

What's Genetic Variation Got to Do with It? Starvation-Induced Self-Fertilization Enhances Survival in *Paramecium*

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Abstract

The pervasiveness of sex despite its well-known costs is a long-standing puzzle in evolutionary biology. Current explanations for the success of sex in nature largely rely on the adaptive significance of the new or rare genotypes that sex may generate. Less explored is the possibility that sex-underlying molecular mechanisms can enhance fitness and convey benefits to the individuals that bear the immediate costs of sex. Here, we show that the molecular environment associated with self-fertilization can increase stress resistance in the ciliate *Paramecium tetraurelia*. This advantage is independent of new genetic variation, coupled with a reduced nutritional input, and offers fresh insights into the mechanistic origin of sex. In addition to providing evidence that the molecular underpinnings of sexual reproduction and the stress response are linked in *P. tetraurelia*, these findings supply an integrative explanation for the persistence of self-fertilization in this ciliate.

Key words: sex, reproduction, stress response, dietary restriction, trade-off, heat shock proteins, ciliates.

Introduction

One long-standing hypothesis for the evolution and maintenance of sex is that it advantageously shuffles paternal and maternal alleles thereby facilitating adaptation to changing environments (Weismann 1889) and enhancing coping with fast-evolving parasites (Jaenike 1978; Hamilton 1980; Bell 1982). This hypothesis fits well with the observation that a shift from asexual to sexual reproduction is initiated in many species upon exposure to environmental challenges (Ram and Hadany 2016). However, a “sex as diversity generator” hypothesis might be less compelling when one considers self-fertilization. Although self-fertilization can also be triggered by stressful conditions, it may give rise to offspring that are completely homozygous and genetically identical to the parent (e.g., in the ciliate *Paramecium*; Sonneborn 1974). This implies that the selective advantage of sex may not lie only in the generation of different, potentially adaptive allelic combinations. If so, then why would selfing species—those that generate F1 copies of themselves, in particular—engage in sex upon exposure to environmental challenges? Any answer

to this question needs to consider the molecular mechanisms that underlie sex as well as existing empirical findings and theoretical arguments that sex can respond to stress (Nedelcu and Michod 2003; Mitchell et al. 2004; Bernstein and Bernstein 2013), is maintained upon exposure to parasites (Altermatt and Ebert 2008; Salathe et al. 2008; Jokela et al. 2009; King et al. 2009; Morran et al. 2011), is often condition-dependent (Hadany and Otto 2009), may be induced in response to a host immune system (Gemmill et al. 1997; West et al. 2001), may have higher fitness than asex (Agrawal 2001), and increases the rate of adaptation (Colegrave 2002) upon exposure to a harsh (but not more favorable) environment (Goddard et al. 2005).

Manifold investigations have attempted to characterize the possible benefits of sex focusing either on how sex has become widespread despite its costs (Agrawal 2001; Otto and Barton 2001), or on the population genetic mechanisms that drive its evolution (Becks and Agrawal 2012; Luijckx et al. 2017). The possibility that sex-underlying molecular mechanisms per se (i.e., regardless of the concomitant effect of sex

on genetic diversity) provide a fitness advantage remains largely neglected (Bernstein et al. 1985). This is an important oversight given that the properties of sex reflect the interplay of intracellular and selective processes. If the capacity for sex and the organismal stress-response overlap, that is, have molecular components in common, then sex could be triggered when there are environmental challenges and enhance survival at the same time.

