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Beyond animals and plants: dynamic maternal effects in the fungus *Neurospora crassa*

K. C. K. ZIMMERMAN*, D. A. LEVITIS† & A. PRINGLE‡

*Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA, USA
†Department of Biology, Bates College, Lewiston, ME, USA
‡Departments of Botany and Bacteriology, University of Wisconsin-Madison, Madison, WI, USA

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Abstract

Maternal effects are widely documented in animals and plants, but not in fungi or other eukaryotes. A principal cause of maternal effects is asymmetrical parental investment in a zygote, creating greater maternal vs. paternal influence on offspring phenotypes. Asymmetrical investments are not limited to animals and plants, but are also prevalent in fungi and groups including apicomplexans, dinoflagellates and red algae. Evidence suggesting maternal effects among fungi is sparse and anecdotal. In an experiment designed to test for maternal effects across sexual reproduction in the model fungus *Neurospora crassa*, we measured offspring phenotypes from crosses of all possible pairs of 22 individuals. Crosses encompassed reciprocals of 11 mating-type 'A' and 11 mating-type 'a' wild strains. After controlling for the genetic and geographic distances between strains in any individual cross, we found strong evidence for maternal control of perithecia (sporocarp) production, as well as maternal effects on spore numbers and spore germination. However, both parents exert equal influence on the percentage of spores that are pigmented and size of pigmented spores. We propose a model linking the stage-specific presence or absence of maternal effects to cellular developmental processes: effects appear to be mediated primarily through the maternal cytoplasm, and, after spore cell walls form, maternal influence on spore development is limited. Maternal effects in fungi, thus far largely ignored, are likely to shape species' evolution and ecologies. Moreover, the association of anisogamy and maternal effects in a fungus suggests maternal effects may also influence the biology of other anisogamous eukaryotes.

Introduction

The development of an offspring is the product of two key factors – the genes inherited from its parents and its developmental environment. The developmental environment is often controlled by the maternal phenotype, for example through the environment provided by the mother during embryogenesis (Ashworth *et al.*, 2009) and inheritance of cytoplasmic components (including organelles and their genetic material) through cytoplasmic provisioning from the mother (Galloway, 2001; Li & Albertini, 2013; Rando & Simmons, 2015). Influences such as these are named maternal effects (Roach & Wulff, 1987). The consequences of maternal effects for evolution are diverse. Maternal effects can alter the direction and strength of trait selection by introducing sources of variation outside of an individual's genes (Kirkpatrick & Lande, 1989). Maternal effects enable the environmental influences on mothers to alter the development of her offspring, effectively transferring environmental influences across generations (Galloway, 2005; Badyaev & Uller, 2009; Donohue, 2009). In clonal organisms, environmental maternal effects can increase offspring heterogeneity (Sakwinska, 2004).

Maternal effects are well documented in animals and plants, but not in other groups of organisms. However, the basic mechanisms causing maternal

Correspondence: Kolea C. K. Zimmerman, Department of Organismic and Evolutionary Biology, Harvard University, 16 Divinity Ave – Biological Laboratories 2112, Cambridge, MA 02138, USA.
Tel.: 1 617 496 5540;
fax: 1 617 495 5854; e-mail: kzimmerman@fas.harvard.edu

effects in animals and plants – anisogamy leading to disproportionate cytoplasmic inheritance from the mother (Westneat & Craig Sargent, 1996; Birky, 2001; Gosden, 2002) and offspring development in maternally derived environments (Roach & Wulff, 1987; Lindström, 1999) – are also found among fungi (Jinks, 1963; Billiard *et al.*, 2010; Debuchy *et al.*, 2010). Within the fungi, the prerequisites for maternal effects are apparent among filamentous species in the phylum Ascomycota (Ascomycetes) and, more rarely, in the phylum Basidiomycota (Basidiomycetes) (Billiard *et al.*, 2010). Most Ascomycetes have distinct male and female structures, whereas there is very little morphological specialization of sex organs among Basidiomycetes. Anisogamy is also found in an early diverging phylum, the Chytridiomycota (James *et al.*, 2006). The wide distribution of anisogamy across the fungal kingdom suggests that anisogamy is ancestral in the fungi, with isogamy being a derived state (Billiard *et al.*, 2010).

Experiments testing for maternal effects among fungi are long overdue. Fungi are critical drivers of ecosystem functions, with roles as pathogens, mutualists and decomposers. The diverse evolutionary consequences of maternal effects documented among animals and plants are likely equally relevant to anisogamous fungi. The majority of fungal pathogens are Ascomycetes (Guarro *et al.*, 1999), and many of the most damaging plant diseases in this group, including rice blast (*Magnaporthe oryzae*) and grey mould (*Botrytis cinerea*) (Dean *et al.*, 2012), are anisogamous (Faretra *et al.*, 1988; Saleh *et al.*, 2012). If maternal effects play a role in the evolutionary trajectories or ecological niches of these economically relevant species, documenting the phenomena involved may provide novel tools to understand pathogen biology and adaptation to new hosts. Moreover, testing for maternal effects among fungi provides an opportunity to extend theory on the evolutionary impact of maternal effects generated from two groups, animals and plants, to diverse eukaryotes.

To test whether maternal effects occur in a fungus, we mated genotyped strains of the hermaphroditic fungus *Neurospora crassa* in a fully and reciprocally crossed experiment. Strains were originally isolated from a diversity of sites across the globe. We used generalized linear mixed-effects models to quantify the effects of crossing distances (the genetic and geographic distances between parents in any particular cross) and strain identities, as mothers or fathers, on traits (Table 1) associated with the sexual ontogeny of the fungus. Geographic and genetic differences among strains may influence reproductive success because, for example, crossing closely related parents results in inbreeding depression or crossing distantly related parents results in outbreeding depression (Lynch, 1991). Our analyses document clear maternal effects on offspring phenotypes, but the presence and strength of these effects vary dynamically across development.

Table 1 Target traits.

Trait	Description	Characteristic of:
Perithecia count	A number of protoperithecia fertilized: perithecia are specialized structures housing zygotes; zygotes immediately undergo meiosis to form spores.	Mother
Spore count	The spores produced by a given cross; each zygote has the potential to result in eight spores.	Zygotes of a particular cross
Proportion pigmented	Proportion of spores that are pigmented. Analogous to measuring the potential gametic viability of the F1 generation; pigmented spores are mature and potentially viable whereas all unpigmented spores are inviable.	Spores of a particular cross
Pigmented spore size	Mean length of pigmented spores.	Spores of a particular cross
Pigmented spore germination	Proportion of pigmented spores that germinate within 150 min. after activation of spores.	Spores of a particular cross

Materials and methods

Why test for maternal effects in *N. crassa*?

We use *Neurospora crassa* (Fig. 1) as a model because it is anisogamous (female gamete-forming organs can be 100 μm in diameter and the male gametes range from 2.5 to 8.5 μm in diameter) and offspring development takes place in maternally derived reproductive structures. Furthermore, *N. crassa* is hermaphroditic (Fig. 1), enabling reciprocal crosses. Any *N. crassa* strain, regardless of mating type, can produce male or female sexual structures, depending on the nutritional content of the culture medium. For example, strain 1 with mating-type 'A' could be grown on low nitrogen media to induce the formation of *female* sexual structures and then fertilized by the conidia (asexual spores/*male* gametes) of strain 2 with mating-type 'a'. To perform the reciprocal cross, strain 2 would be induced to form *female* structures and then fertilized by conidia from strain 1.

