Sex allocation pattern of the diatom 
*Cyclotella meneghiniana*

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Sex allocation is one of the most successful applications of evolutionary game theory. This theory has usually been applied to multicellular organisms; however, conditional sex allocation in unicellular organisms remains an unexplored field of research. Observations at the cellular level are indispensable for an understanding of the phenotypic sex allocation strategy among individuals within clonal unicellular organisms. The diatom *Cyclotella meneghiniana*, in which the sexes are generated from vegetative cells, is suitable for investigating effects of phenotypic plasticity factors on sex allocation while excluding genetic differences. We designed a microfluidic system that allowed us to trace the fate of individual cells. Sex allocation by individual mother cells was affected by cell lineage, cell size and cell density. Sibling cell pairs tended to differentiate into the same fates (split sex ratio). We found a significant negative correlation between the cell area of the mother cell and sex ratio of the two sibling cells. The male-biased sex ratio declined with higher local cell population density, supporting the fertility insurance hypothesis. Our results characterize multiple non-genetic factors that affect the phenotypic single cell-level sex allocation. Sex allocation in diatoms may provide a model system for testing evolutionary game theory in unicellular organisms.

1. Introduction

Sex allocation is one of the most successful areas in evolutionary game theory. In theoretical analyses it can predict an offspring’s sex ratio in response to environmental changes [1–4]. Although the theory is usually applied to organisms with obligatory sexual systems, such as higher animals and plants, it has not been applied to sex allocation in unicellular organisms (except in the case of malaria [4]) with a facultative sexual cycle, which is assumed to be the ancestral sex system [5,6]. Furthermore, conditional sex allocation [4] based on cell state remains an unexplored field.

In this study, we investigate phenotypic sex allocation in a unicellular organism, the diatom *Cyclotella meneghiniana*. We address how each sex is generated from vegetative clonal cells following induction of meiosis, using an on-chip single-cell microfluidic system [7,8] that made it possible to trace the fate of individual cells. Furthermore, we characterize the intrinsic and extrinsic factors that affected sex allocation by individual mother cells to two sibling cells.

Diatoms undergo a cyclic process of size diminution and size restoration, which is used to time the length of the life cycle and, therefore, the frequency of sexual reproduction [9,10]. There is a vegetative phase, lasting months to years, of size diminution [9], followed by a relatively short sexual reproduction phase, during which the large cell size is restored. In centric diatoms, the cell diameter diminishes during the vegetative phase of mitotic division because of the unique silicified structure of the cell wall [10]. It is only when cells are below a given size threshold (usually 40–50% of the maximum diameter) that sexual reproduction can be induced, usually in response to a species-specific environmental cue [10]. Centric diatoms are monoecious; therefore, both sperm and non-motile eggs can be formed within a single clone [10]. The *C. meneghiniana* species complex [11] is sexualized by increased salinity, and each of its vegetative cells differentiates into four sperm cells or one egg [12].

Studies of sex allocation have suggested that individuals within a population should not adopt the same universal strategy when individual traits are...
conditionally heterogeneous [3,4]. The effects of phenotypic plasticity on sex allocation can be investigated by using a clonal population because genetic differences are excluded between individuals to the extent possible. In this study, we investigated three factors that could affect sex allocation by individual mother cells—cell lineage, cell size and cell density—using an on-chip single-cell microfluidic system [7,8]. First, we investigated whether the sex allocation of individual mother cells is distorted, whereby individuals specialize in producing either male or female offspring (split sex ratio) [3,4]. Second, we investigated the effects of the size of individual mother cells on conditional sex allocation under the Trivers–Willard hypothesis (i.e. if a physiological condition, such as body size, differs among individuals, then, the fitness of the male and female also becomes different; consequently, selection favours conditional sex allocation), as shown across broad taxonomic groups [3,4]. As the average cell size of a diatom population decreases, the population becomes more male biased [10]. However, the individual diatom strategy has not been elucidated; for instance, in what case does an individual diatom cell choose its cell fate (egg, sperm, undifferentiated)? Third, we examined the effect of local cell population density on the disproportionate male ratio (the ‘fertility insurance hypothesis’ [13,14]).