This possibility aligns with evidence that highly conserved components of the cellular stress-response machinery such as heat shock proteins (HSP) play crucial roles in reproductive development and sex. Although HSP are probably best known as major players in the counteraction of proteotoxic damage, these proteins can play other biological functions, for example, they regulate mitotic cell division (Truman et al. 2012) and stimulate the immune response (Binder 2014; Abdel-Nour et al. 2019). HSP genes are also expressed physiologically during oogenesis and/or spermatogenesis across a wide range of species (Ambrosio and Schedl 1984; Heikkila et al. 1985, 1986, 1997; Allen, O'Brien, and Eddy 1988; Allen, O'Brien, Jones, et al. 1988; Yue et al. 1999; Christians et al. 2000; Metchat et al. 2009; Bierkamp et al. 2010). Members of the HSP70 family are required for the process of meiosis: HSP70 is key to the formation of the synaptonemal complex in mice and hamsters (Allen et al. 1996; Dix et al. 1996, 1997) and in the apicomplexan *Eimeria* (del Cacho et al. 2008). Furthermore, HSP70 is required for fertilization in bovines (Matwee et al. 2001) and the ciliate *Tetrahymena* (Fukuda et al. 2015). Because of the strong molecular ties that link HSP to sexual reproduction, if sex-related intracellular processes can truly enhance fitness in the context of environmental alterations, then HSP with meiotic/fertilization functions may contribute to the advantage of sex.

But what might this advantage be? For one, HSP with meiotic/fertilization functions (HSP_{MF}) might help activate HSP with stress-related functions (HSP_S) upon exposure to environmental stress, benefitting either the individuals that undergo sexual reproduction, or their resulting offspring, or both. This hypothesis is consistent with observations in the ciliate *Tetrahymena thermophila*, where the cytosolic HSP70 SSA5 chaperone has a crucial role in pronuclear fusion during fertilization and is also expressed in response to heat stress—without directly contributing to cell survival however (Yu et al. 2012; Fukuda et al. 2015). In a nonmutually exclusive fashion, the expression of HSP_{MF} might also enhance survival by reducing sensitivity to environmental alterations. These circumstances are reminiscent of the diapause: a widespread and reversible state of dormancy that is achieved through the involvement of HSP and insulin/insulin-like signaling (Tatar and Yin 2001; Walker and Lithgow 2003; Morley and Morimoto 2004; Marucci et al. 2009; Kamei et al. 2011; Sim and Denlinger 2013; King and MacRae 2015). Finally, HSP_{MF} might facilitate sex in stressful environments. Elevated levels of environmental stress in early life might prematurely activate

HSP_{MF} (letting alone HSP_S), and in so doing ultimately accelerate sexual maturation. The resulting association between early life stressors and accelerated sexual maturation is consistent with a wealth of observations (Hales and Barker 2001; Gems and Partridge 2008; Gluckman et al. 2009) and involves, once again, the modulation of insulin/insulin-like signaling (Roa and Tena-Sempere 2010; Tennesen and Thummel 2011). It is worth emphasizing that stress over an organismal lifetime can be experienced even without ecological changes. Mechanisms and molecular products that are commonly associated with a stress response and induce expression and accumulation of HSP70 (e.g., apoptosis, reactive oxygen species) have been linked to development and sexual reproduction (Thorburn 2008; Akematsu et al. 2012; Dokladny et al. 2013; Kurusu and Kuchitsu 2017; Park et al. 2017; Wagner et al. 2019).