Although *N. crassa* is a classic genetic model (Roche *et al.*, 2014), no breeding or quantitative genetics experiments have investigated maternal effects in *N. crassa* or any other fungus. However, *post hoc* evidence of reciprocal differences in fertility among *N. crassa* strains suggests strong maternal control over reproductive development. There are mutant *N. crassa* strains with phenotypes that are only apparent when the strain acts as the maternal parent; these kinds of mutations cause

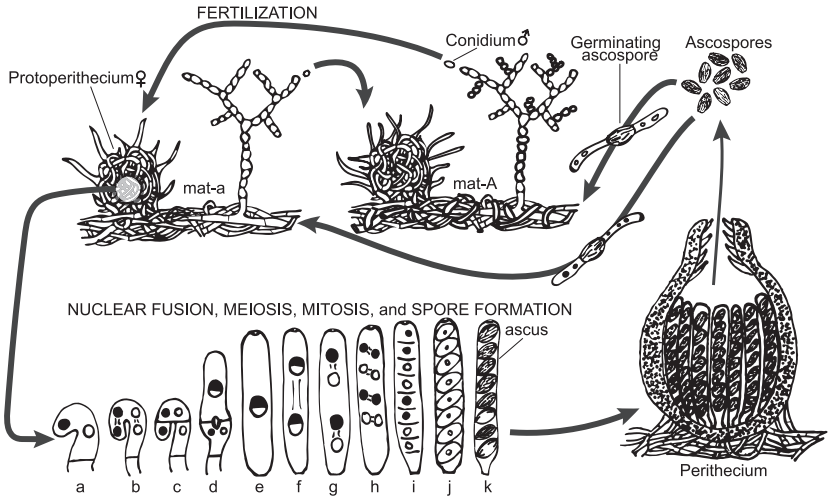

Fig. 1 Life cycle of *Neurospora crassa*. *N. crassa* is a haploid filamentous fungus capable of reproducing both sexually and asexually (Davis, 2000). Sexual reproduction is restricted to parent strains different at a single two-allele mating-type locus and a single strain is either mat-a or mat-A. However, both mating types can produce female and male gametes; all strains are hermaphroditic. For example, reciprocal fertilization between mat-a and mat-A strains is shown at the top of the figure. Nitrogen starvation induces the production of protoperithecia, which are female reproductive structures that house female gametes termed ascogonia. Asexual spores termed conidia (or potentially other cells from a compatible mate) act as male gametes. Fertilization follows the growth of a trichogyne – a sexually receptive hyphae emanating from the female sexual structure – and its fusion with a conidium (the male gamete) of the opposite mating type. After fusion, the paternal nucleus migrates to an ascogonium within the protoperithecium. The division and segregation of maternal and paternal nuclei results in the proliferation of cells each containing one maternal and one paternal nucleus (a–c). Within these cells, each of the two nuclei fuse to form a diploid zygote (d, e) that rapidly undergoes meiosis I (f) and meiosis II (g), producing four haploid nuclei. These nuclei then undergo mitosis, resulting in eight haploid nuclei per ascus (h). Cytoplasmic cleavage occurs (i) and a cell wall develops around each of the eight haploid nuclei, forming eight ascospores (j). In normal ascospores, the cell wall becomes pigmented as it matures; in aborted or undeveloped ascospores, the cell wall remains unpigmented (k). Spores are forcibly ejected from a mature perithecium one ascus at a time. Nuclei are coloured black or white to show that they are derived from the mat-a or mat-A parent, respectively. All nuclei are haploid (1N) except for the diploid (2N) half-black/half-white nuclei shown in steps d, e and f.

defects in female gamete formation or fertilization and result in reciprocal differences in fertility (Raju, 1992). In strains isolated from the wild, reciprocal differences in the ability to form perithecia suggest protoperithecial formation and fertilization are influenced by the maternal parent (Dettman *et al.*, 2003b; Turner *et al.*, 2010). Reciprocal crosses between experimentally evolved *Neurospora* strains also show maternal influence on mating success (Dettman *et al.*, 2008).

These studies proved that female structures (protoperithecia and perithecia) in *N. crassa* are truly female and play a dominant role in the sexual cycle of this fungus. However, by definition, maternal effects act on offspring and can only be determined whether offspring and their traits are measured. Although many mutations affecting the production of sexual spores (termed ascospores, and hereafter referred to as 'spores') have been identified in *N. crassa*, these mutations either segregate after meiosis or are only expressed in crosses homozygous for the allele, indicating they are direct genetic effects and not maternal effects (Leslie & Raju, 1985; Raju *et al.*, 1987; Raju & Leslie, 1992). Offspring viability is easily measured in *N. crassa* based on the pigmentation of spores – generally, pigmented spores are viable and unpigmented spores are not viable.

Mating scheme and strain choice

We used a full diallel cross to quantify parental effects in *N. crassa*. In a full diallel, all possible combinations of parents are mated, along with reciprocals (i.e. each strain is used as a mother, then a father, with every other compatible strain). The mating design maximizes the statistical power for estimating maternal, paternal and direct genetic effects on offspring (Zhu & Weir, 1996). To control for the effects of crossing distances on reproductive output, we first identified a set of 24 mat-A and 24 mat-a wild isolates of *N. crassa*, previously genotyped with RNAseq (Ellison *et al.*, 2011).

We used genomewide SNP data (Ellison *et al.*, 2011) to calculate pairwise genetic distances between all possible pairs of strains, measured as number of differences across all SNP sites. For each of the 48 strains, we obtained collection site information from the Fungal Genetic Stocks Center strain catalog (FGSC, www.fgsc.net) and calculated geographic distances between strain collection sites.

