MDH has been described in other plant species such as maize, barley and rye[12-14].

Since all the *glu* plants analyzed have the same 3 banded pattern, it could be concluded that *glu* plants are homozygous, and therefore, in diploid species, such as *glu*, at least 2 loci are needed to obtain 3 isozymes. *Glu* plants must be homozygous MDH-1_{1g} MDH-1_{1g}, MDH-2_{1g} MDH-2_{1g}, the fastest migrating subunit being coded by the MDH-1_{1g} allele and the lowest by the MDH-2_{1g}, isozyme 2 being the heterodimer.

Two possible hypotheses can be postulated for the explanations of the *sua* pattern, admitting its fixed heterozygous nature, due to the fact that it is an amphiploid species[9]:

1) The 2 loci are represented in its genomes, but only active alleles, MDH-1_{1s} and MDH-2_{2s}, are present (table).

2) Only 1 locus is represented by active alleles, MDH-1_{1s} and MDH-1_{2s} (table).

Since the relative staining intensity of hybrid plant isozymes appears to fit a 4:4:1:4:2:1 pattern instead of a 9:6:1:12:4:4 pattern, it is necessary to postulate that the amphiploid species contributes to the hybrid only with 2 active gene doses instead of the 4 expected (see table). Therefore the *sua* species must be fixed for null alleles. An alternative explanation is that chromosome segments have been lost during the evolution of *sua*. According to Goodspeed[15] this species should have lost chromosomes or chromosome fragments until attaining the actual chromosome number. If this is correct, it could be possible that the loci MDH-2 and/or MDH-1 were lost in the first and/or second genome.

Another alternative hypothesis that cannot be ruled out is that the diploid species of *Nicotiana* had in their origin only 1 locus of MDH, and the 2nd MDH locus of *glu* came from a duplication; duplicated genes for MDH have been described in diploid species[13,16,17]. If this last hypothesis was true, it would not be necessary to postulate the existence of null alleles or chromosomal losses in *sua*.

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Gamete formation reflects the sexual pheromone hierarchy of *Dictyostelium giganteum*[1]

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Summary. Sexual development in *Dictyostelium giganteum* begins with the appearance of small, amoeboid gametes that fuse to produce mainly binucleate cells which differentiate into zygote giant cells. The data presented here show that the number of gametes produced by each strain (WS589 > WS606 > WS607 > WS588) is directly related to its position in this hierarchy.

Key words. Mating types; sex pheromone; gametes; zygote formation; *Dictyostelium*.

Sexual development in both homothallic and heterothallic cellular slime molds is regulated by pheromones. The species which have been shown to produce macrocyst-inducing pheromonal activity include *Dictyostelium discoideum*[3-5], *D. giganteum*[6], *D. mucoroides*[7-10], *D. purpureum*[11], and *Polysphondylium pallidum* (unpublished results). In contrast, *D. rosarium* apparently does not produce sex pheromones[12]. The accumulated data reveal that the sex pheromones of the cellular slime molds are volatile but none has yet been characterized. Ethylene can induce macrocyst formation in *D. mucoroides* and inhibitors of ethylene synthesis inhibit macrocyst formation suggesting that ethylene or a derivative may be the pheromone in this species[9]. A very tiny amoeboid cell which appears to represent the gamete phase of *D. discoideum* has recently been discovered[13,14]. These cells fuse producing binucleate and multinucleate cells which form the zygote of this species. Furthermore, these gametes are produced in much higher levels in the secreter (NC4) strain than the responder (V12) strain of this species[13]. This suggests a possible relationship between the pheromone-producing ability of a strain and its competence to produce gametes. The results presented here on early sexual development and on gamete production in single strains of *D. giganteum*, which comprise a unique mating hierarchy[6], supports this concept.