Paramecium is an ideal organism to evaluate the significance of the foregoing hypothetical scenarios. One of the best studied species of this genus, *P. tetraurelia*, reproduces asexually, via binary fission, or sexually via meiosis followed by either cross-fertilization (conjugation) or self-fertilization (autogamy) (Sonneborn 1974). Autogamy in *P. tetraurelia* occurs in unpaired cells, is induced by starvation, and generates fully homozygous individuals (Sonneborn 1954; Mikami and Koizumi 1983; Berger 1986). More specifically, *P. tetraurelia* contains two types of nuclei, a micronucleus (with germinal functions) and the macronucleus (with somatic functions). When exposed to a sufficiently long period of food restriction, the macronucleus of *P. tetraurelia* undergoes fragmentation and is gradually reabsorbed via apoptosis- and autophagy-like processes. The two remaining micronuclei undergo two meiotic divisions producing eight haploid nuclei, one of which is preserved and divides mitotically to form genetically identical gamete nuclei that unite to form a completely homozygous fertilization nucleus. This latter nucleus then divides twice, producing new germline diploid and somatic polyploid nuclei (Sonneborn 1974). Autogamy rejuvenates *P. tetraurelia*, in the sense that it resets the cell's clonal age (Sonneborn 1954). Sex-caused rejuvenation “restores youthful vigor” in *Paramecium* (Maupas 1889) by replacing a possibly damaged somatic nucleus with a pristine, freshly produced somatic nucleus developed from an unburdened micronucleus (Bell 1988). Furthermore, *P. tetraurelia* is more likely to undergo autogamy when sufficiently old: at comparable levels of starvation, ~27 fission-old *P. tetraurelia* is far more likely to undergo autogamy compared with cells that are ~15 fissions away from the last fertilization (Komori et al. 2002). Interestingly, the level of starvation required for initiation of autogamy cells scales negatively with clonal age (Berger and Rahemtullah 1990). Besides, the length of autogamy immaturity is linked to clonal life span and rate of cell division (Smith-Sonneborn 1987; Takagi et al. 1989; Komori et al. 2004, 2005). The reasons for these relationships remain largely unknown in *P. tetraurelia*. In multicellular systems,