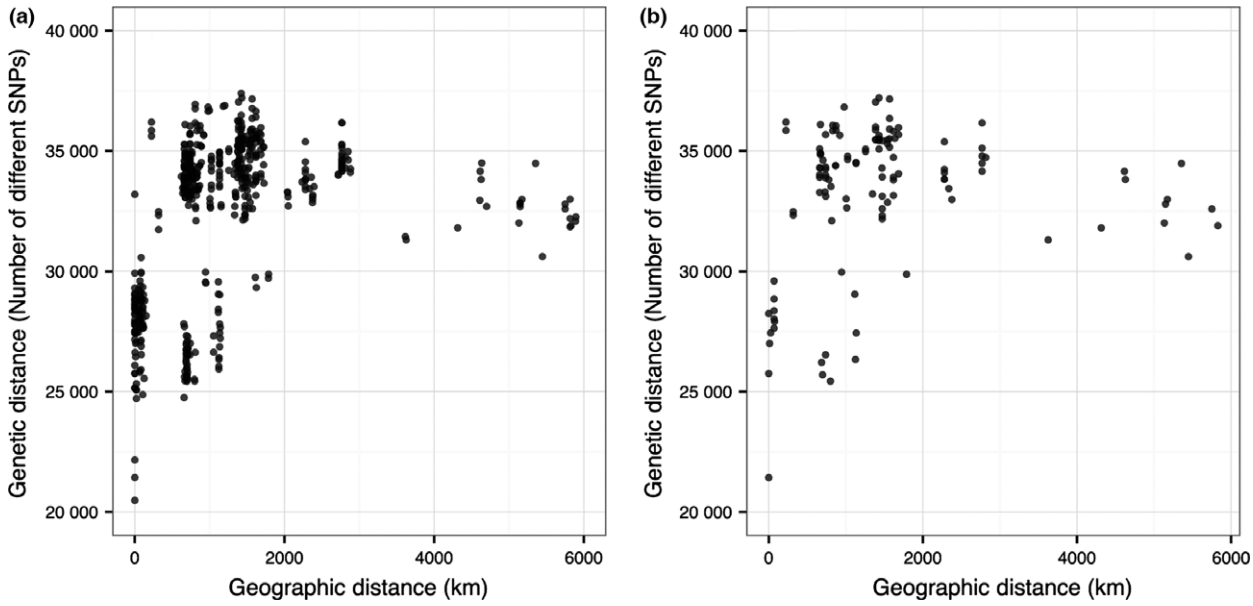

Fig. 2 Experimental crosses chosen from among all potential crosses using the SPREAD algorithm. (a) Genetic distance vs. geographic distance between parents of all possible crosses. (b) The subset of 11 mat-A × 11 mat-a crosses selected with the SPREAD algorithm (Zimmerman *et al.*, 2015). Crosses are plotted as semitransparent dots and darker shading marks overlapping crosses.

The genotyped population included 24 wild isolated strains of each mating type along with a laboratory strain. We could not perform all possible crosses using these 48 strains (excluding the laboratory strain). Using the SPREAD algorithm (Zimmerman *et al.*, 2015), we selected a mating set of 11 mat-a and 11 mat-A strains for use in our study (Fig. 2, Table 2). SPREAD identifies the subset of crosses that most evenly covers the original range of the entire set of all potential crosses. Briefly: SPREAD selects a crossing set from 1000 randomly generated crossing sets (11 mat-a × 11 mat-A) by maximizing the mean nearest neighbour distance of the subset of 121 crosses plotted according to the geographic vs. genetic distances among crosses (Fig. 2). The algorithm effectively maximizes both the range and evenness of the resulting crosses plotted on genetic vs. geographic distance space. We obtained the 22 strains chosen by this algorithm (Table 2) from the Fungal Genetic Stock Center (McCluskey *et al.*, 2010).

Generating conidia for crosses

We inoculated each strain onto a slant of Vogel's medium N (Vogel, 1956) made with 2% sucrose and 1.5% agar. We harvested conidia (asexual spores) from these cultures and preserved them on silica gel for subsequent experiments. To grow conidia for crosses, we used conidial stocks to inoculate 50-mL narrow-neck flasks containing 7 mL of Vogel's medium N with 2% sucrose, 1.5% agar, 0.1% yeast extract and 0.1% casamino acids, incubated at 30 °C with 12 h/12 h light/dark illumination for 7 days. We harvested conidial inocula for crosses with 10 mL diH_2O and filtered suspensions through nonabsorbent cotton to remove extraneous mycelia.

Crossing conditions and spore collection

We grew four replicate reciprocal crosses of each of the 121 strain pairs for a total of (4 replicates) × (121 mating pairs) × (2 reciprocals) = 968 crosses. We performed all crosses using paper crossing media (Pandit & Russo, 1993) with slight modifications: we prepared crossing plates by placing 4 × 3.5 cm gel blot paper squares in 5-cm petri dishes and adding 1 mL of synthetic crossing media (Westergaard & Mitchell, 1947) containing 0.1% sucrose, 0.5% sorbose, 0.1% yeast extract and 0.1% casamino acids. We grew each of the 22 strains as a female by evenly dropping 200 μL of a suspension containing 1×10^6 conidia in diH_2O onto the paper. We incubated plates in their plastic sleeves in the dark, at 25 °C for 48 h, and then fertilized the plates by evenly dropping 200 μL of a suspension containing 1×10^6 conidia in diH_2O onto the filter paper. Because these conidia are dropped onto individuals already growing as females, they will function as the male gametes. We incubated the fertilized crosses at 25 °C in darkness for 20 days, except for occasional exposures to light during routine inspections. After incubation, we harvested spores from lids with 1 mL of diH_2O and deposited spores in deep 96-well plates. To allow for complete maturation of the spores, and to

Table 2 Experimental *N. crassa* strains. Strains used in this experiment are shown in the first column, 'Derived from FGSC ID' = ID of heterokaryotic strain purified to homokaryons by Ellison *et al.*, 2011, 'Other ID' = additional names for the same strain used in other publications.

FGSC ID of strains	Derived from FGSC ID	Other ID	Mating type	Strain origin	Origin latitude	Origin longitude
8783		D23	A	Homestead, Florida, USA	25.468722	−80.477557
8850		D90	A	Uxmal, Yucatan, Mexico	20.361044	−89.770828
851		JW1	A	Coto, Costa Rica	8.535825	−83.036696
1131		JW3	A	Canal Zone, Panama	9.086502	−79.814301
3968		JW28	A	Okeechobee, Florida, USA	27.243935	−80.829783
4713		JW45	A	Merger, Haiti	18.65	−72.3
4716		JW49	A	Puilboreau Mt., Haiti	19.533333	−72.466667
4730		JW52	A	Puerto Ayachucho, Venezuela	5.665781	−67.634697
5910		JW56	A	Digitima Creek, Guiana	5.6	−57.599998
10951	3199	JW70	A	Coon, Louisiana, USA	30.775463	−91.757893
6203		JW77	A	Agudas Rd, Costa Rica	9.529566	−84.45475
10886	8876	D116	a	Franklin, Louisiana, USA	29.79604	−91.5015
8819		D59	a	Carrefour Dufort, Haiti	18.456533	−72.619552
8829		D69	a	Tiassale, Ivory Coast	5.9	−4.833333
8845		D85	a	Kabah, Yucatan, Mexico	20.247697	−89.646391
1133		JW5	a	Canal Zone, Panama	9.086502	−79.814301
1165		JW7	a	Panama	9.086502	−79.814301
10950	3200	JW59	a	Coon, Louisiana, USA	30.775463	−91.757893
10957	p4450	p4450	a	Franklin, Louisiana, USA	29.79604	−91.5015
10959	p4452	p4452	a	Franklin, Louisiana, USA	29.79604	−91.5015
10961	p4455	p4455	a	Franklin, Louisiana, USA	29.79604	−91.5015
10968	p4476	p4476	a	Franklin, Louisiana, USA	29.79604	−91.5015

standardize spore age among crosses, we stored the harvested spores at 4 °C for 16 days before flow cytometry analyses of spore counts, morphology and germination.

Counting perithecia

We photographed all crosses at time of ascospore harvest for subsequent counting of perithecia. Using the photographs, we determined total counts of perithecia per cross by manual counting and marking in ImageJ (Schneider *et al.*, 2012).

Counting and measuring spores using flow cytometry

Due to the large number of spore samples to be analyzed, we used high-throughput flow cytometry to characterize spore samples collected from the crosses. In a single run of a spore sample through the flow cytometer, we counted spores in the sample and measured the size and pigmentation of spores. Offspring viability can be assessed based on spore pigmentation. Viable spores are darkly pigmented and inviable spores lack pigment (and are termed hyaline spores). Using a high-throughput sampling accessory, we were able to analyze spore samples at a rate of one sample/minute.

