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SEXUALITY, INCOMPATIBILITY, SIZE VARIATION, AND PREFERENTIAL POLYANDRY IN NATURAL POPULATIONS AND CLONES OF *SELLAPHORA PUPULA* (BACILLARIOPHYCEAE)[1]

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The capitate and rectangular demes of the freshwater epipelic diatom *Sellaphora pupula* (Kütz.) Mereschk. are dioecious, the first such report for any freshwater diatom. Sexual differentiation, which is probably determined genetically, involves recognition at the cell surface as well as differences in gamete behavior (one gametangium produces an active "male" gamete, the other a passive "female" gamete). In culture, successful sexual reproduction occurs only when compatible clones are mixed. All cells of a clone behave identically in interclonal crosses, being either male or female, regardless of the stage of the life cycle, in contrast to the sequential hermaphroditism of centric diatoms. Males and females have identical frustule morphology. As in other diatoms, there is an upper size threshold for sexual reproduction, below which cells become progressively easier to sexualize. In culture, sexual interactions occur in cells much smaller than those ever seen in natural populations, so that in nature the sexual size range is effectively open. Natural populations almost always contain sexualizable cells; often, most of the cells are below the upper sexual size threshold. Male gametangia are, on average, slightly larger than females in the capitate deme, which may be produced by preferential polyandry, depleting the population of males and making them younger at mating. Rarely, selfing occurs producing zygotes, but these abort before producing initial cells. The sizes of the gametangia and initial cells are correlated but this does not invalidate the use of "cardinal points" of the life cycle in taxonomy. No interbreeding occurs between the rectangular and capitate demes. However, when males of one deme are mixed with females of the other, there is a stimulation of activity, as during the early stages of pairing in compatible intrademic crosses.

Key index words: **auxosporulation; Bacillariophyceae; diatoms; dioecy; life cycle; *Sellaphora*; sexual reproduction**

Until very recently, it was accepted dogma that diatoms are homothallic: starting with a single cell, a culture can be established that will be able to complete and repeat the whole life cycle (Drebes 1977). The sexual phase may involve the formation and fusion of morphologically or physiologically dissimilar gametes (small motile sperm and large sessile egg cells in centric diatoms; morphologically similar but behaviorally diffentiated nonflagellate gametes in many pennate taxa: Round et al. 1990), but in most cases this is not considered to reflect sexual differentiation of the diploid vegetative cells that gave rise to them. In a review of sexuality in both centric and pennate diatoms Drebes (1977: 277) stated that "... the data, especially those obtained from observations on clonal cultures, indicate that the great majority of species are monoecious. In principle, sex determination is assumed to be diplophenotypic" (see also Geitler 1935, 1957a, b, Lewin 1954, Patrick 1954, Köhler 1967).

The dogma is not without support. In many centric diatoms a single clone can produce female and male gametangia, though not necessarily at the same time: generally, as size reduction proceeds, eggs are produced first, then sperm (e.g. von Stosch 1951, 1956, Drebes 1977; contrast *Leptocylindrus danicus* Cleve: French and Hargraves 1985). A few cases have been reported in which sexuality is more complex, such as *Coscinodiscus granii* Gough, where Drebes (1968) reported "subdioecy": some clones are "female," only producing eggs, whereas others are predominantly "male," though with the production of a few oogonia. Otherwise, available data indicate that centric diatoms are predominantly monoecious and self-compatible (von Stosch 1951, 1956, von Stosch and Drebes 1964, von Stosch et al. 1973, Drebes 1974, 1977, Hasle et al.

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1983, French and Hargraves 1985, Edlund and Stoermer 1991).

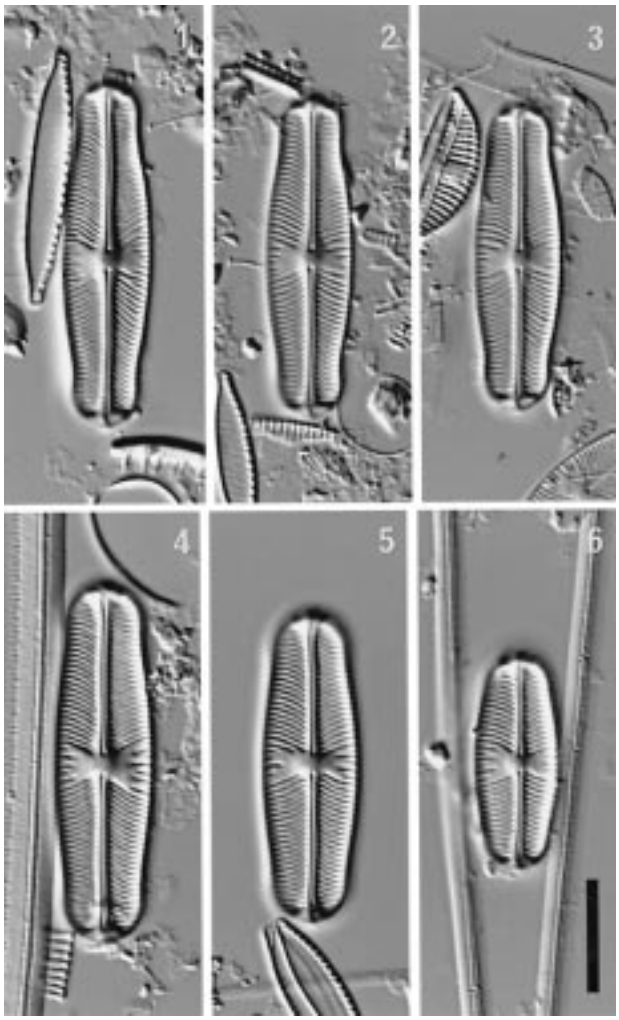
Pennate diatoms have also been regarded as basically monoecious (homothallic), apart from the marine araphid species *Rhabdonema adriaticum* Kütz. and *Grammatophora marina* (Lyngb.) Kütz., which are dioecious (von Stosch 1958, von Stosch and Drebes 1964). Some of the best studies of the life cycle in pennate diatoms were made by Geitler (1932), who demonstrated unequivocally, using clonal cultures, that three species of raphid diatom exhibit monoecious reproduction. However, among the many other reports of sexual reproduction in pennate diatoms (e.g. those summarized by Geitler 1932, 1973, 1984), very few are based on studies of clonal cultures (examples include Wiedling 1948, Mizuno and Okuda 1985, Mizuno 1994). In view of this, it is perhaps surprising that it has been assumed so readily that pennate diatoms are monoecious. The significance of Geitler's work, and the likely reason for assuming that his results can safely be extrapolated to most other pennate diatoms, was that the three freshwater species studied—*Sellaphora seminulum* (Grun.) D. G. Mann (previously *Navicula seminulum*), *Gomphonema parvulum* (Kütz.) Kütz. var. *exilis* Grun. (this identification of Geitler's specimens was offered by Krammer and Lange-Bertalot 1986), and *Eunotia formica* Ehrenb.—belong to distantly related genera and exhibit quite different types of behavior during sexual reproduction. Thus, *Eunotia formica* is morphologically and behaviorally isogamous, whereas in *Gomphonema parvulum* each gametangium produces two gametes, one of which is active (male) and the other passive (female); plasmogamy involves the movement of the active gametes across into the other gametangium (Geitler 1932). In these two species, the gametangia are apparently equivalent in every way—morphologically, behaviorally, and physiologically—so homothally is perhaps unsurprising. However, in the third species studied by Geitler, *Sellaphora seminulum,* the gametangia are visibly differentiated. Each produces only one gamete, as in *Eunotia,* but in one gametangium the gamete is active, while in the other it is passive. After plasmogamy, one gametangium is left empty while the other gametangium contains the zygote (cf. our Figs. 39, 41, 44 and Geitler 1932) and the gametangia thus appear to be differentiated into ''male'' and ''female.'' However, since both types of gametangia are produced within a single clonal culture, leading to successful production of zygotes, *S. seminulum* is homothallic (monoecious) and the ''sex'' of the gametangia cannot be invariable throughout the diploid phase.

The example of *Sellaphora seminulum* is particularly important because the gametangia are more clearly differentiated than in any other pennate diatom (except *Rhabdonema:* von Stosch 1958) yet must apparently owe their differences to environmental effects or developmental processes (including, perhaps, pheromonal interactions between gametangia) or conceivably to some kind of cytoplasmic inheritance, not to a chromosomal sex determination mechanism. Geitler (1957b) therefore returned to this species in a later discussion of sex determination in pennate diatoms, providing new data from natural populations. Here, he reported a tendency for the male gametangia to be smaller than the female gametangia, as also occurs in *Cocconeis placentula* var. *pseudolineata* Geitler (Geitler 1932), which has a similar type of sexual reproduction. These size differences would, of course, be consistent with the correlation of small size and maleness in many centric diatoms (see above) and might similarly imply sequential hermaphroditism.

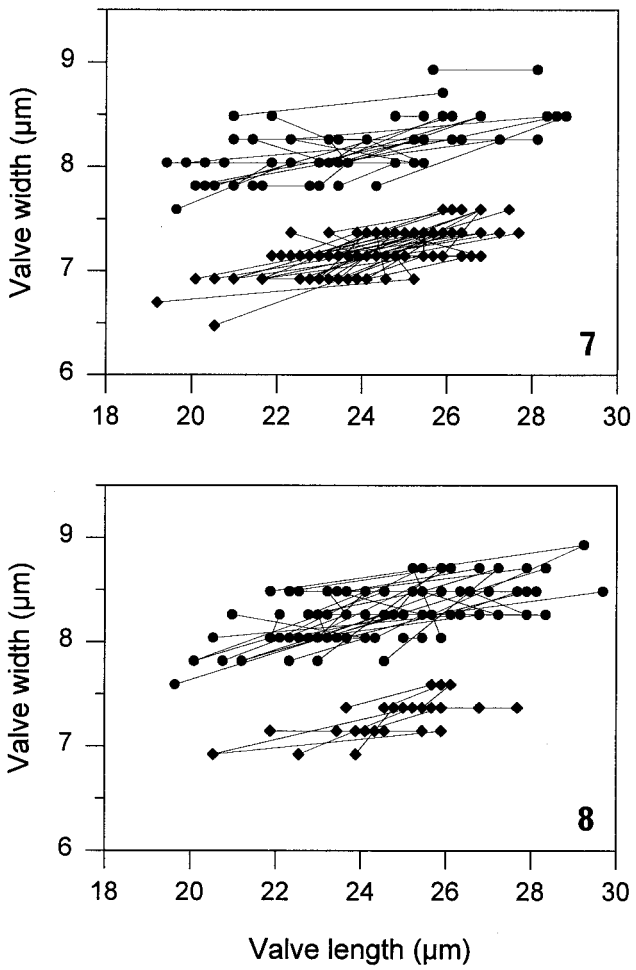
Recent work by Roshchin and Chepurnov, however, suggests that mating systems are much more complex and varied in diatoms than was previously thought. Several marine species have been shown to be dioecious or to contain clones that exhibit different levels of intra- and interclonal sexual reproduction (Roshchin 1987, 1989, 1990, 1994a, b, Chepurnov 1993, Roshchin and Chepurnov 1994). In *Achnanthes longipes* C. A. Ag., for example, there are monoecious, unisexual, and bisexual clones (Chepurnov and Mann 1997), whereas *Nitzschia longissima* (Bréb. *ex* Kütz.) Grun. is dioecious (Roshchin 1994a). None of the pennate species studied by Roshchin and Chepurnov have proved to be simply monoecious, contrasting sharply with the freshwater diatoms studied by Geitler (1932). Dioecy also has been demonstrated in the marine planktonic *Pseudonitzschia multiseries* (Hasle) Hasle and *P. pseudodelicatissima* (Hasle) Hasle (Davidovich and Bates 1998).