The aims of this study were (i) to quantify how C. meneghiniana forms male and female subtypes at the single-cell level using the microfluidic system, and (ii) to investigate phenotypic sex allocation within a clonal population from the perspective of evolutionary game theory.

2. Material and methods

(a) Culture conditions

The C. meneghiniana clone used in this study was maintained in a freshwater medium. The clone was deposited as NIES-2364 at the Natural Institute for Environmental Studies. The GenBank accession number for the D1–D2 regions of the nuclear 28S rDNA is JN854149 and the slide number of the clone deposited in the National Museum of Nature and Science (Tokyo) is TNS-AL-57092. Sterile freshwater medium was used under conditions of 20°C, 16 L: 8 D illumination, and a light intensity of approximately 30 μmol photons m⁻² s⁻¹ in a 1.5 ml centrifuge tube to prevent cell division as much as possible (preservation stock). Freshwater medium was prepared by adding 10 mg L⁻¹ Na₂SiO₃ 9H₂O, 20 μg L⁻¹ thiamine to one-fifth diluted BBM [15]. For the study, the preservation stock was reactivated by transferring to the new media at 25°C and under continuous illumination at a light intensity of approximately 100 μmol photons m⁻² s⁻¹ until the mid-to-late log phase, and then cultivating again under the same conditions until the mid-log growth phase. Sexual reproduction was induced by changing the medium from freshwater to dilute seawater by dissolving 10.8 g L⁻¹ Daigo artificial seawater (Waiko Pure Chemical Industries, Osaka, Japan) in the freshwater medium. Note that the seawater medium used in this study contained only 30 per cent of the amount of artificial seawater powder prescribed in the manufacturer’s protocol.

(b) On-chip single-cell cultivation system

An on-chip single-cell cultivation system [16] was constructed to follow a particular single-cell sexual differentiation process. This system contained a microchamber array plate, medium exchange unit and microscope (see the electronic supplementary material, figure S1). The microchamber array plate was a cover glass of 0.1 mm thickness on which we constructed a 15 × 15 array of micrometre-sized structures called microchambers using SU-8—a thick, negative, photoresistive material (Microlithography Chemical, Newton, MA). Each microchamber was approximately 100 × 100 × 20 μm. After the sample cells were placed in the array, the array was sealed with a semipermeable membrane (molecular weight cut-off, 25,000; Spectrum Laboratories, Irving, TX) using an avidin–biotin attachment to prevent the cells from escaping [17]. Microchambers comprising various cell numbers were prepared using stochasticity for the process of cell application and sealing with the membrane. A PDMS (polydimethylsiloxane) plate of approximately 2 × 2 × 0.5 cm was mounted against the microchamber array plate using an adhesive flame (Frame-Seal incubation chambers, 25 μm capacity; MJ Research Inc., Waltham, MA). Medium pumped from the medium tank at 2 ml h⁻¹ passed through the space between the PDMS plate and the semipermeable membrane, and flowed to a waste tank. The freshwater medium flowed for approximately 24 h and was then changed to seawater to induce sexual differentiation. Images were obtained using time-lapse microscopy. For a detailed description of the microscopy conditions, see the materials and methods in the electronic supplementary material.

(c) Morphological criteria for sex distinction

Morphological and nuclear patterns that occur during oogenesis and spermatogenesis have been reported in many centric diatoms [10]. The process of meiotic division and sperm release from the cell walls of C. meneghiniana [18,19], and the egg morphology and nucleus pattern [20] after sexual induction by increasing salinity, have been described.

On the basis of the results of previous studies [10] and our preliminary observations, we focused on two notable differences between egg and sperm production. The first is that the egg cytoplasm was continuously attached to the cell walls, whereas the sperm cytoplasm became detached from the cell walls after a short time. The second difference is that the exposed cell membrane of the egg expanded more than the cell membrane of the released primary spermatagonia. The sperm that entered the first meiotic cytokinesis (represented as a typical sperm by blue triangle in the electronic supplementary material, figure S2) had qualitatively distinct cytoplasmic dynamics, where the cytoplasm formed into a sphere and then split into two parts (figure 2a). However, the other cells (black hollow circle in the electronic supplementary material, figure S2a), which could be either eggs or aborted sperms before the first meiotic cytokinesis, required a quantitative criterion for sex distinction. The duration of attachment of the spherical cytoplasm to the cell wall and the maximum amplification of membrane expansion were measured to create the criterion. Sex distinction of cells was performed according to this criterion (electronic supplementary material, figure S2).