We prepared spores for flow cytometry by first centrifuging the 96-well plates with harvested spore suspensions at 500 rpm for 10 min. We pipetted away the supernatant and resuspended ascospores in 500 μL of 1% wt/v polyvinylpyrrolidone molecular mass 360,000 (PVP-360) in diH_2O. We transferred 50 μL of this ascospore suspension, along with 100 μL of diH_2O to new 96-well U-bottom plates. To wash PVP-360 out of suspensions, we centrifuged the plates for 5 min at 1000 rpm, then removed 125 μL of the supernatant and added 125 μL diH_2O. Next, we added Spherotech AccuCount 7.7-μm fluorescent beads (Spherotech Inc., Lake Forest, IL) to the spore suspension in 50 μL of 0.4% Triton X-100 (Sigma Chemical Co., St. Louis, MO), resulting in a final spore and bead suspension in 200 μL of 0.1% Triton X-100 in diH_2O. Spore samples were analyzed by a BD LSRII flow cytometer with a high-throughput sorter attachment (BD Biosciences). The sampling parameters for high-throughput sorting were as follows: sample flow rate: 3.0 μL/s, sample volume: 50 μL, mix volume: 100 μL, mix speed: 75 μL/s, number of mixes: 5, wash volume: 300 μL. We collected data on three parameters: forward scatter (to measure pigmentation), side scatter (to measure size) and fluorescence (to measure bead size). Using FACSDiVa software (BD Biosciences), we recorded data on height, width and area of forward scatter; height, width and area of side scatter; and height, width and area of fluorescence for every spore in the sample volume (50 μL). The data from each sample were saved in the flow cytometry standard (FCS) 3.0 format.

Measuring spore germination with flow cytometry

To measure the relative germination abilities of spores collected from all crosses, we used flow cytometry to measure the length of spores in spore samples from each cross before and after exposure to germination triggers (nutrient media and heat shock). As spores germinate, spore length increases because germ tubes emerge from each end of the spore. For each sample, we transferred 50 μL of the spore suspension in 1% PVP-360 to duplicate U-bottom 96-well plates, each representing either pregermination or post-germination treatments. Each well contained 100 μL of diH_2O and plates were centrifuged for 5 min at 1000 rpm. We removed 100 μL of supernatant and added 50 μL of 2 × Vogel's medium N containing 4% sucrose, resulting in a final concentration of 1 × Vogel's medium N containing 2% sucrose. To control for the effects of heat shock on spore size (pregermination plate) and activate germination (post-germination plate), we heat-shocked both plates in a forced air incubator at 60 °C for 1 h. We fixed the pregermination plates immediately following heat shock. Post-germination plates were incubated with shaking for 150 min at 34 °C to promote germ tube growth and then fixed.

We fixed spores by adding 100 μL of cold 95% EtOH and 0.2% Triton X-100 in diH_2O directly to the spore suspensions and incubating overnight at 4 °C. Just before flow cytometry, we transferred spores to 96-well PCR plates and centrifuged them at 2000 rpm for 10 min. We removed 175 μL of the supernatant and added 175 μL of diH_2O, resuspended spores by gentle pipetting and transferred the resuspended spores to new 96-well U-bottom plates along with 25 μL of a Spherotech AccuCount 7.7-μm fluorescent bead solution containing ~ 2550 beads in 0.4% Triton X-100. Flow cytometry parameters were the same as above.

Flow cytometry data processing

Our initial analysis of the flow cytometry data indicated the presence of both conidia and ascospores, with ascospores exhibiting two clear subpopulations of pigmented and hyaline (unpigmented) ascospores (Fig. S1). Hyaline ascospores are demarcated by greater forward-scatter height values, compared to pigmented ascospores; because they are clear, they scatter light differently and exhibit higher light transmittance. There was some overlap of conidia and ascospores when plotted on forward-scatter-height vs. side-scatter-width axes, and the overlap prevented manual two-dimensional gating. To differentiate the conidia and ascospores, we performed multidimensional clustering analyses with all measured flow cytometery parameters, using a custom clustering and sorting pipeline in R with the following packages: *flowCore* (Ellis *et al.*, 2016), *flowClust* (Lo *et al.*, 2008, 2009) and *flowStats* (Hahne *et al.*, 2016).

Flow cytometry standard files were imported into R using *flowCore* and sets of FCS files for each plate were merged into a single data set to facilitate clustering by increasing the density of measurements. To distinguish the populations of conidia, pigmented ascospores and hyaline ascospores, we performed k-means clustering using the *flowClust* package. We manually adjusted the number of clusters to be identified for each data set to obtain clustered data sets whose clusters matched subpopulation characteristics obtained from analysing pure conidia or pure ascospores. We then computed summary statistics using *flowStats* for each measured sample including the total number of ascospores, the number of pigmented spores, the number of hyaline ascospores and the size of pigmented ascospores. Fluorescent bead populations were identified based on their high fluorescence parameter values (fluorescence area). To standardize ascospore counts in subsequent analyses, we used the flow cytometry sample measurement time of each sample. To standardize spore size across samples, we used the mean side-scatter width of the fluorescent beads for each sample.

To calculate ascospore germination, we first drew rectangular gates around populations of spores from pregermination ascospore samples plotted on forward-scatter height vs. side-scatter width. We applied these gates, representing the size of pregermination ascospores, to the post-germination ascospore samples and counted the ascospores outside of the previously drawn gates as germinated ascospores. For each pregermination sample, we used our clustering pipeline to determine the proportion of conidia and ascospores, excluding fluorescent beads. Using these proportions, we calculated the total ascospore number in each post-germination sample (including germinated ascospores) by multiplying the proportion of total ascospores in the pregermination sample by the total number of particles (excluding fluorescent beads) in the post-germination sample. Finally, we calculated the proportion of germinated ascospores by dividing the number of germinated ascospores (determined by gating) by the estimated total number of ascospores in the sample (determined by the clustering analysis of the pregermination sample).

Verification of flow cytometry methods

We verified the ability of our flow cytometry analysis to measure ascospore pigmentation and germination by performing traditional counting and plating assays for a subsample of the crosses used in the full crossing experiment. In a separate experiment, we replicated a subset of crosses and measured ascospore production, pigmentation and germination using flow cytometry as

described above. Then, using the same ascospore samples, we obtained counts of pigmented and hyaline ascospores by dropping 10 μL of a spore suspension onto plates (Vogel's medium N with 1% sucrose and 1.5% agar) and examining the drop under a dissecting microscope. Next, we spread the spores using a bent Pasteur pipette (to minimize spore loss), heat-shocked the plates for 1 h at 60 °C and incubated them overnight at 30 °C. To measure the number of germinated spores, we counted growing colonies using a dissecting microscope. We determined the strength and nature of the relationship between measurements obtained from flow cytometry and manual methods using asymptotic nonlinear least squares regression, with the SSasymp function from the *stats* package in R. Analyses confirm a strong relationship between the two different methods of measuring spores. The slope (log of the rate constant) of the relationship between spore pigmentation measurements by manual counting vs. flow cytometry is significant ($P < 2e\text{-}16$) (Fig. S2), as is the slope of the relationship between spore germination measurements ($P = 0.0301$) (Fig. S3). Comparisons ground-truth the flow cytometry and confirm it can be used as a proxy for manual counting.