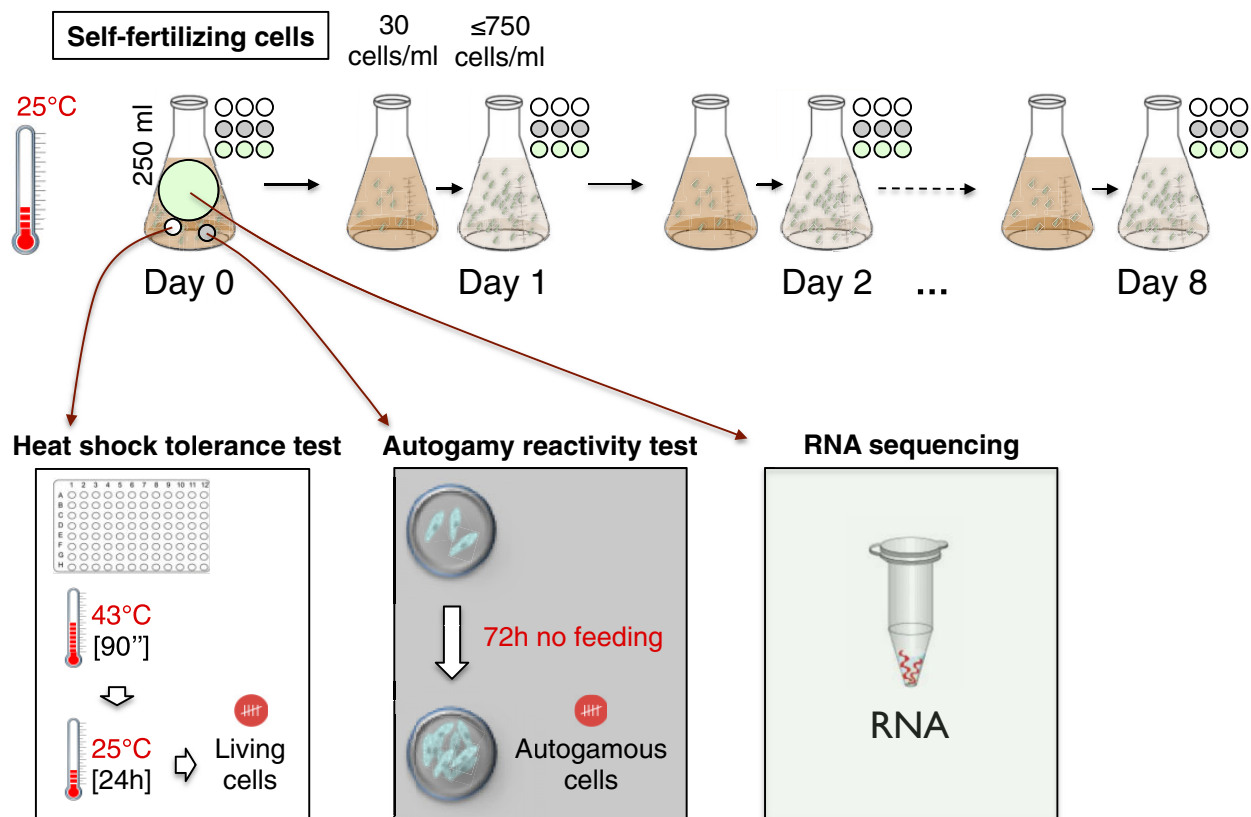


FIG. 1.—Time-course experimental design.

the insulin/IGF-1 signaling pathway plays a key role in shaping similar relationships (Arking 2019).

Here, we leverage *P. tetraurelia* to ask whether self-fertilization can enhance survival probability in the absence of new genetic variation. We show that fully homozygous self-fertilizing *P. tetraurelia* cells display an increased heat shock resistance, and so do also cells that have recently achieved competence for self-fertilization. A time-course transcriptomics study alongside additional phenotypic essays provides first insights into the molecular mechanisms that underlie this survival advantage. We find 1) that the observed enhanced stress resistance is negatively coupled with cell proliferation—a relationship that may be partly shaped by the withdrawal of growth factor(s)—and 2) that self-fertilization, onset of sexual maturation, stress response, and cell proliferation are interlinked in *P. tetraurelia*.

These findings suggest that the molecular mechanisms that contribute to the response to nutritional deprivation also promote sex in *P. tetraurelia*. Not only does this molecular environment couple sex with a substantial survival advantage in the absence of new genetic variation but it also enhances the cell's protection during meiosis, a fundamental requirement for rejuvenation. We propose that this molecular setting contributes to the persistence of self-fertilization in *P. tetraurelia*.

Materials and Methods

Time-Course Experiment

Paramecium Strain and Culture Conditions

Three independent polycaryonidal mass cultures of the *Paramecium tetraurelia* stock d12 (Rudman et al. 1991) were used in this experiment. These cultures were made of fully homozygous cells, which grow on Cerophyl Medium (CM; Taylor and Van Wagtendonk 1941, 0.25% w/v Wheat Grass Powder, GSE-Vertrieb GmbH) bacterized with *Enterobacter aerogenes* Hormaeche and Edwards (ATCC 35028TM). For bacterization, CM was inoculated (1:100 v/v) with a dense suspension of *E. aerogenes* ($OD_{600}=0.5$) and incubated at 30°C over night under gentle shaking (120 rpm). Culture medium was prepared every 48 h and supplemented with 0.5 µg/ml stigmaterol (Art. No. 9468.1, Carl Roth GmbH) before use. To ensure monoxenic conditions, cells that were used for the experiment were taken from a stock, transferred into depression slides, and washed nine times in mineral water (Volvic) before the beginning of the time-course study.

We produced autogamous mass cultures as starting material for the time-course experiment (day 0, fig. 1). Briefly, stocks cells of unknown age were taken through additional ~30 divisions to maximize autogamy reactivity and allowed to

starve naturally at 25 °C for 3 days to stimulate mass autogamy. Following a first expansion phase and unless otherwise indicated, log-phase cells were mass-cultured daily in 250 ml of bacterized CM at the standard cultivation temperature of 25 °C. Aliquots of cells (30 cells/ml) from the previous day were used daily to seed the progressively (clonally) older mass cultures. Cell density never allowed to exceed 750 cells/ml. For each of the 27 mass cultures (9 days × 3 replicates), aliquots of progressively older cells were obtained daily to perform a heat shock tolerance test and an autogamy reactivity test between 0 and ~30 divisions postautogamy (DPA) (fig. 1). Cell age expressed in DPA was calculated as the cumulative daily growth rate up to day n according to the following equation:

$$\text{DPA} = \sum_{i=1}^n \log_2(d_i/s_i),$$

where d is the final cell density, s is the seeding cell density, and $\log_2(d_i/s_i)$ is the growth rate for day i expressed in divisions per day.