(d) Measurement of sex ratio and cell size, cell density and cell lineage

The field of view was calibrated using a Zeiss micrometre, and areal projections of cells were determined using IMAGEJ software [21]. Cell diameter and cell area were measured in the girdle (lateral) view (see the electronic supplementary material, figure S3). The lateral view of the cell area changed during one cell cycle; therefore, we focused on the cell area immediately after cell division (see the electronic supplementary material, figure S3). The sex ratio of each sibling cell pair that included at least one differentiated cell was determined from all the sibling cell pairs that appeared during a cell division event in the microchamber (corresponding to the sibling pairs ‘a’, ‘c’ and ‘d’ in the electronic supplementary material, figure S4). The sex ratio of two sibling cells was regarded as the number of cells differentiated into sperm divided by the number of differentiated cells among the two sibling cells. For a single pair of cells, the ratio could
therefore be 1 (both sibling cells are male, or one sibling cell is male and the other is undifferentiated), 0.5 (one sibling cell is male and the other is female) or 0 (both sibling cells are female, or one sibling cell is female and the other is undifferentiated). Data were collected from 344 mother cells in 49 chambers to analyse sex allocation by individual mother cells according to cell size or cell density. Cells that could not be assessed for fate or size because of overlap with other cells were excluded. The cells were followed until they differentiated or until the measurement time ended. All cell-tracking procedures were conducted manually to ensure accuracy. The cell density within the microchamber was the number of vegetative cells within the chamber at the sexual induction cue.

(e) Split sex ratio by the bootstrap test
Information from 722 cell pairs in 55 chambers was used to investigate the effects of cell lineage on sex ratio distortion. Cells whose fate could not be assessed because of overlap with other cells were excluded. The determined cell fate pairs were used from all sibling cell pairs that appeared in the microchamber (corresponding to the sibling pairs ‘a–d’ in the electronic supplementary material, figure S4). The bootstrap method was used as described later. The frequency of each combination of cell fates was constructed by random pair choice from the observed data and was duplicated 10 000 times. The observed number of each cell fate combination of the two sibling cells was compared with the 99 per cent confidence limit of this virtual frequency distribution of the randomly chosen pair. The calculations were performed using the original code of R software v. 2.13.0 (R Foundation for Statistical Computing; http://www.R-project.org).

(f) Statistical analysis
Values are reported as the mean and standard error. A post hoc power calculation was performed using G^Power v. 3.1.3.1. Statistical tests including analysis of variance (Welch’s ANOVA) with unequal error variances using oneway.test function, Tukey’s HSD test and a generalized linear model (GLM) were performed using R software. We examined Akaike’s information criterion (AIC) [22] to determine the best-fitting model. A GLM with binomial errors and logit-link function was used to determine whether cell area and cell density had an effect on sex ratios of the two sibling cells. The significance of independent variables was evaluated by comparing the models using log-likelihood ratio tests, following stepwise model selection using AIC [22] (see the electronic supplementary material, table S1). McFadden’s R^2 was used to estimate the degree to which cell area differences contributed to differences in the fate of the two sibling cells.

3. Results
(a) Induction of sexual reproduction in a microfluidic device
We designed an on-chip single-cell microfluidic system [16,17] for tracking differentiation processes at the single-cell level. Cells were randomly seeded on arrays of 100 × 100 μm microchambers fabricated on a glass slide (figure 1). Cells were enclosed in each microchamber by a semipermeable membrane. Multipoint time-lapse microscopy enabled tracking of multicell lineages within microchambers simultaneously. The medium was supplied to the microchambers through the semipermeable membrane. A freshwater medium was used for the first 24 h, after which seawater was used to induce sexual differentiation.