Assuming that all the published reports are accurate, two explanations might be offered for the discrepancy between Roshchin and Chepurnov's observations and Geitler's: it could be an accident of sampling, or there could be real differences between marine and freshwater diatoms, as suggested by Roshchin (1994a). We are attempting to establish which of these is correct by studying a variety of unrelated freshwater diatoms, including *Sellaphora* and *Eunotia* species.

Mann (1984a, 1989a) demonstrated that the kind of sexual reproduction exhibited by *Sellaphora seminulum* (Geitler 1932) is also present in other *Sellaphora* species. Within *S. pupula* there are many morphologically distinct entities, several of which can coexist in the same lake with significant barriers to gene flow between them (Mann 1984a, 1989b, Mann and Droop 1996, Mann et al. 1997). In order not to prejudge their taxonomic status, we will refer to such populations as ''demes,'' a deme being ''any assemblage of taxonomically closely related individuals'' (Gilmour and Gregor 1939: 333). Here we report work on two demes of *S. pupula* from Blackford Pond, a small eutrophic lake in Edinburgh, referring to them by the informal names we have used



FIGS. 1–6. *Sellaphora pupula* valves from natural populations, Blackford Pond. FIGS. 1–3. Capitate deme. FIGS. 4–6. Rectangular deme. Scale bar (Fig. 6) = 10 μm.



FIGS. 7, 8. *Sellaphora pupula* sexualized populations from Blackford Pond. Gamograms showing mating (connecting lines) between cells characterized by their width and length. Measurements were made from fixed, stained cells. ● rectangular; ♦ capitate. FIG. 7. August 1987. FIG. 8. March 1988.

previously: "rectangular" and "capitate" (Figs. 1–6). The demes have been partially characterized already, but only from observations of seminatural populations (Mann 1984a, 1988a, 1989b, Mann and Droop 1996).

MATERIAL AND METHODS

Sampling of natural populations. Fine muds containing epipelic diatoms were collected from Blackford Pond, Edinburgh (U.K. National Grid reference NT 253709, 70 m alt.) and processed as described by Mann (1989b). Populations of *S. pupula* were studied on coverslips used to harvest epipelon, either immediately after removal from the mud or after incubation for a few days in lake water enriched with 12%–30% WC medium with silicate (Guillard and Lorenzen 1972). Incubated coverslips were maintained at 16°–18° C under cool-white fluorescent lights, either in continuous light or with 16–18 h light per day. Supplements of WC medium (1 part in 8–10) were added every 3–4 days. As previously (Mann 1984a, 1989b), sexual reproduction was often induced when mixed seminatural populations of *S. pupula* were transferred from Blackford Pond mud (at 2°–18° C) to continuous illumination in the laboratory (at 15°–25° C). Pairing was generally observed within 2–3 days of collection and stages of auxosporulation occurred thereafter, up to around 10 days after collection, when most pairs had formed initial cells. Populations of sexualized *S. pupula* (and other epipelon) were preserved either by mounting in Naphrax, after incineration at 550° C for 20 min in a muffle furnace to remove organic matter, or by fixing and staining. For the latter, the protocols were: (1) fixation with 3:1 or 4:1 ethanol:acetic acid, containing enough ferric chloride (as mordant) to give a pale straw color, followed by staining with acetocarmine; (2) fixation with Dyer's (1979) solution, staining with Harris' hematoxylin; (3) fixation with Flemming's weak solution (Johansen 1940), staining with Harris' hematoxylin (cf. Mann 1987, Mann and Stickle 1995a, b). Following dehydration, preparations were mounted in euparal or canada balsam.

Identification of the demes. Representative valves of the capitate and rectangular demes collected from Blackford Pond are shown in Figs. 1–6; others are illustrated by Mann (1989b). There are no qualitative (presence/absence) distinctions between these demes; all differences are matters of degree. For any given length, capitate valves are consistently narrower than rectangular valves (compare Figs. 3 and 5, both ca. 28 μm long, but 8 and 9 μm wide, respectively), leading to complete separation in length–width plots (Figs. 7–9, Mann 1988a: fig. 1); rectangular deme exhibits a wider range of valve length between initial cell and gametangium (see Results and Mann 1989b). In addition, capitate valves have a slightly less sinuous raphe, striae that are slightly more geniculate near the poles (Figs. 1–6), and polar bars that

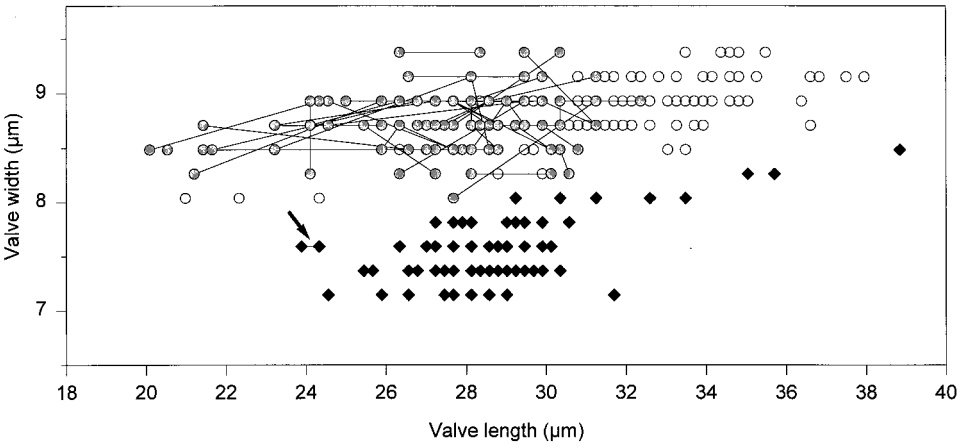
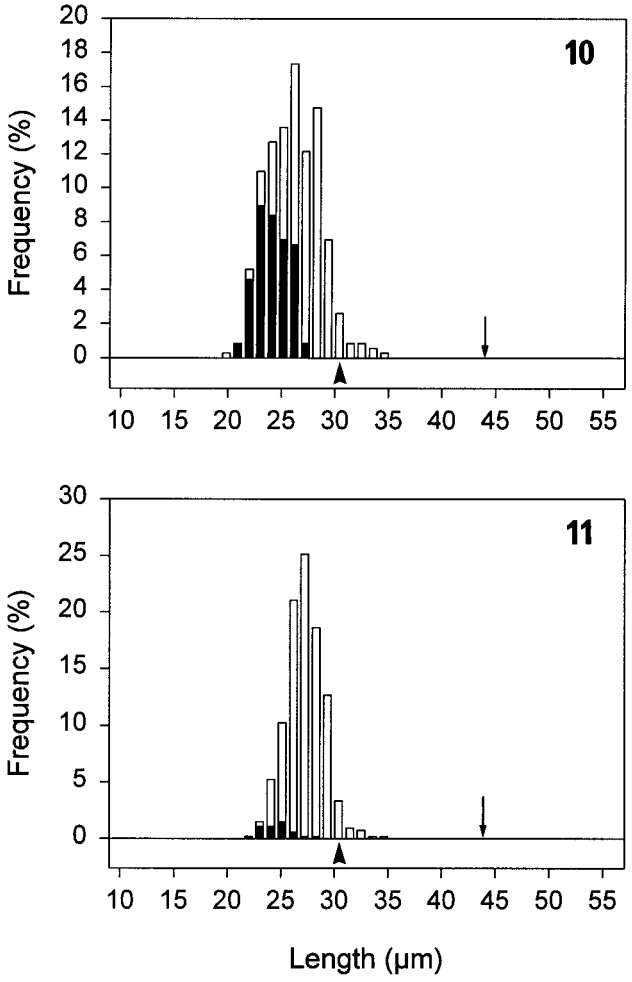


FIG. 9. *Sellaphora pupula.* Length–width variation in a population from Blackford Pond, January 1988, with superimposed gamogram. Note the imbalance between the demes in the numbers of sexualized cells (the single capitate pair is arrowed). ○ rectangular (white, vegetative; grey, sexualized); ♦ capitate.



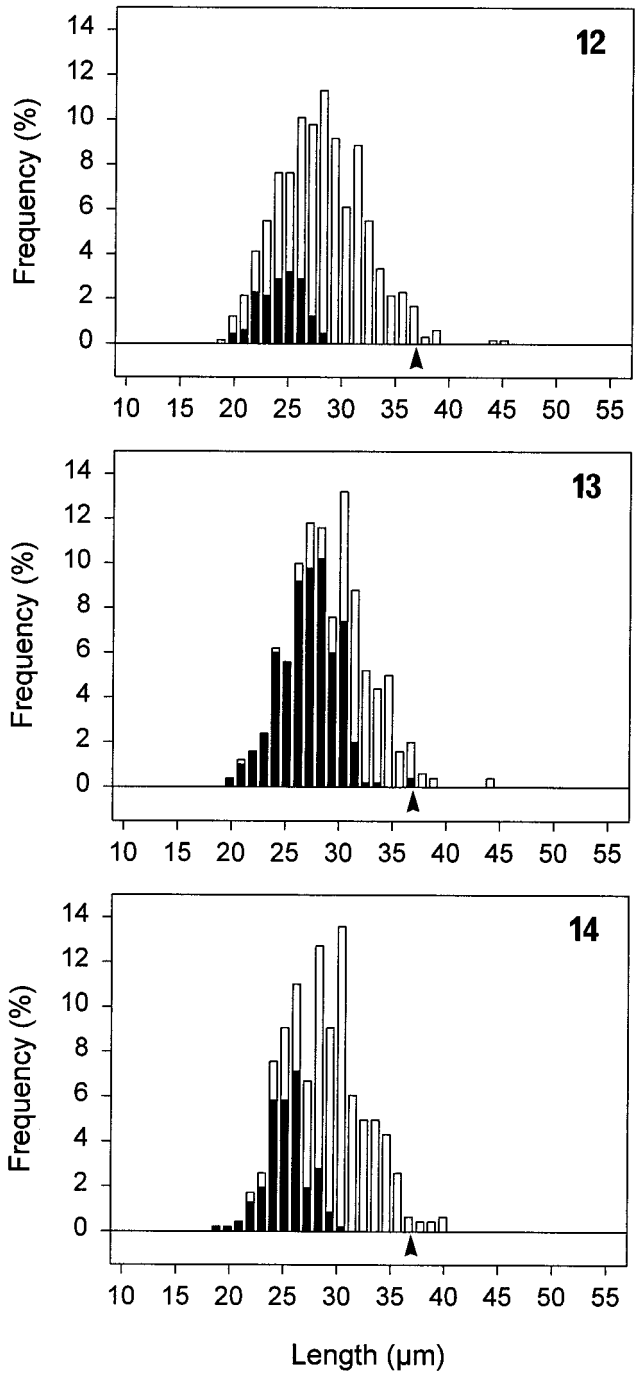
FIGS. 10, 11. *Sellaphora pupula* capitate deme. Size spectra in seminatural populations with vegetative (white) and sexualized cells (black) differentiated. The sexual size threshold (arrowhead, 30.5 μm) and the maximum recorded initial cell size (arrow, 44 μm) are indicated. FIG. 10. August 1987 (346 cells). FIG. 11. March 1988 (537 cells).

are perpendicular to, rather than inclined to, the apical axis (Mann 1989b: fig. 1g–j, m–o; possession of these bars next to the polar raphe endings is characteristic of *S. pupula* and a few other species of *Sellaphora*). The valves of the capitate deme have thinner walls than those of rectangular demes (note the thicker outline in Figs. 4–6). The capitate and rectangular demes exhibit similar ranges of shape during the life cycle (e.g. Figs. 1–6). Longer valves of both demes have capitate poles (Figs. 1, 2, 4, 5), but this feature is gradually lost during size reduction (Figs. 5, 6; Figs. 21, 23, 29–35). The similarity in shape makes it difficult to distinguish the demes quickly by eye, unless both are present in the same field of view. However, with care, identification is unambiguous. During mating, the gametangia lie close together, in the same orientation (Figs. 36–39, 41–44), so that it is simple to compare their size, stria pattern, and polar bar inclination. As a result, mating between demes would be easily detected if it occurred.