Heat Shock Tolerance Test

Mass-cultured, log-phase cells were tested daily for heat shock as they progressed through the clonal life cycle (fig. 1). The assay included three independent biological replicates, each consisting of 90 cells. Single cells in 1 μ l were transferred to a 96-well PCR plate (Art. No. 710880, Biozym Scientific GmbH) preloaded with 70 μ l/well of bacterized CM and incubated at 43 °C for 1.5 min in a thermocycler (Eppendorf Mastercycler pro S). Cells were randomly assigned to plate wells for both control (no heat shock) and treatment (heat shock) groups. Following the heat shock bout, the plates were kept at 25 °C and scored for survival after 24 h. Each well was labeled according to a binary classification as alive (presence of actively swimming cells) or dead (absence of actively swimming cells).

Autogamy Reactivity Test

Mass-cultured, log-phase cells were also tested daily for autogamy reactivity (fig. 1). The assay included three independent biological replicates, each consisting of 56 cells on an average. Briefly, 10-ml aliquots were collected from triplicate mass cultures and routinely challenged with nutritional deprivation at 25 °C for 3 days to stimulate autogamy. From each starved aliquot, cells were collected in 20 μ l, fixed in 0.5% (w/v) paraformaldehyde, and stained with 5 μ g/ml DAPI to label the nuclei. To determine the fraction of autogamous cells in each sample, at least 40 cells per replicate were screened on an epifluorescence microscope (ZEISS Axioskop 2, light source LQ-HXP 120). Cells showing intact macronuclei were scored as nonautogamous (n), whereas cells with extensive macronuclear fragmentation were scored as autogamous (a). The

ratio $a/(a+n)$ was taken as the fraction of autogamous cells inducible at a given age.

Total RNA Extraction and Sequencing

Total RNA was collected in triplicates for each time point (fig. 1). Briefly, $\sim 10^5$ log-phase cells from 200-ml mass culture were concentrated on hydrophilic nylon membranes (Nylon-Netzfilter, 10 μ m pore size, 47 mm, Merck KGaA), rinsed in mineral water (Volvic), and pelleted by low-speed centrifugation at 800×g for 2 min. The pellet was immediately lysed in 1 ml of TRI Reagent (Sigma-Aldrich, Inc.) and stored at –80 °C until extraction. Total RNA isolation was performed according to the manufacturer instructions and yielded high-quality, DNA-free RNA with an average RIN of 8.3. A total of 27 RNA samples were sequenced, using strand-specific RNA sequencing library preparation and the HS4000 Illumina technology.

Quality Control and Reads Preprocessing

We analyzed Illumina Pair End sequences of polyA-RNA (~55 Mb; 2×150 bp) obtained for all the 27 RNA samples. Quality control of the RNA-seq data was performed using the FastQC (version 0.11.57; Andrews 2010). Low-quality reads were removed using default options of trim galore version 0.4.5 (<https://github.com/FelixKrueger/TrimGalore>, last accessed March 30, 2020). On an average, more than 25 million reads per samples were produced from the sequencing.

Reference-Guided Transcriptome Assembly

The RNA sequence reads were mapped with STAR version 2.5.0a (default parameters; Dobin et al. 2013) onto the *Paramecium tetraurelia* genome (Aury et al. 2006), with the annotation of the P51 version 2 (Arnaiz et al. 2017) obtained from the *Paramecium* database (ParameciumDB) (Arnaiz and Sperling 2011). On average, for each sample 90% of the reads aligned to the reference genome. Transcript abundance was measured in terms of read counts using the same annotation file used for the transcriptome assembly, leveraging the featureCounts (Liao et al. 2014), R Bioconductor package, default parameters. The count matrix was used as input for gene differential expression analysis.