An example of the time course of C. meneghiniana sexual differentiation is shown in figure 1a–d and in the electronic supplementary material, movie S1. After changing the medium to seawater, spermatogenesis occurred more rapidly than oogenesis (figure 1c,d; electronic supplementary material, figure S5). Figure 2 presents a depiction of identifying each cell type and the time course of differentiation. During spermatogenesis (around 15 h after induction; figure 2a; electronic supplementary material, figure S5), we observed that the cells released spermatogonia from their cell walls and then underwent the first meiotic cytokinesis (figure 2a). Meiotic cytokinesis occurred within the cell walls before extrusion in approximately 40 per cent of sperm (figure 2a). Individual clone cells produced four sperms. Oogenesis occurred relatively later than spermatogenesis (approx. 30 h after induction; figure 2b; electronic supplementary material, figure S5). We observed that an egg cell opened its cell wall and expanded the spherical cytoplasm attached to the cell wall (figure 2b). The nuclei dynamics by meiosis and insemination were investigated by DAPI (4',6-diamidino-2-phenylindole) staining (see electronic supplementary material, figures S6 and S7). Expansion of the spherical cytoplasm of egg cells occurred even without insemination (see electronic supplementary material, figure S7b–c). In addition, we confirmed insemination within the clone (see electronic supplementary material, movie S2 and figure S8), and the clone was able to generate vegetative cells of the next generation (see electronic supplementary material, figure S7g,h); however, the relationship between insemination and formation of the next vegetative cell was not determined.

Cells that did not open their cell walls until termination of the recording (60.8 h after sex induction) were identified as ‘undifferentiated cells’ (figure 2c). The majority of these

Figure 1. Sexual differentiation in Cyclotella meneghiniana using a microfluidic system. (a,b) Cells within a microchamber under freshwater media. (c) The cell started spermatogenesis (arrowhead) under seawater. (d) Cells differentiated into male gametes (arrowheads) or female gametes (arrows). Scale bar, 10 μm.
undifferentiated cells tended to terminate cell division 20–30 h after sex induction.

On the basis of our observations (figure 2; electronic supplementary material, figure S2) and a previous report [10], the processes of diatom oogenesis and spermatogenesis appear to have morphologically distinct patterns. The sex of each cell can be distinguished by a differentiation pattern.

We focused on the following three notable differences between egg and sperm:

(i) after sex induction, spermatogenesis started faster than oogenesis by approximately 15 h;
(ii) the egg cytoplasm was continuously attached to the cell wall, but the sperm cytoplasm was detached from the cell wall after a short time (figure 2a,b); and
(iii) the exposed cell membrane of the egg was more expanded than the primary spermatogonia that were released (figure 2a,b).

We recorded the start time of a cell’s sexual reproduction, and determined sex discrimination by the duration of attachment of the cytoplasm to the cell wall and by the maximum amplification of cytoplasm expansion (see the electronic supplementary material, figure S2). The proportional cell fate of the population was as follows: egg, 6.5 per cent \( (n = 103) \); sperm, 61.45 per cent \( (n = 974) \); and undifferentiated cells, 32.05 per cent \( (n = 508) \) (total \( n = 1585 \)).

(b) Distorted cell fate allocation based on cell lineage
To examine the dependence of sex ratio distortion on cell lineage, we investigated whether two sibling cells were likely to experience the same fate (egg, sperm or undifferentiated). We determined the cell fates of terminal sibling pairs of a cell lineage (see electronic supplementary material, figure S4). Figure 3 illustrates that sibling cell pairs tended to experience the same fate (i.e. the frequencies of egg–egg, sperm–sperm and undifferentiated–undifferentiated pairs were significantly higher, and the frequency of egg–undifferentiated, sperm–undifferentiated pairs were significantly lower, than the 99% confidence limit of a virtual random-pairing distribution estimated by the bootstrap method). Therefore, the two sibling cells demonstrated the split sex ratio.

**Figure 2.** Cell-type identification and differentiation process observed by movies. (a) Typical spermatogenesis process. Spermatogenesis started approximately 15 h after sexual induction. The cytoplasm extruded for a short time after cell wall attachment. Two male gametes (asterisks, *) after the first meiotic cytokinesis and four sperm (arrowhead) resulted from the second cytokinesis. (b) Typical oogenesis process. Oogenesis started approximately 30 h after sexual induction. Cytoplasm expansion occurred with the cell wall attached. (c) Cells that did not expose the cytoplasm out of the cell wall were identified as undifferentiated cells.