Neither deme corresponds exactly to any previously described infraspecific taxon within *S. pupula,* although Krammer and Lange-Bertalot (1986: 3, fig. 68) illustrate a valve that resembles the rectangular deme, as a form ''mit Konvergenz zu [*Sellaphora*] *laevissima*.'' Lange-Bertalot and Metzeltin (1996: pl. 82, figs. 4, 5) illustrate two valves, which they identify as ''syn. Blackford Pond deme rectangular sensu Mann 1989,'' but their dimensions (43 × 10.5 μm and 45.5 × 11 μm) do not agree with the Blackford deme; for the same valve lengths, Blackford rectangular valves are ca. 1 μm narrower, which is probably significant, judging by the differences between the rectangular and capitate demes.

Cultures. Rough cultures of Blackford Pond epipelon were established by transferring cells harvested by coverslip or lens tissue into WC medium. Single cells were then isolated by micropipette into 3–4 mL of WC medium with silicate, adjusted to pH 7, in repli dishes. Cultures were inspected for contamination by other eukaryotes and reisolated from single cells if necessary (no attempt was made to free the cultures of bacteria), before being transferred to 50–55 mm diameter polystyrene or glass petri dishes to establish stock cultures. In Edinburgh, stocks were grown in incubators at 15° or 20° C with 16:8 or 14:10 h light:dark cycles using cool-white fluorescent lights and photon flux densities of 20–25 $\mu mol \cdot m^{-2} \cdot s^{-1}$. During periods when the cultures were required for experimental crosses, stocks were inoculated into new medium every 2–3 weeks. For long-term maintenance of cultures, cultures were transferred to 15° C and ca. 2 $\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$ with 14:10 or 12:12 h light:dark cycles. In these conditions, stocks could be kept for several months without transfer. In the Crimea, procedures were similar, except that cultures were grown at 18°–20° C in diffuse natural light from a north-facing window. Clones are referred to here by the abbreviated name of the deme and the clone number. Nine clones of the capitate (cap) and two of the rectangular (rect) deme were used (Figs. 15–35, listed in Fig. 45). All clones were isolated on 2–4 May 1996, except rect-13B, which was isolated on 8 October 1996.

Experimental crosses. As with the species studied by Roshchin and



FIGS. 12–14. *Sellaphora pupula* rectangular deme. Size spectra in seminatural populations with vegetative (white) and sexualized cells (black) differentiated. The length axis spans the full range within the deme (9–57 μm), and the sexual size threshold is indicated (arrowhead, 37 μm). FIG. 12. August 1987 (654 cells). FIG. 13. January 1988 (500 cells). FIG. 14. March 1988 (463 cells).

Chepurnov (Roshchin 1994a), sexual reproduction was most vigorous when the stock cultures used for experimental crosses were growing actively in exponential phase. Whatever the past history of the stocks, therefore, new stocks were usually grown whenever crossing experiments were planned. They were then monitored regularly to check for vigorous growth. Crosses were initiated by transferring small aliquots of stock cultures, in pairs, into 3–4 mL of fresh WC medium in repli dishes. The mixed cultures were examined daily thereafter using a Zeiss Axiovert inverted microscope to check for any sign of sexual activity. Occasionally, for photography, mixed cultures were initiated in 90 mm diameter petri dishes, in which coverslips had been placed; the cover slip could then be removed, wiped clean on one side and mounted on a drop of WC medium for examination.

Preparation of cleaned valves and microscopy. Frustules were cleaned by boiling natural populations or cultured diatoms with a mixture of concentrated nitric and sulphuric acids. After washing with deionized water, specimens were mounted in Naphrax and observed using a Reichert Polyvar photomicroscope; photographs were taken on Kodak Technical Pan film. Measurements were made using the drawing attachment of the Polyvar (Mann 1988a). Cleaned material of natural populations and all clones and fixed and stained seminatural populations are preserved in the herbarium of the Royal Botanic Garden, Edinburgh.

RESULTS

Characteristics of the Capitate and Rectangular Demes in Natural Populations

Further observations of natural populations from Blackford Pond have not extended the morphometric data provided by Mann (1989b). The minimum length recorded in natural populations is the same in both demes: 19 μm. The upper limits differ, however, with initial cells of 34–43 μm recorded in the capitate deme and 40.5–57 μm in the rectangular deme.

As in other diatoms (Geitler 1932, von Stosch 1951, 1956, Drebes 1977), *S. pupula* cells above a certain critical size threshold cannot reproduce sexually (Fig. 9). The largest gametangium of the capitate deme recorded among seminatural populations was 30.5 μm long; the largest of the rectangular deme, 37 μm. However, observations of sexualized populations (Figs. 10–14) suggest that the size threshold for sexual reproduction is not ''all-or-nothing.'' In both capitate (Figs. 10, 11) and rectangular (Figs. 12–14) demes within the size range where any cells become sexualized (i.e. below 30.5 and 37 μm, respectively), the proportion of cells sexualized increases with smaller size. This might reflect variation in the sex threshold itself so that some cells pass the threshold before others. However, although it is likely that there is some genetic heterogeneity in any large population with respect to quantitative traits, our data suggest that a second factor also operates—an increasing tendency to become sexualized within any particular cell lineage as cell size decreases. The data plotted in Figures 13 and 14 were derived from material collected from Blackford Pond in early 1988. Overall, these two populations of the rectangular deme have very similar size spectra and insignificantly different variances (14.047 and 13.419; $F = 1.047$; 499, 462 = df) and means (29.038 and 28.962; $t = 0.32$, 961 df). However, in the first sample to be taken (Fig. 13), a far higher proportion of cells became sexual than in the later sample (Fig. 14)—62.4% as opposed to 28.7%—and the sexual cells were on average longer (27.130 vs. 25.530; $t = 6.99$, 329 df; significant at $\gg$99.9%). This suggests that, unless there was a re-

markable shift in the composition of the population in less than 2 months without any detectable change in the size distribution, cells within the sexual size range are differentially susceptible to induction. Larger cells only become sexual if very strongly stimulated, as reflected in the high percentage of sexual cells in Figure 13 (unfortunately, we do not know what the stimulus is).

In spite of the morphological similarity between the demes, over 10 years observation of sexual reproduction in mixed seminatural populations has failed to reveal a single case of interbreeding between them. Intrademic mating is displayed in Figures 7–9 via gamograms—plots of pairing between cells characterized morphometrically (here, by length and width). Between January 1987 and March 1989, populations were transferred to the laboratory at approximately monthly intervals to test for sexualization. The results (Table 1) show that sexualizable cells of both rectangular and capitate demes are present at virtually any time of the year; for contrast, Table 1 also contains data on another Blackford deme, ''small,'' which was sexualized much less frequently. Even when sexualized cells of one deme were greatly outnumbered by those of the other deme (e.g. Fig. 9), no interbreeding occurred.

Clonal Cultures

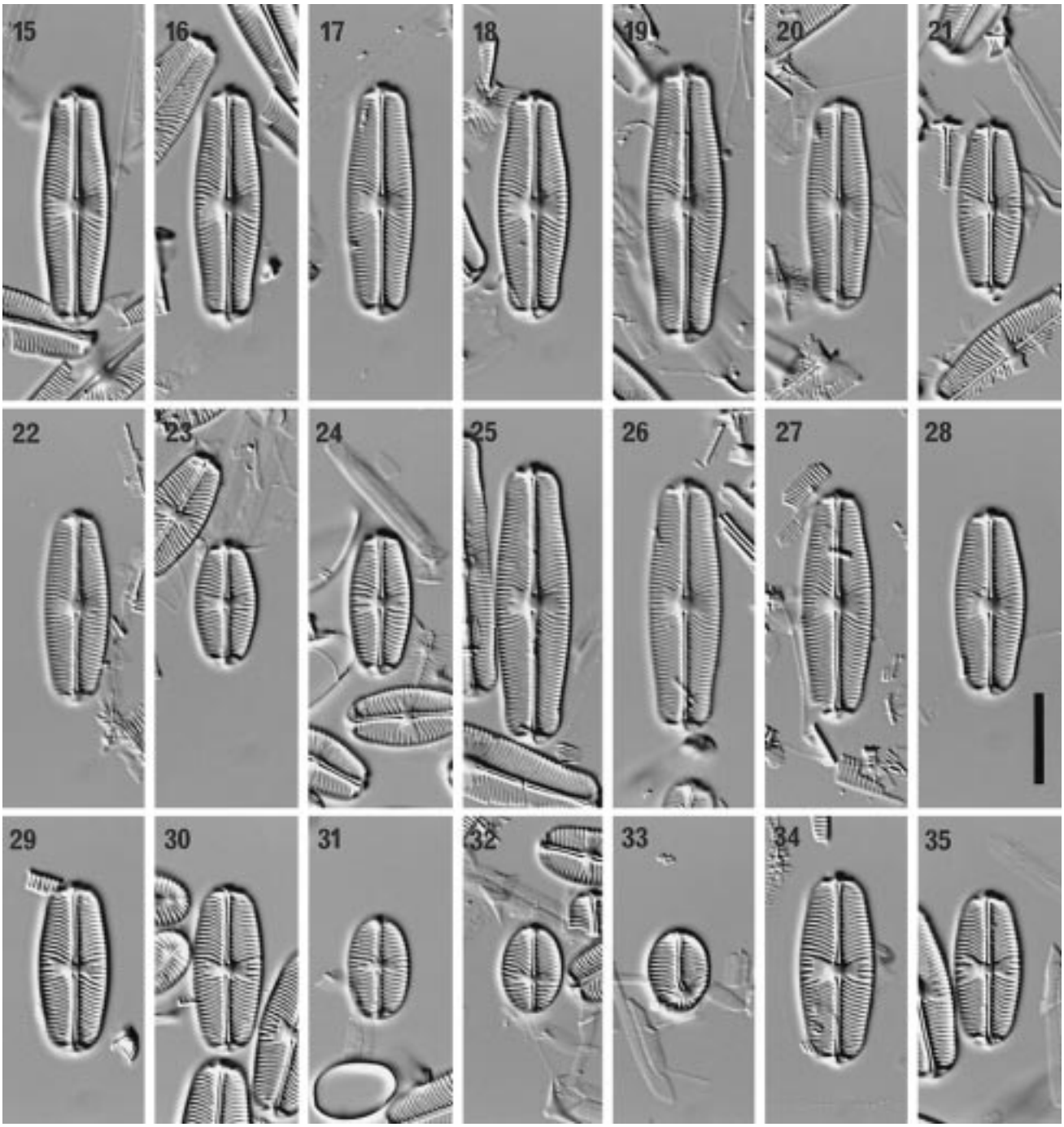
One of the consequences of the mating system in the capitate and rectangular demes, which we describe below, is that it is impossible to retain clones (or inbred lineages) for longer than a few months or years. At the time of writing (January 1998), only cap-2 and cap-6 are still growing actively, and rect-13B is very small-celled and moribund; three clones have already died through becoming too small to auxosporulate (cap-11, cap-12, cap-38), and others have been lost during subculturing (cap-3) or destroyed (cap-5, cap-10, cap-25, cap-26). Valves of all the clones used in this study are therefore illustrated in Figures 15–35. Visual comparison reveals a close resemblance between cultured material and wild-collected valves (e.g. compare Figs. 2, 3 with 15, 16 and Fig. 6 with 29, 34), but in culture the demes are more variable in outline and stria pattern. For example, the valve of cap-25 illustrated in Figure 25 is broader and less capitate than a wild-collected valve of equal length (Fig. 1). The central areas are almost always bow-tie-shaped or rectangular in nature (Figs. 1–3) but are sometimes much smaller in culture (Fig. 22), and striation density also can vary (Figs. 23, 24). The valves shown in Figures 15–35 (except Figs. 24, 32–34) were not selected to show the extremes of variation in culture, which will be the subject of a subsequent paper. At present, we would only note that the capitate and rectangular phenotypes are more plastic in culture than in natural populations from Blackford Pond (Mann 1984a, 1989b).