Materials and methods. Four mating type strains (WS588, WS589, WS606, WS607) of *D. giganteum* were maintained as stock fruiting body cultures on SM agar plates with *Escherichia coli* as a food source. For mixed mating type cultures spores of WS588 and WS589, which represent the opposite ends of the pheromonal hierarchy[6], were treated as detailed previously for

The cytoplasmic and nuclear volumes of cell types in sexual cultures of *Dictyostelium giganteum*

Cell type	Cytoplasmic volume (μm^3)	Nuclear volume (μm^3)
Amoebae	123.73 ± 3.94	13.80 ± 0.72
Gametes	43.16 ± 1.63	5.67 ± 0.16
Zygotes	450.88 ± 19.19	54.28 ± 2.47
Macrocysts	565.59 ± 29.51	NA

A mixed mating type culture (WS588 × WS589) was made as detailed in 'Materials and methods'. Cell aliquots were removed at specific times, placed on slides, fixed and stained with the nuclear fluorochrome Hoechst 33258. Photographs were made and the diameters of each cell type were measured with calipers coupled to a microcomputer which calculated the cell volumes[13]. The data represent the means and standard error as determined from measuring at least 24 cells of each type. NA, not applicable.

D. discoideum[15]. The spores were heat shocked and mixed together before being placed in 50-ml aliquots of 0.1% lactose-proteose peptone. A small aliquot of *E. coli* was added and the cultures were shaken at 150 rpm at 22 ± 1 °C in 250-ml Erlenmeyer flasks which had been covered with black, plastic tape. Small aliquots (200 µl) were removed at selected intervals and placed on gelatin-subbed slide to allow the cells to settle. The cells were then fixed and stained with Hoechst 33258 and observed using a fluorescence microscope. Unmated cultures were treated in an identical way to mated cultures except spores of only one strain were added to the culture flask. Cell types were defined as detailed previously[13,15]. The volumes of the cytoplasms and nuclei of the different cell types were determined as outlined by McConachie and O'Day[13].

Results and discussion. The morphological events of early sexual development of *D. giganteum* (WS589 × WS588) are reminiscent of those occurring in *D. discoideum*[13–15]. Early cultures consist of large, typical amoebae (fig. 1, a) and small presumptive gametes (fig. 1, b). Cell fusion results in the formation of binucleate cells (fig. 1, c). As the pronuclei of the binucleate cells swell, migrate together and fuse, the cytoplasmic volume of the cells increases dramatically (fig. 1, d). The end-product of this differentiation process is a zygote giant cell (fig. 1, e) which will serve as the focus for further development. Multinucleated cells appear in low numbers in sexual cultures of *D. giganteum* (fig. 1, f).

Precise quantification of the cell sizes again emphasized the similarity between the morphological events of early sexual development in *D. giganteum* and *D. discoideum* (table). Gametes have been defined on the basis of their very small size, the presence of a tiny nucleus and their fusion to form binucleates which differentiate into zygote giant cells[13]. In *D. giganteum*, the gametes are approximately ⅓ the size of typical uninucleate amoebae while the zygote giant cells are 10 times the size of the gametes and 4 times the size of amoebae. Although the zygote giant cell will endocytose hundreds of amoebae as it differentiates into a mature macrocyst, little increase in cell size occurs during later development.

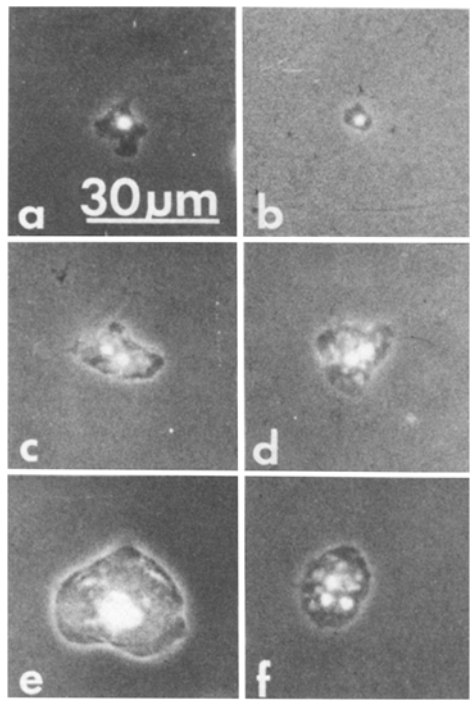

Figure 1. Cell types from mated (WS588 × WS589) cultures of *Dictyostelium giganteum*. The cells have been fixed and stained with Hoechst 33258 and photographed using simultaneous phase and fluorescence microscopy. *a* Amoeba; *b* gamete; *c* young binucleate; *d* swollen binucleate giant cell; *e* zygote giant cell; *f* multinucleated cell. Note the differences in nuclear and cytoplasmic size of the different cell types.