Data Preprocessing and Gene Expression Visualization

A total of 31,830 genes with count per million >1 in ≥ 1 of the 27 samples were retained for differential gene expression analysis. We transformed the data counts using the variance stabilizing transformation function as in the Deseq2 package (Love et al. 2014). We used principal component analysis to visualize the between-sample distance based on gene expression data.

Differential Gene Expression and Gene Set Enrichment Analysis

To minimize any possible approach-specific bias, we performed pairwise differential gene expression analysis using Deseq2 (Love et al. 2014) and EdgeR (Robinson et al. 2010). The raw count matrix was employed as input for the Deseq2 package. Concerning the EdgeR tool, the input data matrix was normalized using the TMM (trimmed mean of M values) method. After obtaining the differential gene expression tables from Deseq2 and EdgeR, we extracted the differentially expressed genes with adjusted P value <0.05 based on the Benjamini and Hochberg method (Benjamini and Hochberg 1995) and considered only genes that were identified as differentially expressed (after correction for multiple testing) by EdgeR and Deseq2 at the same time. The functional annotation tool DAVID (Huang da et al. 2009) and the PANTHER classification system (Mi et al. 2019) were used for functional enrichment analyses.

Extreme Gene Expression Levels and Gene Set Enrichment Analysis

For each gene, we computed the maximum (MAX) and minimum (MIN) expression values across all the replicates and time points, from TMM-based normalized gene expression data. For this analysis, we considered genes with the sum of normalized read count in all 27 samples > 30 . Within a given time point, a gene was included in the MAX list if its expression value is the highest value across all time points or differ from the highest value for $<10\%$. Genes that for a given time point have at least two replicates in the MAX_{10%} list were used to investigate the “most” active biological processes at a given time point with DAVID (Huang da et al. 2009) and PANTHER (Mi et al. 2019). The same logic and tools were used to create a MIN_{10%} list of genes and investigate the “least” active biological processes at a given time point.

Cell Proliferation and Sexual Maturation

Outside of the time-course experiment, we performed a meta-analysis of data obtained from independently propagated cell lines of three different *P. tetraurelia* strains, d12, d4-2, and 51, fed with either *Alcaligenes faecalis* (KL) or *E. aerogenes* (EA) Hormaeche and Edwards (ATCC 35028TM) (three strains \times three biological replicates for EA + two strains \times greater than or equal to three biological replicates for KL). For all lines, we determined the cell proliferation rate and the age at autogamy maturity. The mean proliferation rate in the 24 h was determined over a period of 7–8 days. A linear regression was fitted to the autogamy ~ age values. Age at (auto)maturity was extrapolated from the regression equation and taken as the age (expressed in day postautogamy, DPA) at which 90% of the cells were capable of undergoing autogamy when challenged with nutritional

deprivation. The data were used to evaluate the relationship between cell proliferation rate and time at autogamy maturity.

Dietary Restriction and Survival to Stress

Outside of the time-course experiment, we also assessed the effect of dietary restriction on survival upon prolonged exposure to lethal levels of heat. Briefly, mass cultures of postautogamous cells grown in 50 ml Falcon tubes were randomly assigned to two dietary regimes, one consisting of nutritionally deprived cells and the other of cells fed with a nutrient-rich diet (bacterized medium). Nutritionally deprived cells were starved at the age of six divisions postautogamy for 3–5 days. Inability to undergo autogamy following starvation was confirmed with DAPI staining. Fed cells were kept in exponential growth (log phase) by daily transfer in fresh, nutrient-rich medium. During the assay, both groups were exposed to 40 °C in a water bath until total cell death. The experiment was performed on 12 independent biological replicates for each dietary group and cell density measured at 10-min intervals throughout the assay. Three aliquots (technical replicates) of 50–100 μ l were taken from each mass culture and cells were counted manually under a dissecting microscope to estimate cell density. Culture density was normalized over the initial cell density recorded before the test.