In culture, both demes exhibited size reduction (Figs. 22, 23, 27, 28, 29–35), as in nature (Figs. 1–6, 9). However in culture, much smaller cells were produced than have ever been seen in nature, where the limit seems to be 19 μm (see above). Cap-11 continued to reduce in size until some cells were only 10 μm, whereas rect-13 reached 9 μm (Fig. 33). In the smallest cells of both demes, the raphe slits were often of unequal length (Fig. 32), and sometimes there was only one slit with a disturbed pattern of striae (Fig. 33); despite these changes, the raphe still tended to lie in the apical plane (Figs. 31–33). The growth rate decreased markedly in cells less than 12 μm long. Size reduction was more rapid in the rectangular than in the capitate deme. Between 26–28 May and 23 July 1996, the average decrease in mean lengths of the capitate clones (except cap-10, which was not measured until 14 June) growing actively in WC medium and subcultured at similar intervals was 1.172 μm (±0.383, 95% confidence limits), whereas rect-13, grown alongside the capitate clones, decreased by 2.1 μm in the same interval. Later, in just under 2 months (22 October–18 December 1996), the mean length of rect-13B declined by 3.05 μm, whereas in 3 months (18 September–18 December 1996), cap-2 and cap-6 only declined by 0.75 and 2.25 μm, respectively. However, in other diatoms the rate of size reduction is known to vary during the life cycle (e.g. Jewson 1992) and so the differences we observed between the two demes may not be maintained over the whole course of size reduction. *A priori,* however, rectangular cells might be expected to reduce in size more per division as a result of their more robust, thicker frustule (Figs. 1–6).

There were no consistent differences in morphology between clones of a single deme, apart from those associated with size reduction (Figs 15–35).

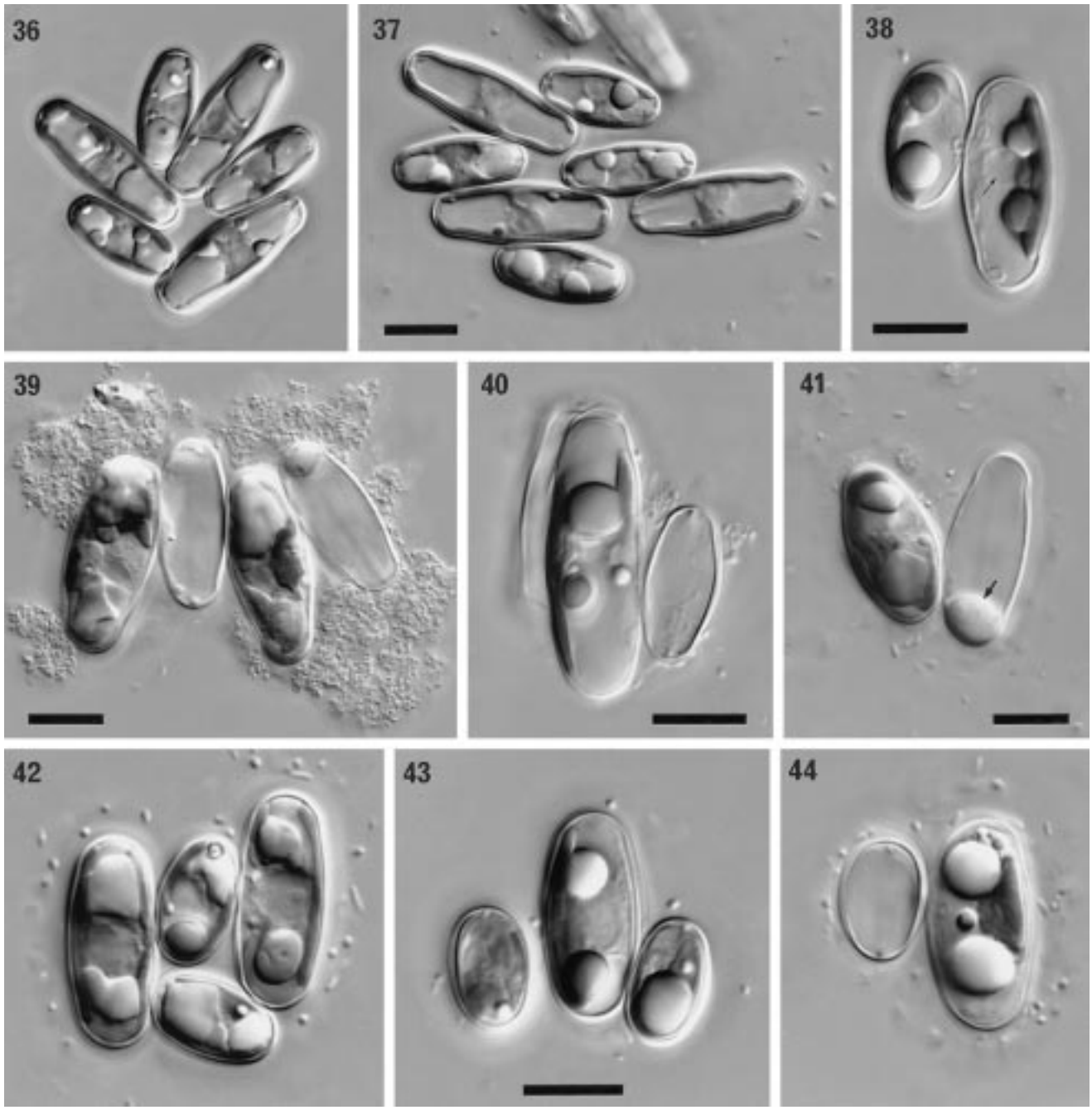
Characteristics of Sexual Reproduction in Culture

Sexual reproduction in *Sellaphora pupula* has been described by Mann (1989a), based on natural and seminatural populations; cells behaved identically in culture. Briefly, sexualized cells cluster in pairs or larger aggregates (Figs. 36, 37). At this stage they have the same protoplast configuration as during mitotic interphase, with a single H-shaped chloroplast lying against the epitheca and a nucleus slightly to one side of the cell at the center. The formation of clusters appears to involve active, directional movement, possibly in response to a pheromone. Mating cells move backward and forward near each other, before bonding firmly to each other via their girdles. Aggregates such as those in Figures 36 and 37 are initially mobile, the whole mass moving around without displacement of individual cells. Later, the pairs or aggregates become stationary and meiosis begins. Because large clusters are common during the early stages of sexual reproduction but are rare later when most sexualized cells are present



FIGS. 15–35. *Sellaphora pupula* valves from clonal cultures used in crossing experiments from May–July (S) or October–December (A) 1996. FIGS. 15–28. Capitate deme. FIG. 15. Cap-2 (S). FIG. 16. Cap-2 (A). FIG. 17. Cap-3 (S). FIG. 18. Cap-6 (A). FIG. 19. Cap-10 (S). FIG. 20. Cap-11 (S). FIG. 21. Cap-11 (A). FIG. 22. Cap-12 (S). FIGS. 23, 24. Cap-12 (A). Note the variation in striation density and pattern between these valves. FIG. 25. Cap-25 (S). FIG. 26. Cap-26 (S). FIG. 27. Cap-38 (S). FIG. 28. Cap-38 (A). FIGS. 29–35. Rectangular deme. FIGS. 29, 30. Rect-13 (S). FIGS. 31–33. Rect-13 (A), showing a valve of more or less normal appearance (Fig. 31) and two very small valves with abnormal raphe systems. FIGS. 34, 35. Rect-13B (A). Scale bar (Fig. 28) = 10 μm.

as pairs or triplets (Figs. 38, 40, 41, 43, 44), clusters must often break up. This process has not been studied. During meiosis, the chloroplasts of paired cells (gametangia) move away from each other and the nuclei move toward each other, to produce the configuration shown in Figure 38. At meiosis I, each gametangium divides unequally into a large cell, containing the single undivided chloroplast, and a small apochlorotic cell, which lies next to the epitheca. The larger cell becomes the single gamete, following degeneration of one of the two haploid nuclei from the second meiotic division. A small



FIGS. 36–44. *Sellaphora pupula* sexual reproduction in mixed clonal cultures. FIGS. 36–40. Cap-11 (smaller, male) × cap-38 (larger, female). FIGS. 36, 37. Motile clusters of sexualized cells, still with the interphase arrangement of nucleus and chloroplast. Note the alternation of small and large cells within the clusters. FIG. 38. Pair after meiosis II. In the cap-38 cell (right), the haploid gametic nucleus is visible (arrow); note also that the chloroplasts lie outermost in the pair. FIG. 39. Quadruplet, after fertilization: cap-11 cells appear empty, following the emigration of the active gametes, while the zygotes lie within the cap-38 cells (larger). FIG. 40. Expanding auxospore, still partially contained within the cap-38 frustule. FIG. 41. Cap-38 (smaller, female) × cap-2 (larger, male): a pair, after fertilization. Cap-38 contains the zygote, whereas cap-2 appears to contain only a residual bleb of cytoplasm (at arrow; it also contains the expanded apochlorotic cell produced during gametogenesis). FIGS. 42–44. Rectangular deme: rect-13 (smaller, male) × rect-13B (larger, female). FIG. 42. Quadruplet in ring configuration, early meiotic prophase; each cell is bound to two cells of the other deme. FIG. 43. Triplet, meiotic prophase. The left-hand rect-13 cell, although committed to meiosis, is superfluous; it has disengaged and will die. FIG. 44. Pair, after fertilization. The zygote lies in the rect-13B cell (right). Scale bars = 10 μm.