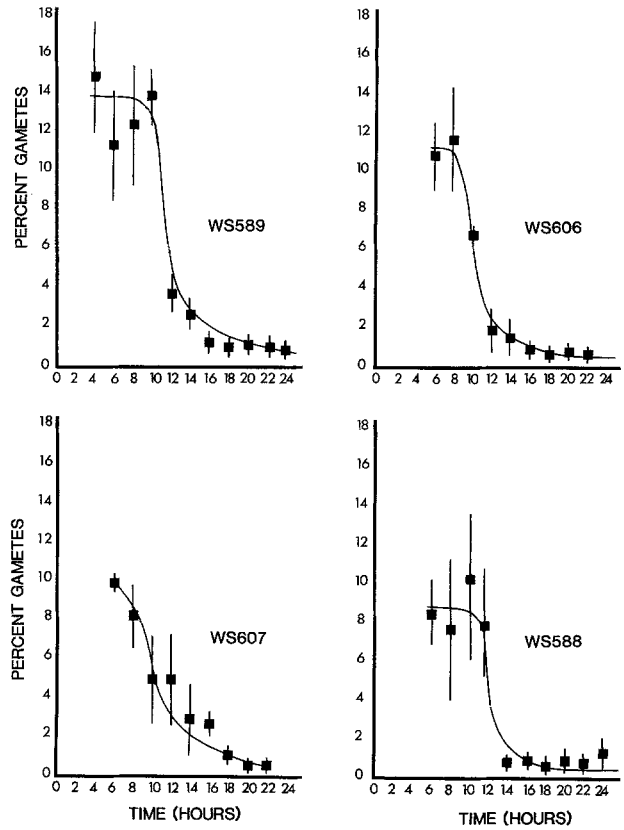

Figure 2. Kinetics of gamete production in unmated strains of *Dictyostelium giganteum*. Single mating type strains were grown under conditions which favor sexual development. At the indicated times cell aliquots were removed, fixed and stained with Hoechst 33258. The percent gametes (plus SE) was then determined.

When mating types of *D. discoideum* are cultured separately under macrocyst forming conditions they retain the capacity to form gametes but the gametes do not fuse[13]. Instead the gametes increase in number, reach a plateau and maintain it. In such cultures, NC4, which secretes pheromone, produces about 4 times more gametes than the responding strain V12 does. When the individual mating type strains of *D. giganteum* were cultured separately under dark, moist conditions, gametes were also produced (fig. 2). The gametes appeared in maximal numbers as soon as spore germination occurred (between 4 and 6 h depending upon the strain) and thereafter decreased in number so that by 18 h they constituted 1% or less of the cell population (fig. 2). The reason for the difference in gamete kinetics in *D. giganteum* and *D. discoideum* is not known but the fragility of these cells may play some role (unpublished results).

When the initial number of gametes that are produced by each strain are compared it becomes clear that there is a great disparity in gamete formation (fig. 2). If we examine the levels of gametes initially produced by each strain it is evident that WS589 (15.2% gametes at 4 h) produces more gametes than WS606 (11.18% at 6 h) which generates more than WS607 (9.6% at 6 h). WS588 produces the least number of gametes (8.58% at 6 h). In terms of a gamete-producing hierarchy we can arrange the strains thus: WS589 > WS606 > WS607 > WS588. Similarly, an overall comparison of the average amount of gametes present during the first few hours after spore germination under sexual conditions yields the same hierarchy: WS589 (average 14.8%), WS606 (11.4%), WS607 (9.8%), WS588 (less than 8.6%).

Using cell free conditioned medium from cultures of individual mating type strains of *D. giganteum*, Lewis and O'Day[6] showed that each strain produced pheromonal activity that could induce all of the other strains to form macrocysts. This induction of artificial homothallism was strain specific such that some strains were very strong secreters of macrocyst-inducing activity while others were strong responders. The hierarchical arrangement of inducers is: WS589 > WS606 > WS607 > WS588. This corresponds to the hierarchy of gamete formation described above. The hierarchy for responders is the exact opposite: WS588 > WS607 > WS606 > WS589.