Mixed-effects models

We used the R package *glmmADMB* to compute mixed-effects models for each trait (Fournier *et al.*, 2012; Skaug *et al.*, 2013). Based on preliminary analyses of the trait data distributions, we chose to model pigmented spore size with a Gaussian distribution and all other response variables with a negative binomial distribution. Our initial models included the following fixed effects: genetic distance (number of different SNPs between parents in a cross), geographic distance (km between strain locales), the squares of both genetic and geographic distance and an interaction term for genetic and geographic distance. In each model, we also included maternal and paternal parentage as random effects, specifically to test the hypothesis that strains will vary in their ability, as mothers or fathers, to influence the target traits. For all five phenotypic traits, we computed full models and then selected simplified final models using the quasi Akaike information criterion (qAIC) or Akaike information criterion (AIC) for models with negative binomial or Gaussian response variable distributions, respectively.

Best linear unbiased predictors (BLUPs), also referred to as random effect coefficients or conditional modes, are model-based estimates of the relative influence of a strain as a mother or a father on a given phenotype, after controlling for fixed effects. For each trait, we calculated the relative proportions of overall random effect variance attributed to the variance of maternal or paternal BLUPs and assessed correlations between maternal and paternal BLUPs.

R^2GLMM calculations

To determine the relative importance of crossing distances (fixed effects) and the identity and sex of the parents (random effects) in selected models, we calculated the proportion of variance explained by either fixed effects or just random effects for each model using R^2GLMM statistics (Nakagawa & Schielzeth, 2013). For the pigmented spore size model, we used equations for calculating R^2GLMM statistics for models with a Gaussian response distribution listed by Nakagawa & Schielzeth (2013). For the other models that use negative binomial response distributions, we derived the distribution-specific variance (σ_d^2) for negative binomial distributions with a log-link function where α is the negative binomial dispersion parameter following the derivation in Appendix 1 of Nakagawa & Schielzeth (2013). Our derivation is shown in the supplementary information (Derivation S1).

Highest posterior density of parameter estimates

We verified the significance of parameter values in each selected model by calculating HPD intervals for parameter values. HPD intervals, also known as Bayesian confidence intervals, are based on the observed data and indicate the range of values in which the true parameter value is found with a 95% probability. HPD intervals of model parameter values that do not span zero are deemed significant. We calculated HPD intervals of the parameters in each model using the built-in Markov chain Monte Carlo (MCMC) functionality of glmmADMB that uses parameter estimates calculated from the model as priors. We used a chain of 10000 MCMC iterations and verified MCMC chain convergence by plotting the distributions of samples drawn from the chain. Parameter estimates with HPD intervals that did not span zero were deemed significant.

Results

Offspring phenotypes from a full diallel cross are evidence of maternal effects in *N. crassa*

All crosses resulted in perithecia and viable spores, except crosses involving strain FGSC 10968 (mat-a), which was infertile as both male and female and grew abnormally. We excluded crosses involving FGSC 10968 from all subsequent analyses.

Offspring phenotypes varied, and differences between reciprocal crosses were observed for most traits (Fig. 3). When crosses involving a particular strain acting as a female are grouped, the maternal effects of different strains are obvious. For example, when strain 10951 (mat-A) is used as a female, crosses appear to have the same level of spore production no matter the identity of the male strain (Fig. 3c). These kinds of patterns are

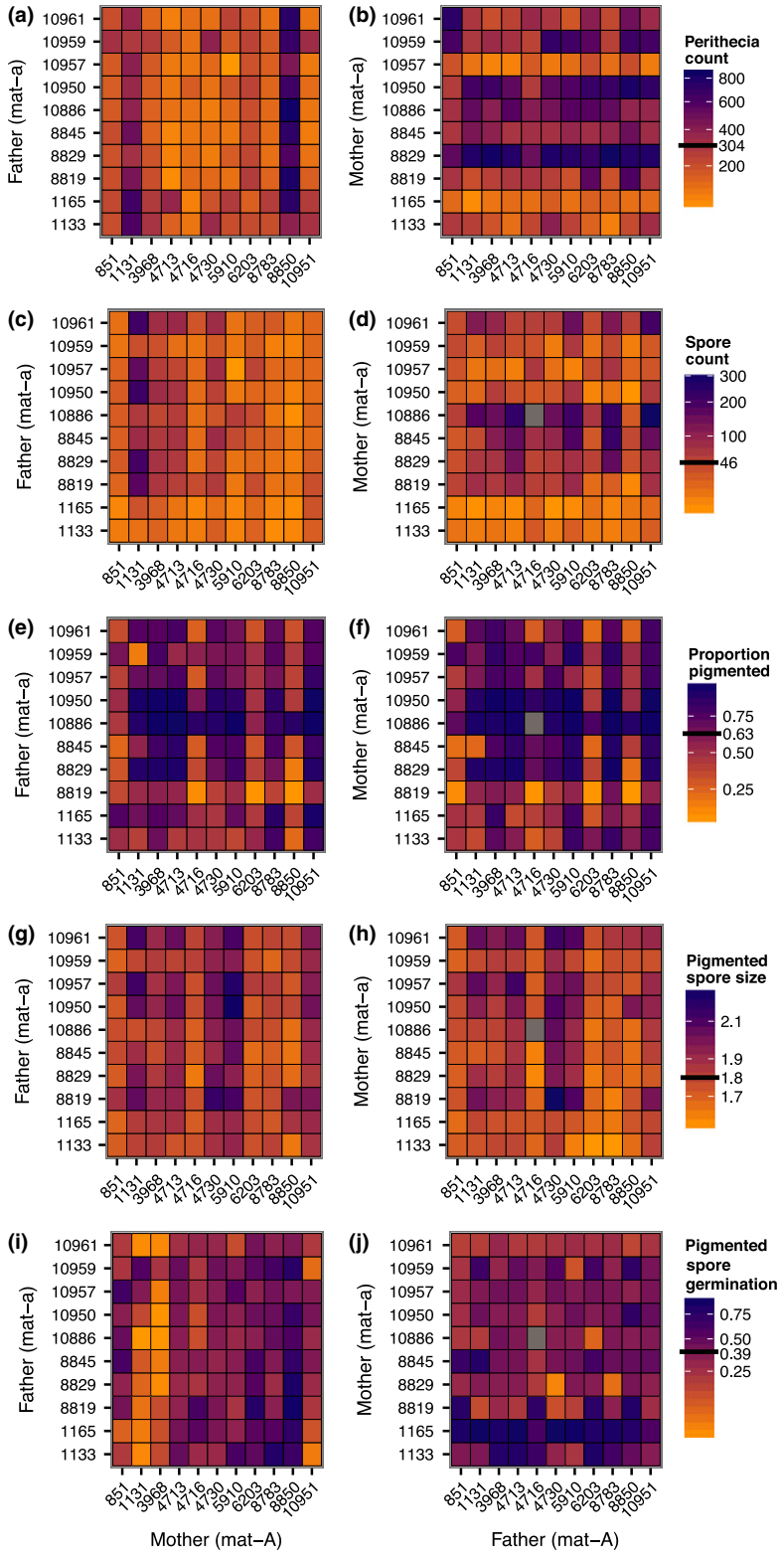

Fig. 3 Trait phenotypes for all crosses. Heat maps show crosses between mat-a fathers and mat-A mothers (panels a, c, e, g and i) and reciprocal crosses between mat-a mothers and mat-A fathers (panels b, d, f, h and j). Scales at right are values for a particular trait; black bars on scales are mean values for the trait, calculated from all crosses. Grey boxes mark missing data caused by instrument error.

also observed for data on pigmented spore germination, see for example crosses involving 8850 (Fig. 3i) or 1165 (Fig. 3j). But when crosses involving a particular strain acting as a male are grouped, patterns are more variable; for example, the influence of strain 8783 (mat-A) when it is used as a male depends on the pairing: when crossed with 8845 (mat-a), many spores are produced, but with 1165 (mat-a), few spores are produced (Fig. 3d). These patterns – consistent maternal influence and variable paternal influence – are not easily observed within the data for proportion pigmented (Fig. 3e, f) or pigmented spore size (Fig. 3g, h), but as predicted, are observed for perithecia count (Fig. 3a, b), a maternal trait.