**Figure 3.** Cell fate similarity according to cell lineage \( (n = 722\text{ cell pairs}) \). The observed number of each cell fate combination of the two sibling cells (red circles) and mean virtual frequency distribution of a randomly chosen pair (black diamonds) were compared. Error bars indicate 99% CI of virtual random-pairing distribution. E, egg; S, sperm; U, undifferentiated cell. The pale red bar shows that the observed frequency of the cell fate combination was significantly higher than the 99% CI of the virtual distribution. The pale green bar shows that the observed frequency was significantly lower.
A homogeneous diameter. The sample size had 34 per cent power to detect the effect of individual diameter on cell area, which can change with the different developmental ability of cells to elongate. The diameter range of the clone was 5.97±0.03 µm and the cell fate (sperm, egg or undifferentiated) did not depend significantly on cell diameter in this clone (ANOVA, $F=1.73$, d.f. = 2, $p=0.18$; figure 4a), because of the use of a single clone with cells of homogeneous diameter. The sample size had 34 per cent power to detect the effect of individual diameter on cell fate, at a $p$-value of 0.05.

The lateral view of the cell area changed during one cell cycle in the vegetative stage; therefore, to unify the timing of cell area measurement, we focused on the cell area immediately after cell division (see electronic supplementary material, figure S3). We measured the cell area of cells whose fate was determined (see electronic supplementary material, figure S4). Our results revealed that individual cell area in the lateral view was significantly different among the groups categorized by subsequent cell fate (ANOVA, $F=20.41$, d.f. = 2, $p<0.0001$; Tukey’s test, for all combinations of cell fates: egg > undifferentiated > sperm, $p<0.05$; figure 4b). The sample size had 100 per cent power to detect the effect of individual cell area on cell fate, at a $p$-value of 0.05. In addition, the lateral view of the cell area of the mother cell affected the combination of two sibling cell fates (see electronic supplementary material, figure S9). Note that the mother cell area is the sum of the areas A1 and A2 described in the electronic supplementary material, figure S3.

Sex allocation is usually evaluated as the ratio of individuals that become males among all offspring [3,4]. In the present study, the sex ratio of the two sibling cells was regarded as the number of cells differentiating into sperm divided by the number of differentiated cells among the two sibling cells. The results showed that the sex ratio of the two sibling cells decreased as cell area increased (average sex ratio, 0.83; $n=344$ mother cells; GLM with binomial error: deviance = 68.63, $p<0.001$; figure 4c; electronic supplementary material, table S1a). Therefore, if the mother cell had elongated to a greater extent before dividing, there was a greater tendency for daughter cells to differentiate into eggs. The sample size had 100 per cent power to detect effects of the mother cell area on sibling sex ratio, at a $p$-value of 0.05.

We found a significant negative correlation between the similarity of cell fate (same = $P$, different = $1-P$; logit) and the difference in cell area between the two sibling cells (GLM with binomial error: deviance = 17.73, $p<0.001$; pseudo $R^2 = 0.05$). Therefore, sibling cells with the same fate are likely to divide equally.

(d) Dependence of the sex ratio on local cell population density

In this system, cells should have experienced different cell densities in the microchambers because they were randomly trapped there. We focused on this isolated, local cell population density, which was thought to be important for mating success. A significant negative correlation was observed between cell density at the time of the sexual induction cue and the sex ratio of the two sibling cells ($n=344$ mother cells; GLM with binomial error: deviance = 15.16, $p<0.0001$; figure 5; electronic supplementary material, table S1a). The sample size had 100 per cent power to detect effects of cell density on sibling sex ratio, at a $p$-value of 0.05.
4. Discussion