With the recent discovery of the presumptive gamete phase of sexual development in heterothallic slime mold species and of the present relationship established between their formation and pheromone production in *D. giganteum*, future work should establish whether the gametes are the source of macrocyst-inducing factors or whether their presence reflects some other genetic component of pheromone production. Continued, critical analysis of pheromone production in other slime mold species and strains and by isolated populations of gametes should divulge the true relationship between macrocyst development, sex pheromone production and gamete formation.

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Effects of the light-dark cycle and scheduled feeding on behavioral and reproductive rhythms of the cyprinodont fish, Medaka, *Oryzias latipes*[1]

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Summary. Medaka were maintained on a 16:8 light-dark cycle and fed once daily on one of 5 different feeding schedules. The daily rhythm of agonistic behavior rapidly entrained to the scheduled feeding time and maintained this entrainment during a 3-day starvation period. In contrast the daily rhythms of egg laying and courtship stayed entrained to the L:D cycle regardless of the feeding schedule. Thus, temporal integration of this fish with its daily environment can involve multistimuli which concurrently and differentially entrain externally expressed circadian systems.
Key words. Circadian rhythms; zeitgebers; *Oryzias latipes;* meal-feeding; behavior; environmental factors.

Behavior acts as a link between an organism's internal physiology and external environment; specific variations in physiological function produce definable and predictable behaviors. The temporal synchronization of these functions and behaviors with daily and seasonal changes in the environment is important to the individual's survival and perpetuation of the species.

The light-dark cycle has long been considered the most important environmental stimuli synchronizing or entraining circadian rhythms in plants and animals[3]. In vertebrates, including fishes, the light-dark cycle can entrain a host of endogenous rhythms including circulating hormones, locomotor activity, and reproduction[3-7]. More recently the daily eat-fast cycle has been demonstrated to be a potent synchronizer of circadian rhythms, in some cases a more potent synchronizer than the light-dark cycle. Moore-Ede et al.[4] detail research on mammals that indicate daily eat-fast cycles are more important than the light-dark cycle in entraining a number of daily patterns. Circulating glucocorticoids and locomotor activity rhythms entrained rapidly to feeding schedules but eventually all rhythms examined entrained to the feeding regime in preference to the light-dark cycle. With fishes, in contrast, although circulating cortisol and locomotor activity rapidly entrain to the daily feeding time, the rhythm of circulating thyroxine appears to remain entrained to the light-dark cycle[7].

The fact that rhythms of different physiological variables entrain to different stimuli or entrain differently (phase shift at different rates) to the same stimulus has been used as one line of evidence to support the concept of a multioscillator system of time keeping (rather than a single biological clock)[4]. It has been suggested that a multioscillator system may initially have evolved in order to provide an endogenous timing system that could simultaneously allow for rapid acclimation to short-term circadian changes in the environment (e.g., temporal change in a food resource) as well as fixed long-term circannual changes (e.g., reproduction)[7]. Presumably, reproductive functions and behaviors would be connected to a different oscillator than behavior associated with day-to-day maintenance functions. We selected an oviparous, freshwater cyprinodont, the Medaka (*Oryzias latipes*), to examine this possibility.

Methods. A population of 6 female and 4 male Medaka were placed into each of 10 35-l glass aquaria. External sexual dimorphism allowed rapid sexing both during experimental set-up and behavioral observation[8]. To avoid the continuous bottom feeding and coprophagy observed during a pilot study a plastic grating covered by $\frac{3}{8}$-inch stretchable nylon fish netting was placed on the tank bottom. This false bottom allowed feces and uneaten food to settle to the bottom of the aquaria and prevented continuous grazing and possible associated behavioral responses.

The top of each aquarium was covered by a light hood with a 25 W incandescent bulb and an automatic feeding device (details of the feeder to be published elsewhere). The light-dark cycle and feeding times were controlled by separate timers. Aquaria continuously received dechlorinated tap water (8 l/h). During a 3-week acclimation period fish were fed at random times and