented in the previous experiment the maximum of pairing obtained at 7 h from cell transfer. When the 2nd examination was performed at 10 h from oxygen addition, no further increase of cell pairing resulted. Instead, a general, though not significant, decrease was observed (cf. white and dotted columns of figure 2). If we consider that conjugation normally takes 12 h to be completed, this decrease of the percentage of paired cells should mean that some recently formed pairs spontaneously separated during the second 5-h interval. It may be that the dramatic increase of oxygen concentration was able to switch on the cellular reactions necessary for the cell mating in a part of the experimental H_2-population which was larger than that thoroughly competent to perform sexually. A pair separation caused by a decreased oxygen level in the cultural fluid consequent to the gas consumption of the cells, seems more unlikely because nearly identical decreases of percent of cells in pairs occurred in most of the subcultures.

In another set of experiments, oxygen was removed from cultures by bubbling nitrogen. In the absence of oxygen, cells did not mate. When oxygen was again added to beakers, cells recovered mating capability. However, this inhibition to pairing is also expected because of the trouble caused to cells by the prolonged gas bubbling necessary to remove oxygen as well as by the anoxia condition produced.

Conclusions. Intraclonal pairing was demonstrated[5] to be favoured in clones of *E. crassus* heterozygous at their mating-type locus by lowering the room temperature from 20 to 12 °C. Our results indicate that this sexual manifestation can also be improved by an increase of the oxygen concentration in the cell culture fluid. It seems likely that a metabolic pathway leading up to the synthesis of the macromolecules responsible for mating of *E. crassus*, that are probably glycoproteins[9], is sensitive to variations of the amount of dissolved oxygen. Oxygen could be relevant also in nature as an external regulating factor of the mating reactivity of *E. crassus*. In fact: a) This ciliate is a mesopsammic sand-dweller commonly living at low depth where, as in ponds and sluggish streams[10], the amount of dissolved oxygen could fluctuate during the day with a maximum early in the afternoon and a minimum early in the morning. b) The mating reaction of *E. crassus* changes in intensity with a circadian rhythm: high mating reaction occurs at dusk, low at dawn[11]. That is just several h after the dissolved oxygen has probably reached, respectively a peak and a fall in the *E. crassus* habitat; mainly in relation to changes in the activity of the photosynthetic organisms.

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2 Address: Institute of Zoology, University of Pisa, I-56100 Pisa (Italy).
3 A.M. Elliot and R.E. Hayes, Biol. Bull. *105,* 269 (1953).
4 P. Luporini, F. Esposito and P. Bracchi, Boll. Zool. *43,* 207 (1976).
5 K. Heckmann, J. exp. Zool. *165,* 269 (1967).
6 K. Heckmann, Z. VererbLehre *95,* 114 (1964).
7 D.M. Prescott, R.F. Kimball and R.F. Carrier, J. Cell Biol. *13,* 175 (1962).
8 P.J. Bruns and T.B. Brussard, J. exp. Zool. *188,* 337 (1974).
9 K. Heckmann and R.W. Siegel, Exp. Cell Res. *36,* 688 (1964).
10 L.E. Noland and M. Gojdics, in: Research in Protozoology, p. 216. Ed. T.T. Chen. Pergamon Press, New York 1967.
11 A. Miyake and R. Nobili, J. Protozool. *21,* 584 (1974).