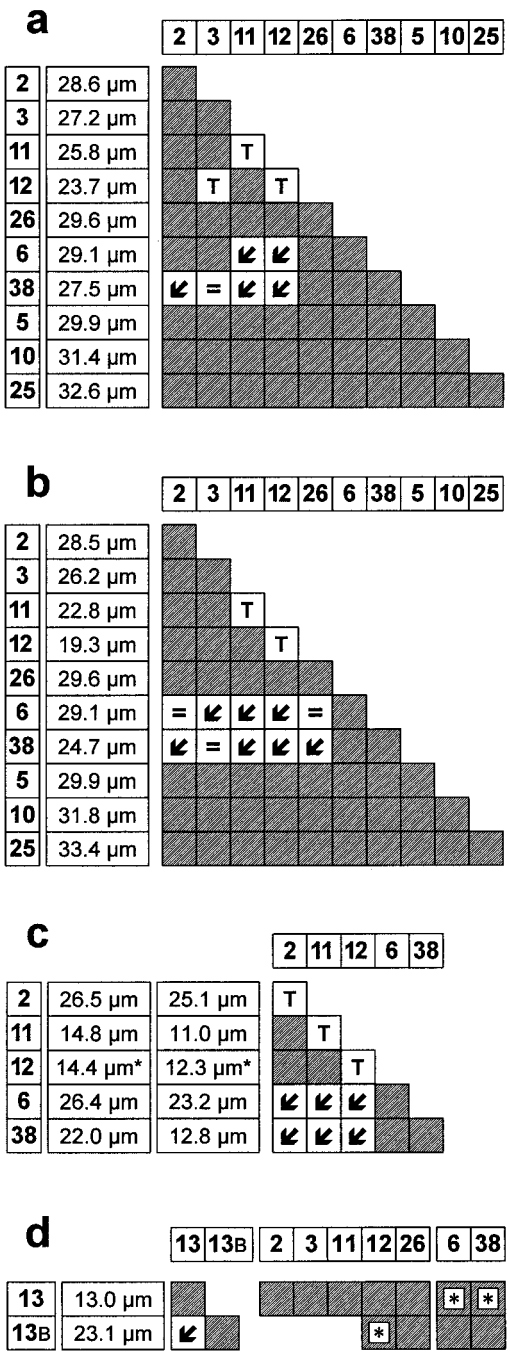


FIG. 45. *Sellaphora pupula* results of crossing experiments and occurrence of intraclonal sexual reproduction. Filled squares: no sexual reproduction; ''T'': trace intraclonal or interclonal reproduction; open square in interclonal crosses: vigorous sexual interaction, producing abundant viable auxospores (an arrow indicates direction of gamete movement; = indicates that the clones were so similar in size throughout the study period that the direction of gamete movement could not be determined). Mean cell lengths at the beginning (also at the end in c) of each study period are given for each clone. a. Capitate. May–August 1996: all lengths ±95% confidence limits <0.36 µm. b. Capitate. September–December 1996: all lengths ±95% confidence limits <0.72 µm. c. Capitate. February–June 1997: all lengths ±95% confidence limits <0.5 µm, except final cap-12 measurement (±0.86 µm) and cap-38 (±1.1 µm and ±0.66 µm) (* unlike other measurements, those for cap-12 refer to late autumn 1996 and 28 April 1997). Note that although cap-2 and cap-6 were initially similar in size, by the end of the study period they had diverged sufficiently for it to be possible to determine the direction of gamete movement. d. Rectangular and capitate. Asterisks in interdemic crosses indicate that, although the clones were incompatible, there was a stimulation of activity in mixed cultures.

opening is formed between the girdles of the gametangia and the gamete from one gametangium moves through to fertilize the gamete produced by the other gametangium. The zygote thus lies entirely within one of the gametangia (Figs. 39, 41, 44), leaving the other gametangium apparently empty, though it is in fact filled by the expanded apochlorotic cell and a residual bleb of cytoplasm containing volutin granules and reserve material (Figs. 39, 41, Mann 1989a). Subsequently, the zygote expands parallel to the apical axis of the gametangium in which it was formed (Fig. 40).

Intraclonal Reproduction

The clones were monitored after every subculturing (and at least weekly in 1996; less frequently during periods of low-level maintenance in 1997) for intraclonal sexual reproduction. No case of successful intraclonal auxosporulation was found. Pairs were found very rarely in some of the clones (cap-11, cap-12), but no viable initial cells were ever produced. In most stock cultures no sexual interactions occurred. In mixtures of pairs of clones, rare intraclonal auxosporulation was observed in cap-11 and cap-12, as in monoclonal culture, and also in cap-2. Again, however, although intraclonal pairs proceeded through meiosis and formed zygotes, these aborted without forming initial cells. No intraclonal sexual reproduction was ever observed in cap-3, cap-6, cap-10, cap-25, cap-26 or cap-38. Neither of the rectangular clones was observed to reproduce intraclonally at any time.

Compatibility

Clones were mated in all possible pairwise combinations. Mixed cultures were sometimes sterile, like the monoclonal cultures from which they were derived, but in some combinations of clones sexual interactions took place, resulting in the formation of many auxospores and viable initial cells. Fifteen sets of experimental crosses were made over the course of a year, the earlier ones involving all the clones (Fig. 45a, b), the later ones involving only a selected subset (Fig. 45c). The results showed that there were two mating types within the capitate clones. One contained cap-6 and cap-38, and the other contained cap-2, cap-3, cap-11, cap-12, and cap-26. With one exception, mating never took place within each group but was usually very vigorous between members of different groups (Fig. 45), often yielding well over 100 pairs in each repli dish compartment. In the few cases where intraclonal

TABLE 1. Occurrence of sexual reproduction in natural populations of the capitate, rectangular, and small demes of *Sellaphora pupula* transferred to laboratory conditions between January 1987 and March 1989.

Date	Capitate	Rectangular	Small
12.1.87	+	+	
26.1.87		+	
9.2.87			
23.2.87	+	+	
16.3.87	+		
20.4.87		+	
4.5.87	+	+	+
18.5.87	+	+	+
15.6.87	+	+	+
6.7.87	+	+	+
3.8.87	+	+	+
17.8.87	+	+	+
31.8.87	+	+	+
14.9.87	+	+	+
28.9.87	+	+	+
12.10.87		+	
26.10.87	+	+	
9.11.87		+	
23.11.87		+	
7.12.87		+	
19.12.87		+	
18.1.88	+	+	
1.2.88	+	+	
15.2.88	+	+	+
29.2.88	+	+	
18.3.88	+	+	
18.4.88	+	+	
10.5.88	+	+	
1.6.88	+	+	+
21.6.88	+	+	+
2.8.88	+	+	+
16.8.88	+		
6.9.88	+	+	
19.9.88	+	+	
3.10.88	+	+	
1.11.88	+	+	+
21.11.88	+	+	
6.1.89	+	+	+
21.1.89	+	+	
6.3.89	+	+	

pairings also occurred in crosses involving compatible combinations of cap-2, cap-11, or cap-12 with either cap-6 or cap-38, the intraclonal pairings were greatly outnumbered by interclonal pairings (by ca. two orders of magnitude). Two clones (cap-10 and cap-25) did not become sexual between their isolation on 2–4 May 1996 and disposal in September 1996. During this period, their mean size remained above 30.5 μm, so they were probably outside the sexual range (see above). A third clone (cap-5), though a little smaller (mean size 29.9 μm), also showed no sexual interactions, except perhaps with cap-38 in September 1996. Rect-13 was compatible with rect-13B (Fig. 45d).

Four clones, cap-2, cap-3, cap-6, and cap-26, although below 30.5 μm when isolated, did not initially show their full range of sexual interactions (compare Fig. 45a, b). For example, within a month of isolation, cap-6 showed itself capable of mating with the small-celled clones cap-11 and cap-12, but no auxosporulation occurred in mixtures of cap-6 with cap-3 until early August (when both clones had decreased in size by about 1 μm), and no crosses between cap-6 and cap-2 occurred until 23 September. No mating was observed between cap-26 and the compatible clones cap-6 and cap-38 before 23 September, suggesting that it was initially above the size threshold. Although no quantitative data were gathered, we gained the clear impression that the intensity and likelihood of mating between compatible strains is inversely related to cell size, below a threshold that probably varies between clones but is never much above 30 μm.

The only anomalous result involved cap-3 and cap-12. In one mixed culture, initiated on 8 June 1996, some monoecious (intraclonal) pairs of cap-12 were formed, but apparently also a few pairs of cap-3 × cap-12, which did not develop beyond the earliest stages. Attempts to cross cap-3 and cap-12 were made on four other occasions (25 May, 28 June, 11 August, 23 September 1996) with no result, despite successful crossing of cap-3 with other clones (cap-6 and cap-38) at the same time and despite further instances of intraclonal pairing in cap-12. The 8 June observations, therefore, remain unconfirmed. It is possible that the mixed culture was contaminated by another clone (cap-38 was of similar size to cap-3), although this is unlikely.

There appeared to be no lower limit of size below which sexual interactions could not take place. Even when reduced to sizes well below those found in nature, cap-11 and cap-12 would still mate with cap-6 and cap-38 (Fig. 45c). For example, on 4 July 1997, cap-11 was mated with cap-38 when cap-11 cells had reached ca. 11 μm in length. Cells paired as usual, underwent meiosis, and formed zygotes, although viable initial cells were not produced. Likewise, by the time it was mated with rect-13B in October and November 1997, rect-13 had reduced to 9–13 μm in length—again, well below the usual minimum in nature. As with cap-11, these extremely small cells could still pair and form gametes (Figs. 42, 43), but zygotes were produced only rarely (Fig. 44), and the auxospores did not expand.

Sexual Differentiation Between Clones

In most cases, clones differed significantly in cell size, so that in mixed cultures, we could identify each individual cell as belonging to one clone or another. As a result, it was possible to determine that, during pairing in compatible combinations of clones, cells of one clone bond only to cells of the other clone (Figs. 36–44). This was particularly obvious in the large clusters often formed in the early stages of pairing. Figures 36 and 37 show two such clusters in mixed cultures of cap-12 and cap-38. In Figure 36, small (cap-12) and large (cap-38) cells alternate strictly within the horseshoe-like cluster; in the more complex cluster in Figure 37, some cells are linked to two or more cells of the other clone

TABLE 2. Sizes of female and male gametangia in sexualized seminatural populations of *Sellaphora pupula* demes.[a]

Date	Pairs (N)	Female gametangia (μm)	Male gametangia (μm)	*t*
Capitate				
24.9.84	61	24.890 ± 1.358 (22.3–30.3)	25.415 ± 1.077 (23.4–28.0)	2.36*
25.2.86	84	25.537 ± 1.383 (22.3–29.7)	26.150 ± 1.272 (22.3–29.1)	2.99**
August 1987	38	23.609 ± 1.055 (21.1–26.3)	23.985 ± 1.624 (19.4–27.4)	1.20
18.3.88	12	24.190 ± 1.428 (21.1–26.3)	25.381 ± 1.388 (23.4–27.4)	2.07*
Rectangular				
24.9.84	107	26.227 ± 1.902 (22.9–32.0)	26.328 ± 1.998 (21.1–31.4)	0.38
24.9.84	100	26.537 ± 2.109 (21.7–31.4)	26.434 ± 2.183 (20.6–32.0)	0.34
early 1988	100	26.731 ± 2.596 (20.6–32.6)	26.303 ± 2.273 (20.6–30.9)	1.24
18.3.88	100	24.994 ± 2.225 (19.4–31.4)	24.783 ± 2.020 (20.6–29.1)	0.70

[a] Measurements are means ± 1 SD (and range). Student's *t*-values are given for the differences between the mean sizes of female and male gametangia with significance levels: ** $P < 0.01$; * $P < 0.05$.

on the same side or both sides of the cell, but again, bonding is only between cells of different clones.