More generally, whether reciprocal crosses result in different phenotypes depends on the traits involved. Spore count and germination data are often different between reciprocal crosses, for example few spores are produced by crossing 10951 as female and 10961 as male (Fig. 3c), but many spores are produced by crossing 10951 as male with 10961 as female (Fig. 3d). However, there is very little difference in the proportion of pigmented spores (Fig. 3e, f) or pigmented spore size (Fig. 3g, h) as shown by the almost identical reciprocal heat maps for these traits.

Mixed-effects models confirm parental effects exert greater influence on trait outcomes than crossing distances

Visual inspection of trait phenotypes suggests that mothers have more control over spore production and spore germination than fathers. But to more carefully explore the relationship between spore characteristics and the multiple influences of parental effects and crossing distances, we constructed generalized linear mixed-effects models for each measured trait (Table 3). Highest posterior density intervals for the selected parameters indicate that parameters for all models are significantly different from zero, except the genetic distance parameter in the spore count model (Fig. 4).

The proportions of variance explained by the fixed effects (crossing distance) and the random effects (maternal or paternal parentage), or not explained by the model, vary widely among traits (Fig. 5). However, in every model, the random effects of maternal and paternal strain identity explain more variance than the fixed effects of genetic and geographic distance (Table 4).

Maternal strain identity explains most of the variation in spore production and germination, but not size and pigmentation

Maternal identity explains most of the random effect variation in models of perithecia count, spore count and pigmented spore germination (Fig. 6). For these traits, the correlations between maternal and paternal BLUPs are weak or nonexistent (BLUPs are the relative influence of a strain as a mother or a father on a given phenotype) (Fig. 7). However, there is no significant difference in the proportion of variance explained by maternal or paternal strains in the models of proportion pigmented and pigmented spore size (Fig. 6) and correlations between maternal and paternal BLUPs for these traits are strong (Fig. 7).

Discussion

Maternal effects emerge as a major feature of the reproductive biology of *Neurospora crassa*. We find strong evidence for maternal effects acting on spore production and spore germination (Fig. 3). While phenotypes are consistent for any particular mother, they vary widely among mothers, and fathers have no consistent influence (Roff, 1998). We find no evidence for strong maternal effects on spore size or the proportion of pigmented spores (Fig. 3).

Table 3 Summary of best models. By default, all models include the random effects of maternal and paternal parentage.

Trait	Equation	Distribution	Link	Offset	Model selection method
Perithecia count	Perithecia count ~ genetic distance + (genetic distance)^2	Negative binomial	log	–	qAIC
Spore count	Total spore count ~ genetic distance + (genetic distance)^2 + geographic distance	Negative binomial	log	Sample run time (FCS)	qAIC
Proportion pigmented	Pigmented spore count ~ genetic distance + geographic distance + (geographic distance)^2 + (genetic distance)^2 + genetic distance: geographic distance	Negative binomial	log	Total spore count	qAIC
Pigmented spore size	Pigmented spore size ~ geographic distance + (geographic distance)^2	Gaussian	–	–	AIC
Pigmented spore germination	Germinated spore count ~ (genetic distance)^2	Negative binomial	log	Pigmented spore count	qAIC

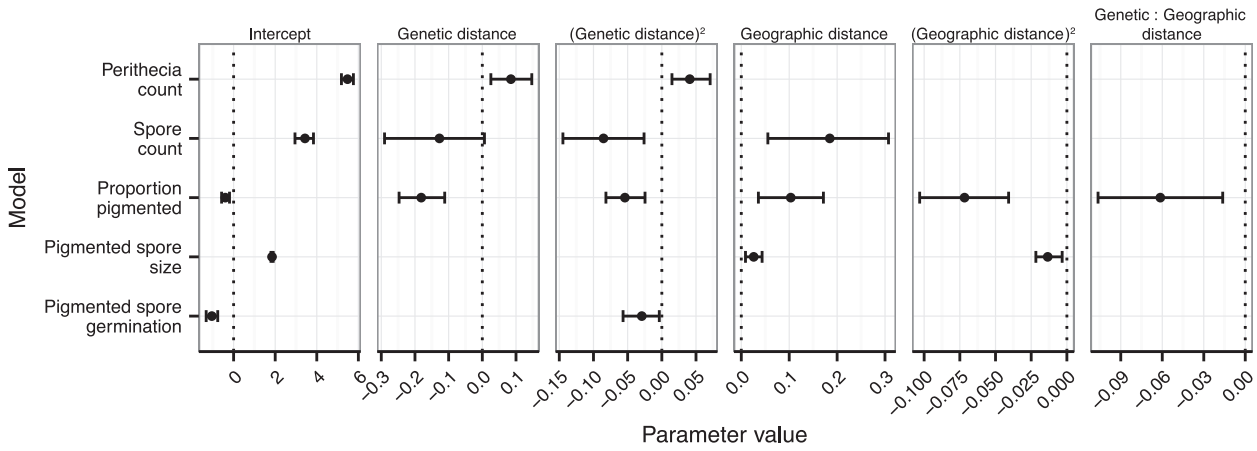

Fig. 4 Summary of model parameter values for each measured trait. Parameter values returned from the mixed-effects models are represented as points with error bars displaying the 95% highest posterior density (HPD) from 10000 iterations of Markov chain Monte Carlo (MCMC) parameter estimation. The absence of points indicates parameters that were dropped from a model based on qAIC or AIC metrics. Dotted lines indicate the zero values. If the HPD interval overlaps with zero, then the parameter is not different from a null model. Only the genetic distance parameter in the spore count model overlaps with zero. Parameter values for all models except 'pigmented spore size' are on a log-link scale. Values are listed in Table S1.

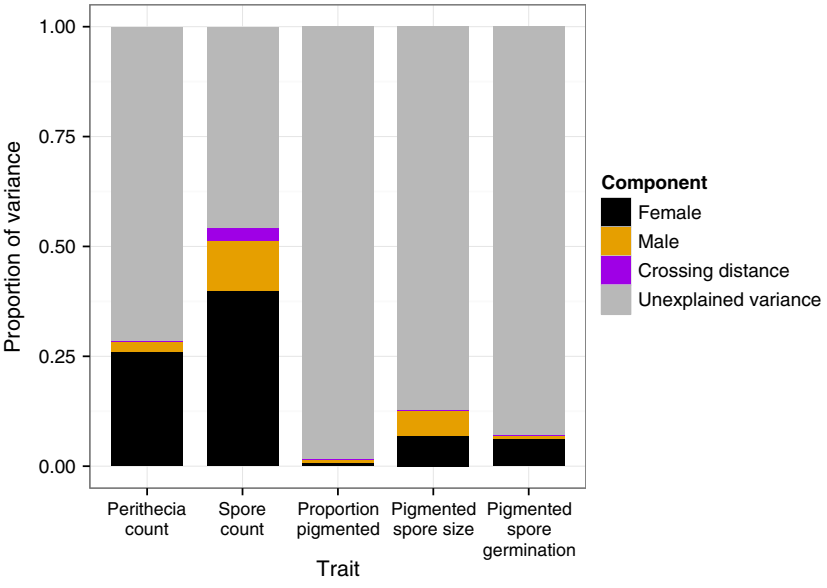

Fig. 5 Proportions of variance explained by maternal and paternal random effects, fixed effects (crossing distances) or other unexplained variance. Proportions were calculated using the R^2GLMM values in Table 4 along with the individual random effect variances returned from the models.