Results

Sex Is Associated with a Survival Advantage in the Absence of New Genetic Variation

We reared three genetically uniform mass cultures of *P. tetraurelia* from a self-fertilization state (day 0) up to ~33 fission-old cells (day 8) in parallel. The vegetative mass cultures were fed constantly and maintained at a density of ≤ 750 cell/ml to minimize stress. In these experimental conditions, the rate of cell proliferation between days (other than day 0) varies to some considerable, but not statistically significant, extent (Kruskal–Wallis test, $P = 0.10$), and amounts to an average of ~4 fissions/day (fig. 2A). We used these clonally aging mass cultures to investigate the association between autogamy competence and stress resistance.

Every day, we screened each of the mass cultures for autogamy competence. We collected aliquots of ~50 cells per cell culture, on an average, subjected them to three consecutive days of starvation, and discarded them once the phenotype had been recorded. The vast majority of day 0 cells show signs of autogamy ($97\% \pm 1.73\%$; mean and SE, respectively). As expected, the fraction of cells that is capable of undergoing autogamy increases with clonal age (fig. 2A). There is a dramatic rise in the percentage of autogamy-competent cells from day 2 to day 3 (6 ± 1 vs. 66 ± 4.6), which is followed by a steady upward trend (day 4: 88.3 ± 2.6 ; day 5: 92 ± 2.1). At day 6, all the cell cultures

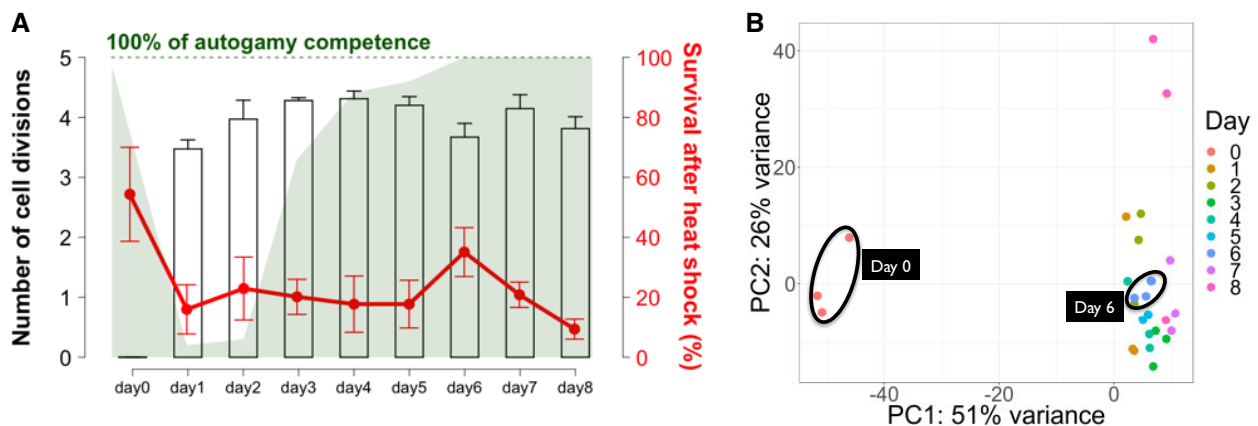


Fig. 2.—Phenotypic and transcriptomic profiles of *Paramecium tetraurelia* cell cultures during nine consecutive days post- and including self-fertilization. (A) Bar plot shows the number of cell divisions per day (average and SEs; three replicates). The per-day degree of autogamy competence is reflected by the height of the green area. Line plot shows cell survival (i.e., active swimming) after a 43 °C heat shock (90 s) and 24-h recovery time in a nutrient-rich medium at 25 °C (average and SEs). (B) Principal component analysis of the *P. tetraurelia* transcriptome (nine successive windows of time \times three replicates).

display 100% autogamy competence, for the first time after the last fertilization. This level of autogamy competence holds for the well-fed day 7 and day 8 cells. It is worth noting that the onset of full sexual competence in nature could be immediately followed by self-fertilization when nutrients are scarce.