The two mating types of the capitate deme differed in the behavior of their gametes, as did rect-13 and rect-13B. Cap-6 and cap-38 always produced passive gametes, whereas when mated with cap-6 and cap-38, cells of other clones always produced active gametes (Figs. 39–41, 45a–c). In a few combinations, cell sizes were so alike that it was not possible to determine the direction of movement of the gametes. However, from the remaining cases, it was clear that gamete behavior is consistent within particular clones and is not dependent on the sizes of the clones being mated (as might have been expected by analogy with centric diatoms; see above and Drebes 1977). Figures 39 and 40 show crosses between cap-12 and cap-38, where the male gametangium (cap-12) is the smaller of the two, whereas Fig. 41 shows the opposite case, where the female gametangium (cap-38) is smaller than the male (cap-2). Five clones, two female and three male (cap-2, cap-6, cap-11, cap-12, and cap-38), were maintained in culture for a year and always exhibited the same sexual behavior when mated with any of the others, no matter their absolute size or their size relative to the other clone (Fig. 45a–c). Thus, cap-6 and cap-38 can be regarded as inherently female, whereas cap-2, cap-3, cap-11, cap-12, and cap-26 are male. Our limited knowledge of rect-13 indicates that it was male (Fig. 45d). When mated with rect-13B, it was already very small and zygotes were formed infrequently (Fig. 44), but always within the frustule of rect-13B. Rect-13B has since maintained consistent behavior (as a female) in crosses with other rectangular clones (Mann and Chepurnov, in prep.).

Size differences between sexes and size selectivity in seminatural populations

The results reported above indicate that there are very strong barriers to inbreeding in capitate clones. The evidence presented for the rectangular deme is less satisfactory as it is based on only two clones. Our observations also show that sexual behavior is constant within a clone and suggest that there is no marked difference in the size at which male and female cells become sexual (e.g. see the behavior of cap-6 and cap-26; Fig. 45a, b). *A. priori,* therefore, there seems no reason to expect a difference in mean cell size between males and females in nature, nor any departure from random mating between cells of different sizes.

However, to facilitate comparison with Geitler's observations of *Sellaphora seminulum* (Geitler 1932, 1957b), we examined the relationship between the sizes of female and male gametangia in seminatural populations collected from Blackford Pond over a 4-year period (Table 2). These revealed a consistent difference between the rectangular and capitate demes. In capitate, although the overall ranges of size were about the same for female and male gametangia, on average, males were significantly larger. No such difference between males and females could be detected in the rectangular deme.

We also performed an analysis of variance to test whether cells were more or less similar in size within pairs (or triplets or larger groups) than would be expected on the basis of random pairing (Table 3). Again, the two demes differed. In the rectangular deme, there was generally less variation within pairs, triplets, etc., than between them: cells tended to pair with cells of similar size more often than can be ascribed to chance. In several pairs in each dataset the gametangia were so similar in size that they could quite easily have been sister cells derived by mitotic division of a single parent. The only exception was one set of data from 18 March 1988 (Table 3), when there was an (insignificant) trend in the opposite direction. The data suggest, therefore, that selfing may occur in natural populations of rectangular demes, although only at a low rate. In the capitate deme, there is no evidence of intraclonal reproduction in seminatural populations. Indeed, as one would expect from the size differences associated with sex (Table 2), there is usually more variation within pairs than between them (Table 3).

TABLE 3. Analysis of size variation within and between pairs of copulating cells in seminatural populations of *Sellaphora pupula* demes, with valance ratio *F* (in square brackets where the variance within pairs is higher than between) and significance levels.

Date	Sum of squares between pairs	Sum of squares within pairs	Trend	*F*
Capitate				
August 1987	94.54 (60)	172.59 (68)	within > between	[1.61]*
August 1987	353.39 (118)	374.23 (132)	between > within	1.07
18.3.88	12.82 (11)	33.07 (13)	within > between	[2.17]
Rectangular				
24.9.84	548.08 (99)	364.73 (100)	between > within	1.52*
August 1987	717.94 (122)	536.07 (127)	between > within	1.39*
August 1987	224.91 (51)	170.29 (54)	between > within	1.40
early 1988	1115.11 (140)	829.92 (161)	between > within	1.55**
18.3.88	227.67 (61)	293.49 (70)	within > between	[0.89]
18.3.88	517.84 (99)	378.61 (100)	between > within	1.38*

[a] Significance levels are $^{**}P < 0.01$; $^{*}P < 0.05$. Sums of squares between and within pairs also include triplets and larger groups; degrees of freedom indicated in parentheses.

Sex Balance and Polyandry in Natural Populations

At present, we know of no marker that would make it possible to determine, before plasmogamy, which cells are male and which are female. Therefore, there is no direct way to determine the sex balance in natural, unsexualized populations. Clearly, among mating pairs, there is an exact 1:1 balance between male and female, and if most of the cells in a size class are sexualized and find mates, as in the rectangular deme in January 1988 (Fig. 13), there cannot be a severe imbalance between the sexes. However, during sexual reproduction, not all cells end up in pairs. In the capitate and rectangular demes, 10% of gametangial clusters are triplets or larger aggregates (Table 4; these results agree well with less extensive data provided by Mann [1984a] for undifferentiated capitate + rectangular demes). Sometimes, the third cell in a triplet reverts, becoming vegetative again (soon afterward it divides mitotically, while the remaining pair of cells are still in meiosis), but frequently it undergoes gametogenesis. This is futile, however, because the third cell never fuses with the other two to form a triploid, in contrast to *Craticula cuspidata* (Kütz.) D.G. Mann or *Dickieia ulvacea* Berk. *ex* Kütz. (Mann and Stickle 1991, Mann 1994), for example. Because quadruplets almost always resolve into two pairs during gametogenesis, quintuplets into a pair and a triplet, etc., we can calculate the maximum effective sex imbalance among mating cells by the proportion of superfluous cells in odd-numbered groups (triplets, quintuplets, etc.). For the rectangular deme, the data in Table 4 give the number of superfluous cells as 176 (175 triplets + 1 quintuplet) out of 4152; that is, a maximum sex imbalance among mating cells of 52.12%:47.88% if all the superfluous cells were of the same sex. For the capitate deme, the maximum imbalance is 56.4%:43.6%.

In order to check further for sex imbalance, we examined triplets in which zygotes had already been formed, and we counted the number of male–female–male and female–male–female types (Table 5). In all cases, male–female–male triplets were predominant, and the 24 September 1984 and early 1988 imbalance was highly (>99%) significant, even if all the indeterminable triplets were counted as female–male–female. The three counts for the rectangular deme are not significantly different from each other ($\chi^2 = 7.75$, 4 df) and combine to suggest a ratio of more than 2:1 between male–female–male

TABLE 4. Frequencies of pairs, triplets, and larger groups of copulating cells in seminatural populations of *Sellaphora pupula*.

Date	Clusters of gametangia		
	Pairs	Triplets	4–6 Cells
Capitate			
3.4.85	18	7	0
1987[a]	25	3	0
1988–1989[a]	19	1	0
1995[a]	101	5	0
Totals	163	16	0
Rectangular			
24.9.84	717	69	0
1987[a]	112	5	0
22.2.88	359	48	15
9.3.89	104	15	3
16.3.89	87	12	1
18.1.95	121	9	1
10.2.95	262	17	5
Totals	1762	175	25

[a] Combined counts for several dates.

TABLE 5. Polyandry and polygyny in *Sellaphora pupula*: numbers of male- and female-biased triplets.

Date	Type of triplet		
	♂♀♂	♀♂♀	Indeterminable
Capitate			
1984–1986[a]	7	1	0
Rectangular			
24.9.84	49	11	8
early 1988	46	18	2
18.3.88	21	12	2

[a] Combined count for several dates.

TABLE 6. Sizes of gametangia and initial cells in crosses between two clones, cap-11 (male) and cap-38 (female), of *Sellaphora pupula* at different stages in the life cycle.[a]

Date	N	Gametangia		Initial cells (μm)
		cap-11 (μm)	cap-38 (μm)	
26.5.96	20	25.75 ± 0.43	27.50 ± 0.47	42.23 ± 1.18 (40–44)
18.12.96	40	20.50 ± 0.53	23.80 ± 1.01	39.73 ± 1.51 (36–42.5)
19.4.97	35	12.15 ± 1.03	19.65 ± 1.20	35.26 ± 1.83 (31–39)

[a] All values are means ± 1 SD (and range for initial cells).

and female–male–female triplets. The capitate and rectangular demes seem, therefore, to be preferentially polyandrous. Superficially, the data suggest a surplus of males in natural populations, but there are alternative explanations (see below).

Interactions Between Demes

Attempts were made to cross rect-13 and rect-13B with various capitate clones (Fig. 45d). No hybridization occurred, consistent with observations of seminatural populations. However, in May–July 1996, before rect-13B was isolated and hence before it became possible to determine the sex of rect-13, it was noted that rect-13 was stimulated by the female capitate clones cap-6 and cap-38; male clones had no effect (Fig. 45d). The stimulation involved increased activity of the cells and close approximation of rect-13 and capitate cells, although without bonding and transition to meiosis. Stimulation by cap-6 and cap-38, but not by cap-2, cap-11, and cap-12, was checked, blind, by one of us (S.J.M.D.). From this it was predicted that rect-13 was a male clone, as was afterward shown in crosses with rect-13B. In autumn 1996, mixed cultures were made of rect-13B with cap-6 and cap-38 (Fig. 45d), all female clones. No stimulation occurred; nor did any occur with cap-26, possibly because cap-26 was still close to the size threshold for sexual reproduction (on 27 September it had a mean length of 28.6 μm). However, when rect-13B was mixed in late October 1996 with the male clone cap-12 (which at the time would pair vigorously with the female clones cap-6 and cap-38), the same kind of stimulation occurred as had previously been observed between rect-13 and female capitate clones.