Our mixed-effects models confirm the visual evidence by controlling for potentially important confounding variables: genetic and geographic distances between parents. Crossing distances account for a small proportion of the overall variation in reproductive outcomes, suggesting that inbreeding and outbreeding depression do not substantially influence the success of crosses among our collection of strains (Fig. 5 and Table 4).

In fact, after controlling for the fixed effects of crossing distances in our models, we find that the random effects of parental strain identity account for most of the explained variance in reproductive outcomes (Fig. 5). Furthermore, variation among strains acting as mothers accounted for most of the explained variation in our models of perithecial production, spore production and spore germination (Fig. 6). Perithecial production appears to be under strong maternal control (Raju, 1992; Turner *et al.*, 2010), whereas maternal effects appear to play a dominant role in spore production and spore germination. We distinguish perithecial production as under maternal control – rather than influenced by maternal effects – because maternal effects can only be determined by measuring offspring phenotypes.

Table 4 Percentage of variance explained by fixed effects (crossing distances) or maternal and paternal random effects for each modelled trait. Percentages are based on the marginal and conditional R^2GLMM values calculated for each model (Nakagawa & Schielzeth, 2013).

	Variance explained	
	Fixed effects	Random effects
Perithecia count	0.24%	28.39%
Spore count	3.03%	51.28%
Proportion pigmented	0.11%	1.52%
Pigmented spore size	0.24%	12.60%
Pigmented spore germination	0.06%	7.00%

Maternal effects do not play a role in spore size or pigmentation; these traits are equally influenced by both parents in a cross, regardless of their roles as mother or father. In fact, the effect of each strain acting as a mother is highly correlated with that strain's effect when it acts as a father (Fig. 7), and mothers exert no more influence than fathers (Fig. 6). Taken together, the analyses suggest that spore size and pigmentation are controlled mainly by direct genetic effects; the large proportion of variance not explained by crossing distance or parental effects for both traits (98.37% and 87.16%) is consistent with direct genetic effects caused by specific allelic variants at one or more loci.

Observed maternal effects are not an artefact of correlations in trait values, and the number of perithecia generated by a cross does not predict subsequent spore phenotypes. Perithecial production, spore production and spore germination are not correlated with each other (Fig. S4).

Mapping maternal effects onto reproductive development: a model

The dynamic maternal effects we document are a clear reflection of *N. crassa* ontogeny and correlate with what is known about the species' reproduction (Fig. 8). Maternal effects influence spore production, but are not a feature of spore maturation; as spores germinate, maternal effects are observed once again. Spore production takes place within maternal tissues, whereas spore maturation occurs after spore walls separate developing spores from maternal cytoplasm. Germination draws on resources provided by the mother.

The apparent lack of maternal effects associated with spore maturation, and the suggestion that the spore cell wall limits contact between the maternal cytoplasm and the developing spore (Fig. 8), is supported by analyses of spore maturation mutants. These mutants suggest that spore maturation is controlled by the genetic material inherited by each spore (Raju & Leslie, 1992). Genes appear to be expressed autonomously within each of the spores of an ascus (Freitag *et al.*, 2004). Both studies suggest mothers have limited control over spore maturation; instead, the genetic material of the spores themselves appears to be in direct control of pigmentation and size.

Mechanisms causing the maternal effects associated with the germination of pigmented spores are less clear, but possible conduits include the carbohydrates and lipids packaged within spores. The raw materials used to form a spore are derived from the maternal parent. Germinating spores use stored materials for energy production during germination, and the amount of lipids in a spore can influence germination (Lingappa &

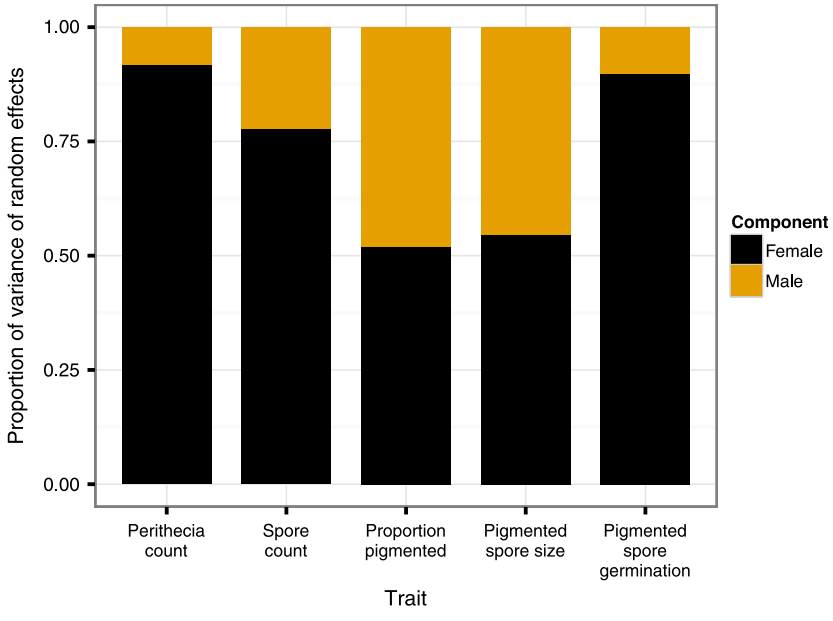

Fig. 6 Proportions of random effect variance explained by strains acting as mothers or fathers. Female vs. male random effect variances: perithecia count ratio of male: female variance = 0.076, $F_{20,20}$ = 0.0756, P = 3.00e-7; spore count ratio of male: female variance = 0.26, $F_{20,20}$ = 0.2582, P = 0.0039; proportion pigmented ratio of male: female variance = 0.92, $F_{20,20}$ = .9226, P = 0.86; pigmented spore size ratio of male: female variance = 0.82, $F_{20,20}$ = 0.8206, P = 0.66; pigmented spore germination male: female variance = 0.087, $F_{20,20}$ = 0.0874, P = 1.05e-6.