(a) Cell fate similarity in a sibling pair

We have demonstrated that the sex allocation of individual mother cells was distorted (figure 3). Creating outbreeding opportunity may be one of the factors that favours the evolution of split sex ratios among mother cells. Although many centric diatoms can reproduce homothallically, self-fertilization deleteriously affects the viability and fertility of offspring in several species [10,23]. Thus, a strategy that distributes the same sex cells as sibling cells that come close to each other may decrease the inbreeding rate depending on population structure. The present sex allocation among individual mother cells suggests that common epigenetic states, such as the number of transcription factors that regulate cell fate, are passed to daughter cells. Cellular states can be passed from one generation to the next without alterations in DNA sequence information by various cis and trans signals [24], and many reports provide examples of this in prokaryotes and single-celled eukaryotes [25]. We found that sibling cells with the same fate are likely to divide equally; therefore, the common epigenetic states may establish a linkage between the symmetry of cell division and cell fate similarity in the two sibling cells. The molecular mechanisms of such epigenetic inheritance are unknown; however, applying genetic engineering tools that have been recently developed for diatoms [26] will help in identifying the mechanisms underlying the observed distorted sex allocation.

(b) Intrinsic and extrinsic factors in sex allocation by mother cells

We demonstrated that larger mother cells tend to differentiate into eggs at a higher frequency (figure 4c). Sex allocation of a maternal intrinsic factor (body size) has been investigated in many organisms, and the size-advantage model of evolutionary game theory predicts a biased sex ratio when one sex gains a greater fitness benefit from increased resources [2,3]. The size of the cell that differentiates into an egg has a positive correlation with the size of the fully expanded auxospore (and hence with the initial vegetative cell size) in several centric diatoms [27]. By contrast, the number of sperm produced by one cell is not affected by cell size in *C. meneghiniana* [12]. These observations suggest that allocation of larger cells to females more effectively restores cell size in the next vegetative cycle.

Furthermore, the difference in cell fate was reflected by cell area, rather than diameter, in the lateral view (figure 4). Our use of a single clone with homogeneous diameter, in contrast to previous studies that used plural clones [28,29], might have resulted in the lack of effect of cell diameter on the sex ratio reported here. Decrease in diameter by vegetative division is remarkably slowed in cells with diameters less than 5 μm in several Cyclotella species, including *C. meneghiniana* [30]. Within homogeneous populations of cells with smaller sexu-alizable diameter, the differential ability of cells to elongate longitudinally would be important for sex allocation. Furthermore, we measured the cell area immediately after cell division; therefore, our results indicate that cell elongation ability in the mother cell generation affects the cell fate decision-making process without waiting for elongation after cell division (figure 4b,c; electronic supplementary material, figure S3).

In this study, the sex ratio became less male-biased as the cell population density increased (figure 5). This extrinsic sex allocation may agree with the fertility insurance hypothesis, which has been previously discussed for sexual reproduction of a malarial parasite [31,32]. The *C. meneghiniana* complex is known to form aggregations in culture condition [33], and exhibits local blooms and dominates in estuaries [34,35]. Therefore, it may be important for these diatoms to adjust their sex ratio by changing local cell density. On the basis of our results and a previous study [12] of *C. meneghiniana*, a vegetative cell that is triggered to become a sperm can differentiate into no more than four sperms. According to the fertility insurance hypothesis, the optimal sex ratio could be affected by the number of gametes (four) released from one male gametocyte [13,14]. Therefore, in the case of this diatom, an insufficient number of gametes released from a male gametocyte may accelerate male bias. As an overall trend, the sex ratio became less male-biased as the cell population density increased. However, this trend was reversed at the highest cell density (figure 5). Further investigation is required to understand the exact mechanism and reason for this response.

(c) Sex allocation in the unicellular organism

We have characterized, for the first time, how non-genetic intrinsic (cell lineage and cell area in the lateral view) and extrinsic (cell density) factors affect sex allocation strategy by individual mother cells to two sibling cells, using an on-chip single-cell microfluidic system. Evolutionary game theory may help explain the sex allocation of diatoms, as well as that of other unicellular organisms. Phenotypic plasticity plays a largely underappreciated role in driving phenotypic diversification and speciation [36,37]. Diatom populations may provide a model system for investigating evolution through phenotypic sex allocation. Theoretical modelling and integrating measurements of the molecular processes at the single-cell level will further reveal the sex allocation mechanism within unicellular organisms.

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