Relationship between Gametangium Size and Initial Cell Size

On the basis of observations of seminatural populations, Mann (1989b) gave a range of 34–43 μm for initial cell length in the capitate deme. New measurements for populations collected on 24 September 1984 yielded similar results: gametangia with a mean length of 24.742 μm (range 22.8–26.8 μm, SD = 1.076, N = 26) gave rise to initial cells measuring 34.2–42.2 μm (mean 38.006, SD = 2.351, N = 17). We also measured the lengths of initial cells formed in culture in crosses between cap-11 (male) and cap-38 (female) (Table 6). The overall range (31–44 μm) was only a little-greater than we found in seminatural populations, in spite of the small sizes of cap-11 and cap-38 on 19 April 1997. The data show a correlation between the lengths of the gametangia and initial cells: small gametangia tend to produce small initial cells (Table 6). However, large changes in gametangium size produce only modest variation in initial cell size. During size reduction in the capitate and rectangular demes, cell width (and also cell depth) changes relatively little (Figs. 1–9, 15–35), so that it is reasonable to use cell length as a proxy for overall size. Table 6 shows that, as the combined mean gametangium lengths of cap-11 and cap-38 declined by 21.45 μm (from 53.25 μm on 26 May 1996 to 31.80 μm on 19 April 1997), mean initial cell length declined by only 6.97 μm.

DISCUSSION

The results of our experiments are consistent with earlier work on seminatural populations (Mann 1984a, 1989a, b), which demonstrated that auxosporulation is an allogamous sexual process in the capitate and rectangular demes of *Sellaphora pupula* and that these demes are reproductively isolated. We have now shown that they exhibit complex breeding systems, involving sexual differentiation and mechanisms that prevent or hinder selfing. These observations contrast sharply with those of Geitler (1932, 1957b), dealing with *Sellaphora seminulum.* However, we do not dispute Geitler's data, which established beyond doubt that *S. seminulum* can complete its life cycle in clonal culture (Geitler 1932: 20–23). We have ourselves isolated clones of small-celled *Sellaphora* species (including a diatom very similar to Geitler's *seminulum*) that can also reproduce vigorously within a clone, producing viable initial cells. We have also observed successful intraclonal reproduction in other demes of *S. pupula* (Chepurnov and Mann, unpubl.). Nevertheless, the majority of demes of *Sellaphora* that we have studied possess mechanisms promoting outbreeding. Some of these mechanisms are more complex than we have reported here, and indeed, the capitate deme, with its clear differentiation into ''males'' and ''females,'' is perhaps the easiest system to understand. It was our good luck to study it first.

Data from seminatural populations and clones all suggest that cells of the capitate deme do not become sexual until they are ca. 30 μm long. Below

this threshold, the propensity of cells to become sexual seems to increase with decreasing size, but their sexual behavior is constant, all cells of a clone always behaving either as males or as females in interclonal crosses (the single contrary result from cap-3 could not be repeated and must therefore be discounted). The size threshold for sexual reproduction seems to be the same for females and males, and male and female clones have identical frustule morphologies. Thus, the sexuality of *S. pupula* is unlike the sequential hermaphroditism of centric diatoms, where clones generally change sex from female to male during size reduction, with a period of overlap when male and female gametangia can both be produced (von Stosch 1951, 1956; the proportion of males and females is sometimes controlled by environmental factors, see Drebes 1977).

The exact nature of sexual differentiation in *S. pupula* cannot be determined from our observations. However, the consistency of sexual behavior within clones, despite deliberate and accidental changes in culture conditions (for example, loss of electric power in the Ukraine, leading to violent temperature fluctutaions over periods of a few hours or days; changes in light intensity and daylength), suggests either that sex determination is genotypic or that there is an irreversible developmental change during size reduction, so that a lineage of cells, initially neuter (sexually undetermined), becomes permanently either female or male.

If sex determination is developmental and phenotypic, one might expect it to be influenced by the proportions of males and females already present in the population so that an optimal sex ratio can be maintained. If so, it would be curious if sex determination took place appreciably before cells reach the size threshold, since otherwise, males might be born into a world in which, through some accident, males were already predominant. In a monoclonal culture, as cells passed the sexual size threshold, one might expect them to become female and male at random. One of our clones (cap-26) did not initially mate with any other clone and remained vegetative for 4 months after isolation, even when mixed with the female clone cap-38, which reproduced very vigorously throughout this period with the male clones cap-2, cap-11, and cap-12. Hence, cap-26 seems to have been above the sexual threshold when isolated. According to our argument, therefore, if sex determination is phenotypic, cap-26 should initially have been neuter and might have been expected to produce female and male cells when it passed the sexual size threshold. However, when cap-26 did become sexual, all cells behaved as males, with no hint of bisexuality, which suggests that the sex of cap-26 or any other clone is predetermined and probably genetically controlled. But we cannot exclude the possibility that the behavior of cap-26 is an artifact, brought about by using only a few cells to inoculate each subculture, leading to the accidental elimination of any cells of cap-26 that had been triggered to become female.

Differentiation between male and female clones, whether diplogenotypic or diplophenotypic, cannot be simple. It is expressed in at least two phases of sexual reproduction: cell–cell recognition during bonding and plasmogamy. These are separated in time (by the whole of gametogenesis), involve different structures and mechanisms, and must be controlled by several different genes. Sometimes, however, the compatibility mechanism breaks down: selfing is rare but does occur, with the formation of zygotes, although these are nonviable in capitate clones. This could not happen unless both aspects of differentiation had been affected. Intraclonal mating requires that bonding takes place between cells that usually reject each other as ''self'' (cf. Figs. 36, 37) and also that one cell in each pair behaves as a female instead of as a male (in cap-2, cap-11, and cap-12: intraclonal mating was not observed in the two female clones used in our experiments, which may be significant). Knowledge of diatom genetics is too scanty to allow interpretation. Interestingly, however, selfing (secondary homothallism) has been reported in a number of oomycetes that are predominantly heterothallic, such as in some *Phytophthora* and *Bremia* species (e.g. Sansome 1980, Michelmore and Ingram 1982). Here, mating type is controlled by alleles located in a section of chromosome involved in a reciprocal translocation. Self-fertile thalli are sometimes unstable, and at least in some cases, this is because they are trisomics and ''possess the potentialities of both . . . mating types due to the presence of the extra chromosome'' (Sansome 1980: 179). In view of the fairly close phylogenetic relationship between oomycetes and diatoms suggested by 18S rDNA data (Medlin et al. 1997) and the fact that both are diploid in the vegetative state, similarities between their mating systems are worth exploring further.

Sexualizable cells of the capitate and rectangular demes are always present in natural populations (Table 1), demonstrating that sexual reproduction cannot be simultaneous and episodic (contrast, e.g. bamboos: Janzen 1976). Related to this, size spectra of natural populations (e.g. Figs. 10–14) show no obvious sign of size classes, in contrast to some other diatoms reviewed by Mann (1988b). Distributions are always biased toward smaller sizes, containing very few cells that can be regarded as the immediate descendants of auxospores; indeed, in Figures 10–14 well over 90% of the cells are within the sexual size range. Mann (1988b, 1991) suggested that this bias reflects the short-term costs of sexual reproduction—in inappropriate copulation, aborted gametes and zygotes, interruption of synthesis and growth during meiosis and auxospore expansion, the extra mitoses required for the formation of the initial valves, and the cost of producing initial valves and perizonium elements. Several of these certainly op-

erate in *S. pupula*. Triplets are formed in around 10% of all copulations. The third cell sometimes resumes vegetative growth but often aborts, producing appreciable losses. Furthermore, as in other diatoms, there is ''no significant antagonism between factors promoting vegetative growth and those eliciting gametogenesis'' (Drebes 1977: 274). In culture, pairing was generally most vigorous a few (2–4) days after compatible clones were mixed in fresh medium. Thereafter, while copulating cells spent several days in meiosis, gametogenesis, plasmogamy, and auxospore expansion, unsexualized cells continued to grow rapidly so that the short-term penalty of sex in lost growth and cell division (interruption of synthesis: Lewis 1983) was considerable. Abortion of gametes and zygotes was often observed, even when cultures otherwise appeared healthy. In combination, these factors seem adequate to explain the excess of medium and small cells in natural populations.

In part, abortion of gametes and young (still dikaryotic) zygotes probably reflects the exposure of lethal recessive genes. This may also explain why the very rare cases of intraclonal pairing observed in cap-2, cap-11, and cap-12 were unsuccessful. In all of these, the zygotes produced by selfing aborted before forming initial cells. Because vegetative cells in the same cultures remained healthy and because the progeny of outcrossing were generally viable, the failure of selfed offspring to develop can perhaps be regarded as extreme inbreeding depression. Muirhead and Lande (1997) predict that inbreeding depression will be especially high in organisms where self-fertilization is rare and sexual reproduction is accompanied (sequentially or simultaneously) by high levels of asexuality. The capitate deme satisfies both of these criteria, since size reduction is slow (so that sexual events are separated by long periods of asexuality) and outbreeding is particularly strongly enforced. The rectangular deme may prove different. Although the two clones studied here were outbreeding, our analysis of seminatural populations suggests that sister cell pairing is not infrequent, and we have observed high rates of selfing in a few of our more recent isolates of the rectangular deme (Mann and Chepurnov, unpubl.). Theory predicts that this should be accompanied by lower inbreeding depression, and indeed, our unpublished observations demonstrate that selfing can produce viable offspring in the rectangular deme. Rectangular and capitate demes may differ too in the degree of asexuality as a result of the higher rate of size reduction in the rectangular deme. However, further observations are needed to determine the true balance of asexuality and sexuality in these demes, since the ''refractory'' size ranges differ, during which cells cannot be induced to become sexual. The rectangular deme is asexual between 40.5–57 μm (the observed range of initial cell size) and ca. 37 μm (the sexual size threshold), whereas the capitate deme is asexual between 31 – 44 μm and 30.5 μm. Thus, the size range for asexuality is higher in the rectangular deme and this could compensate for the higher rate of size reduction.

Geitler (1957b) found that male gametangia tended to be smaller than female gametangia in *Sellaphora seminulum* and *Cocconeis placentula* var. *pseudolineata*. The capitate deme of *S. pupula* shows the opposite trend, with males larger than females, even though all three species exhibit the same overall pattern of sexual reproduction (Mann 1989a). One explanation for the tendency for male gametangia to be smaller in *C. placentula* var. *pseudolineata* and *S. seminulum,* discussed by Geitler (1957a, b) is that smaller cells might tend to complete meiosis faster, so that their gametes would begin movement earlier and become *de facto* males. Geitler discounted this explanation for *C. placentula* var. *pseudolineata* because other varieties of *C. placentula* behave isogamously, whether or not the copulating cells are equal in size. The behavior of the capitate deme is also inconsistent with the maturation hypothesis.

What then causes the size differences between male and female gametangia in *S. seminulum, C. placentula* var. *pseudolineata,* and the capitate deme of *S. pupula*? Two explanations occur to us. There could be slight differences in the sexual size thresholds for males and females: male thresholds higher than female thresholds in the *Sellaphora pupula* demes and the reverse in *C. placentula* var. *pseudolineata* and *S. seminulum.* Or the thresholds may be the same but the frequencies of males and females may be unequal, bringing about size differentiation between the sexes.

To disprove a difference in thresholds would be difficult, involving determination of the threshold in hundreds of clones by repeated mating with oppositely sexed test clones known to be well within the sexual size range. Furthermore, negative results would be difficult to interpret if we are correct that the size threshold is not all-or-nothing, but the point at which cells begin to show some sexual response if stimulated strongly enough. Any difference in thresholds must in any case be very slight, since the observed upper size limits for males and females were essentially the same in each of the two demes of *S. pupula.* Geitler's (1957b) data show too that the thresholds are similar, if not identical, in *S. seminulum.* We have argued above that, if sex determination is phenotypic, determination should take place more or less simultaneously with sexual maturity; that is, when the sexual size threshold is passed. A difference in size thresholds, however, would require predetermination of the cells as male or female and would thus be more plausible if sex determination is diplogenotypic. But diplogenotypic sex determination is impossible in *C. placentula* var. *pseudolineata* and *S. seminulum,* since these species exhibit intraclonal reproduction (Geitler 1932, 1957b).