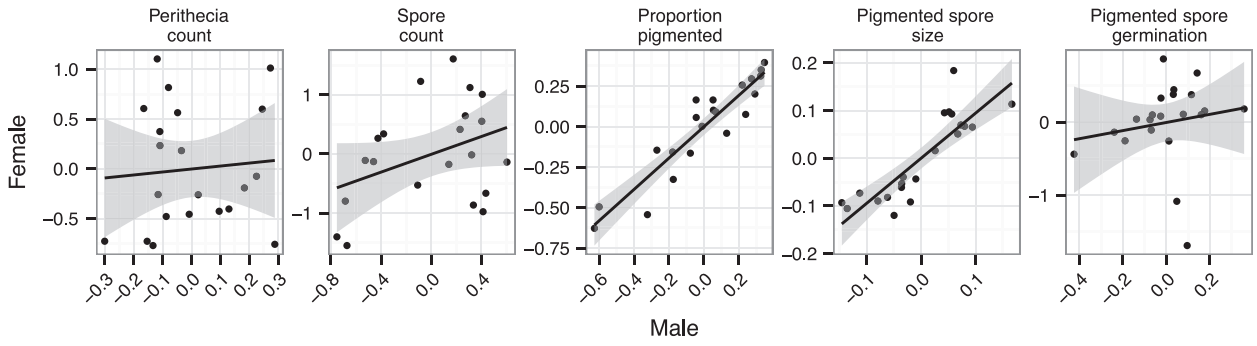

Fig. 7 Correlations between male and female BLUPs for all models. Points represent strains. Values are a measure of the relative influence of strains (as mothers or fathers) on phenotype outcomes. Shading shows the 95% confidence interval of the line. Correlations: perithecia count r^2 0.0067, $F_{1,19} = 0.1287$, $P = 0.72$; spore count $r^2 = 0.15$, $F_{1,19} = 3.257$, $P = 0.087$; proportion pigmented $r^2 = 0.86$, $F_{1,19} = 119.5$, $P = 1.23\text{e-}09$; pigmented spore size $r^2 = 0.73$, $F_{1,19} = 52.38$, $P = 7.18\text{e-}7$; pigmented spore germination $r^2 = 0.027$, $F_{1,19} = 0.5259$, $P = 0.48$.

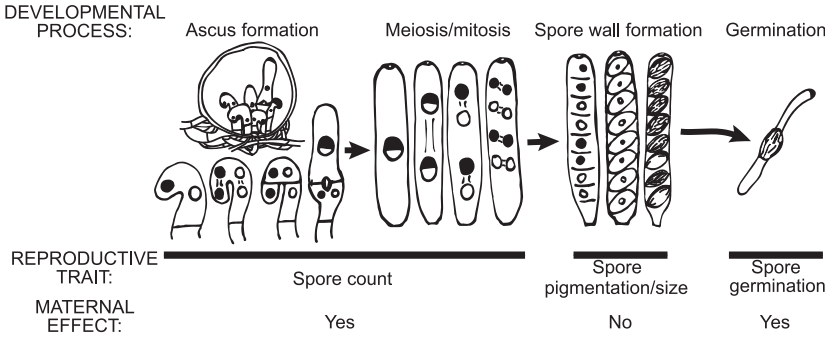

Fig. 8 Maternal effects vary dynamically across *N. crassa* reproductive development. Spores are generated within maternally derived asci, and maternal effects play a role in the total number of spores produced. Maternal effects do not influence spore pigmentation or size; these phenotypes develop after spore walls separate the spores from maternal cytoplasm. Maternal effects influence germination; germinating spores use maternally supplied nutrient stores.

Sussman, 1959; Budd *et al.*, 1966). Maternal strains may differ in their ability to supply carbohydrates and lipids to developing spores, resulting in maternal effects. Maternal effects on ascospore germination may also be mediated by mitochondria.

Mitochondria and maternal effects

Maternal effects caused by the expression of maternally inherited organellar genomes are often discussed as cytoplasmic genetic maternal effects (Roach & Wulff, 1987). To test whether observed maternal effects on spore germination were partly mediated by mitochondrial genetic variation, we tested for associations between mitochondrial DNA polymorphisms and one key component of our data, the maternal BLUPs for pigmented spore germination (Analysis S2). We found very low levels of mitochondrial DNA polymorphism in our collection of strains and no polymorphisms were associated with ascospore germination (Analysis S2).

Although we found no evidence of an association between mitochondrial DNA polymorphisms and spore germination, we note that mitochondria may still mediate maternal effects in a non cytoplasmic-genetic manner. For example, in germinating spores of *Neurospora tetrasperma*, a species closely related to *N. crassa* (Dettman *et al.*, 2003a), the maternal mitochondria – built entirely from maternally derived membranes and proteins – are responsible for all energy synthesis until 90 min after germination initiates (Hill *et al.*, 1992). After 90 min, a germinating spore begins to synthesize its own mitochondria using both its own mitochondrial genome and nuclear mitochondrial genes. Therefore, germination success may be influenced by the supply of mitochondria or other organelles provided directly from the maternal cytoplasm as well as the maternal provision of nutrients.

Anisogamy and maternal effects in other fungi and diverse eukaryotes

Anisogamy is commonly referenced as the basis for maternal effects. Although anisogamy is widespread among eukaryotes, maternal effects are thoroughly

studied in only two kingdoms – animals and plants. Our discovery of maternal effects in *N. crassa*, an anisogamous fungus, suggests anisogamy as a strong predictor of maternal effects across the fungi and the eukaryotic domain.

Within the fungi, maternal effects are likely to influence the life histories of numerous anisogamous species within the Ascomycota (Billiard *et al.*, 2010), as well as species of *Allomyces* and *Monoblepharis* in the Chytridiomycota (James *et al.*, 2006). While the majority of Basidiomycetes do not produce specialized sexual structures, maternal effects may play a role in the life histories of these fungi. When small and large haploid mycelia fuse, cytoplasm can be disproportionately inherited from the larger mycelium (Griffiths, 1996).

Anisogamy also occurs in groups besides animals, plants and fungi. Anisogamous eukaryotes include apicomplexans, dinoflagellates, diatoms, parabasalans and red algae (Dacks & Kasinsky, 1999). The life cycles of red algae in particular are very similar to life cycles of fungi such as *N. crassa* (Demoulin, 1985). Cytoplasmic inheritance in the malaria parasite *Plasmodium falciparum*, an anisogamous apicomplexan, is predominantly female (Vaidya *et al.*, 1993). We hypothesize that maternal effects occur in, and have important implications for the evolution of, these anisogamous taxa.

Conclusions

A simple crossing experiment suggests maternal effects profoundly influence fundamental aspects of individual fitness in the genetic model *N. crassa*. The maternal influence on spore production and viability is stronger than the influence of genetic divergence or geographic isolation – two essential aspects of any cross thought to influence reproductive success and speciation in all sexually reproducing organisms. Our experiment increases the number of eukaryotic clades known to house organisms influenced by maternal effects from two, the plants and animals, to three – the plants, animals and fungi. Given the distribution of anisogamy across many other eukaryotic clades, and the correlation between anisogamy and maternal effects, we hypothesize that maternal effects influence the evolution and ecology of additional eukaryotic organisms.

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Conflict of interest

The authors declared that they have no conflict of interest.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Example plot of forward-scatter height (FSC-H) vs. side-scatter width (SSC-W) of clustered flow cytometry data from a pooled set of 96 analysed ascospore samples.

Figure S2. Asymptotic nonlinear least squares regression of the proportion of pigmented spores measured by flow cytometry vs. counting.

Figure S3. Asymptotic nonlinear least squares regression of the proportion of germinated spores measured by counting vs. flow cytometry.

Figure S4. Correlogram showing all possible pairwise comparisons between the five measured traits.

Table S1. Summary of model parameter values for each measured trait.

Analysis S1. PDF showing R code and output for all analyses and figures in main text.

Analysis S2. Mitochondrial DNA variant identification and association test.

Derivation S1. Derivation of distribution-specific variance for the negative binomial distribution.

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