Alternatively, the size differences between males and females in the capitate deme could reflect the frequencies of the sexes in the population. Let us assume, as the data suggest, that males and females in the capitate and rectangular demes do have the same sexual size thresholds. Then the size of any particular gametangium is a reflection of time and the frequency of cell division since the size threshold was passed. The larger average size of the males in the capitate deme would therefore indicate that they passed the size threshold more recently and are in general ''younger'' than females. This in turn would imply that males are in short supply relative to females, since on average they must go through fewer cell divisions before they mate. It is in this context that our observations of the behavior of triplets are interesting. At first sight, the preferential polyandry of *S. pupula* (the imbalance of male–female–male triplets over female–male–female triplets) suggests that there is an excess of males. However, this assumes that the relative frequencies of polyandry and polygyny are controlled only by the frequencies of the sexes in the population. If so, since diatoms are holocarpic—the whole of each vegetative cell becoming a gametangium and being used up in the production of gametes—polyandry would require an imbalance in the production of males and females (males being produced in excess). But if the imbalance between different types of triplet is caused by the production of excess males and is in any case self-correcting through polyandry, there would then be no reason to expect size (age) differences between the sexes.

We suggest, therefore, that polyandry is not a passive consequence of a male-biased sex ratio but the cause of a female-biased sex ratio. This in turn could generate size differentiation between the sexes for the following reason. In *Sellaphora* species, each initial cell is produced following mating between a male and a female gametangium, which not only contribute equally to the next generation in terms of genes but also contribute roughly equal amounts of cytoplasm, photosynthetic machinery, reserve materials (except volutin granules: Mann 1989a), etc. The gametangia are both active during pairing, and the vegetative cells also move around, so that extreme local variations in the sex ratio are unlikely. And natural populations are enormous. In these circumstances, if sex determination is genotypic, one might expect a 1:1 ratio between males and females (Williams 1966, Maynard Smith 1978, Charnov 1982) at conception (the initial cells) and at maturity (at the sexual size threshold). Given this starting point, preferential polyandry would deplete the population of males leaving an excess of females. As a result, males would be younger on average; that is, larger at mating.

Our attempts to cross capitate and rectangular clones in culture were unsuccessful, as expected from population studies (Figs. 7–9). Hybridization, if it occurs at all, must be extremely rare. On the other hand, we did observe interactions between males of one deme and females of the other, suggesting that the capitate and rectangular demes may have similar pheromone signalling systems that stimulate directional movement of cells during pairing. Preliminary molecular genetic analyses using 18S rDNA (Mann et al. 1997, unpubl.) indicate that the capitate and rectangular demes are more closely related to one another than to other *S. pupula* demes found in the Edinburgh area, including, for instance, the 'small' deme from Blackford Pond (Mann 1989b: fig. 1p–r) or the 'elliptical' deme from Threipmuir reservoir (Mann and Droop 1996: fig. 28); it may be significant that we have not yet detected stimulation of the capitate or rectangular demes by any of these other demes. Even though capitate cells of opposite sex often congregate in large clusters during intrademic pairing, male cells only ever bond to female cells (Figs 36, 37), except in the very few, always unsuccessful instances of monoecious reproduction. Highly selective self–nonself recognition systems must therefore exist at the cell surface, producing (or inducing) the strong bonding that occurs between compatible cells of opposite sex. It is at this stage in the sexual process that reproductive isolation is ensured between capitate and rectangular cells, since they never bond. However, the interaction between capitate and rectangular cells suggests that their life cycle dynamics may be directly linked in nature, since sexualizable cells of both are present simultaneously in natural populations at most times (Table 1).

Very small capitate and rectangular cells (<14 μm) grew slowly and exhibited abnormal striation patterns and raphe structure. Similar changes in small cells have been reported by Geitler (1932) and Hostetter and Hoshaw (1972). The smallest cells were unable to complete sexual reproduction successfully, although many would still pair with a compatible partner. Geitler (1932, 1935) noted a lower, as well as an upper, threshold for sexual reproduction in *Sellaphora seminulum* and *Gomphonema parvulum,* and this pattern of behavior—a ''closed'' size range for sexual reproduction—is accepted to be characteristic of pennate diatoms (Drebes 1977). Drebes (1977: 272) noted that the size range for females in centric diatoms is also closed and suggested that ''this may prove to be of importance . . . when the phylogenetical relationships between the sexual cells of both diatom groups are further studied,'' implying that the one or two large isogametes of pennate diatoms may be ontogenetically and phylogenetically equivalent to the egg cells of centric diatoms, all developmental trace of true maleness (involving spermatogenesis) having been lost. Although this idea is interesting, it must be pointed out that, in nature, the sexual size ranges of many pennate diatoms may effectively always be ''open.'' Even though capitate and rectangular clones can

achieve lengths of 12 μm or less in culture, cells smaller than 19 μm are extremely rare or absent in natural populations. In the marine *Achnanthes longipes,* a closed life cycle can be demonstrated in culture. The smallest cells (less than 20–25 μm) are incapable of sexual reproduction but can enlarge vegetatively, until they are once again within the sexual size range (Roshchin and Chepurnov 1992, Chepurnov and Mann 1997). However, such small cells must be extremely rare, judging by the size ranges recorded in floras (e.g. Hustedt 1927–1966, Proshkina-Lavrenko 1963). Hendey (1951, 1964) studied this species particularly intensively yet recorded no valves smaller than 36 μm. The closed nature of the sexual size range in pennate diatoms may therefore be a cultural artifact, with no evolutionary significance.

Davidovich (1994) found a correlation between the sizes of gametangia and initial cells in *Nitzschia lanceolata* W. Sm., *Tabularia tabulata* (C.A. Ag.) Snoeijs, and *Licmophora ehrenbergii* (Kütz.) Grun. and noted that similar correlations had previously been reported in *Melosira moniliformis* (O. F. Müll.) C.A. Ag., *Skeletonema costatum* (Grev.) Cleve, and *Coscinodiscus granii.* To these species (all marine) we can now add *Sellaphora pupula.* Because of such correlations, Davidovich (1994) questioned the validity of the concept of cardinal points in the life cycle, formulated by Geitler (1932, 1935), in which species or clones can be characterized by the sexual size threshold, the maximum cell size (in the initial cells), and the lower size threshold, below which vegetative growth may continue but sexual reproduction is impossible. The reality of cardinal points has long been accepted (e.g. Patrick 1954, Drebes 1977), and differences in the cardinal points are often used to help justify taxonomic decisions and microevolutionary interpretations (e.g. Geitler 1958, 1968, 1975, Mann 1989b). There is no doubt that variation in gametangium and initial cell size is often underestimated. In *Coscinodiscus granii,* size restitution can take place (via auxosporulation) in two or more steps, small cells giving rise to medium-sized initial cells, whose vegetative progeny can then expand again to form even larger initial cells (Lutsenko et al. 1971, Roshchin 1994a). The range of initial cell size is thus enormous, from less than 130 μm to 248 μm, and overlaps considerably with the sizes of the cells giving rise to the auxospores (up to 188 μm). Schmid (1994) also has reported an overlap between gametangium and initial cell size in *C. granii,* although here there is an interesting extra complication, since North Sea clones of *C. granii* attain much larger sizes than were ever found in Roshchin's material from the Black Sea. In both demes of *Sellaphora pupula* studied here, the smallest initial cells observed were only just above the sexual size threshold.

Nevertheless, it is wrong to dismiss the idea of cardinal points, at least for pennate diatoms. Geitler's experimental data and observations of natural populations (1932, 1957b) and ours indicate that the sexual size threshold is real. If so, there must automatically be an upper limit to initial cell size if there is a correlation between the sizes of gametangia and initial cells. Furthermore, in pennate diatoms, Davidovich's (1994) data and ours show that initial cell length is remarkably well buffered to differences in gametangium length. In outbred *Tabularia tabulata,* Davidovich (1994) found no significant difference in initial cell length despite four-fold variation in the combined lengths of the gametangia. Inbred *Tabularia, Nitzschia lanceolata,* and *Licmophora ehrenbergii* showed roughly 1.4-, 1.8-, and 1.1-fold variation in initial cell length for eight-, four-, and three-fold variation in combined gametangium length, respectively (Davidovich 1994 and estimates from Davidovich's graphs), whereas for cap-11 × cap-38, 1.7-fold variation in the gametangia corresponds to only 1.2-fold variation in initial cell lengths. Furthermore, as our data for the capitate and rectangular demes show, the smallest gametangia, and hence the smallest initial cells, that can be produced in culture do not necessarily exist in natural populations. Altogether, therefore, the concept of cardinal points has not been seriously undermined. Size data can and should be used in taxonomy, though with appropriate caution when experimental data are not available.

We posed the question at the beginning of this article as to whether there is a fundamental difference between freshwater and marine pennate diatoms in their breeding systems or whether the contrast between Roshchin and Chepurnov's data (showing dioecy and sexual differentiation in marine species) and Geitler's (showing homothally in freshwater species) is an accident of sampling. Differences might be expected, perhaps, because of the more fragmented nature of freshwater habitats, which might make homothally advantageous since successful colonization would, in principle, require the arrival of only a single cell. Heterothally is well known in other freshwater algae, such as *Gonium pectorale* O. F. Müll. (Stein 1958), *Pandorina morum* (O. F. Müll.) Bory (Coleman 1959), and *Closterium ehrenbergii* Meneghini (Ichimura 1981), but in these species of Chlorophyta each clone is potentially immortal, whereas diatom clones are not, as a result of size reduction. However, our observations of *Sellaphora pupula* show that freshwater diatoms can indeed exhibit sexual differentiation.

Furthermore, several other freshwater raphid diatoms probably also possess mechanisms promoting outbreeding, involving a kind of sexual differentiation like that present in *Sellaphora.* In *Amphora* cf. *libyca* Ehrenb., *Caloneis silicula* (Ehrenb.) Cleve, and *Neidium ampliatum* (Ehrenb.) Krammer, each gametangium produces two gametes (Mann 1984b, 1989c, unpubl.), which rarely if ever fuse with each other, indicating some kind of self-incompatibility

mechanism. Gametangia are usually paired, but a minority of cells form triplets instead, producing three pairs of gametes. If these diatoms were homothallic, there would seem no reason why the three pairs should not, from time to time, fuse to give three zygotes, but we have only ever observed the formation of two zygotes per triplet (although these are occasionally polyploid, following multiple fusion of gametes). Similar observations were made by Subrahmanyan (1947:263–4). It is noticeable too that sexual reproduction has not often been reported in pennate diatoms from clonal cultures, the bulk of our knowledge coming instead from natural populations (reviewed by Geitler 1932, 1973, 1984). This suggests that simple homothally, like that found by Geitler (1932) in *Sellaphora seminulum* and *Gomphonema parvulum,* may be the exception, not the rule, in pennate diatoms, regardless of whether they are freshwater or marine.

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