Additional aliquots of 96 cells were collected daily from each of the mass cultures for a stress test. These cells were exposed to a heat shock of 43 °C for 1.5 min, and then stored separately for 24 h in a nutrient-rich medium before being screened for survivors and ultimately discarded. We detected a considerable level of variation among *P. tetraurelia* cells collected over the nine experimental time intervals. More than 50% of day 0 cells ($54.3\% \pm 15.7$) survived the thermal shock, >2.5 times more than the average fraction of survivors recorded for the vegetative samples ($\sim 20\% \pm 13.0$; Wilcoxon rank sum test, $P < 0.05$) (fig. 2A). Among the cells in vegetative state, day 6 cells display a percentage of survivors (35.1 ± 8.2) that is 1.5 times lower compared with day 0 cells, but approximately two times higher than the counterpart calculated for the remaining vegetative samples (17.8 ± 11.6) (Wilcoxon rank sum test, $P < 0.05$) (fig. 2A). The enhanced resistance to heat displayed by day 6 cells is associated neither with genetic variation nor with clonal age, as evidenced by the unremarkable survival rates of day 7 and day 8 cells. It is instead associated with the onset of full competence for sex; at day 6, all the cell cultures display 100% autogamy competence for the first time after the last fertilization (fig. 2A).

Transcriptional Changes during Sexual Development Are Coupled with Increasing Levels of Endogenous Stress

To gain insight into the association between heat resistance and autogamy competence, we investigated the transcriptomes of the 27 mass cultures (9×3 replicates) that were

screened for these traits. A principal component analysis reveals differences in the transcript abundance of self-fertilizing and vegetative cell cultures, as expected (fig. 2B). We also detect a tendency for day 0 and day 6 cell cultures to contain more genes with comparable levels of expression relative to other pairwise combinations (supplementary fig. S1, Supplementary Material online). Of 32,252 genes with non-zero expression values across each of the experimental days, 1,037 and 1,145 have increasing and decreasing levels of expression, respectively, over the eight vegetative time points (Pearson's r , $P < 0.05$). These genes are candidates for regulating sexual maturation and reproductive processes as well as lifespan in *P. tetraurelia* (supplementary table S1, Supplementary Material online). “Cell redox homeostasis” is among the most enriched biological processes for the genes with steadily increasing expression levels (supplementary table S1 and fig. S2, Supplementary Material online). Among these genes, Hsp70Pt04 has alongside Hsp70Pt01 and Hsp70Pt03 the highest level of sequence similarity to the ciliate *T. thermophila*'s HSP70 SSA5 (supplementary fig. S3, Supplementary Material online). SSA5 is a key protein for pronuclear fusion during fertilization and is also expressed during development and in response to heat stress (Fukuda et al. 2015). Similarly, SSA5 homologs in *P. tetraurelia* are expressed throughout development (supplementary fig. S3, Supplementary Material online) and are inducible after heat shock exposure (Hsp70Pt03 and Hsp70Pt04; Krenek et al. 2013) or upregulated after a 3-week-long period of starvation (Hsp70Pt01; Cheaib et al. 2015). Hsp70Pt01 also displays levels of expression that vary significantly during the process of self-fertilization (i.e., this gene is upregulated during meiosis compared with later stages) (Arnaiz et al. 2017). Different levels of expression during self-fertilization are detected for seven other members of the HSP70 family (fig. 3). These observations raise the possibility that in *P. tetraurelia*, like in

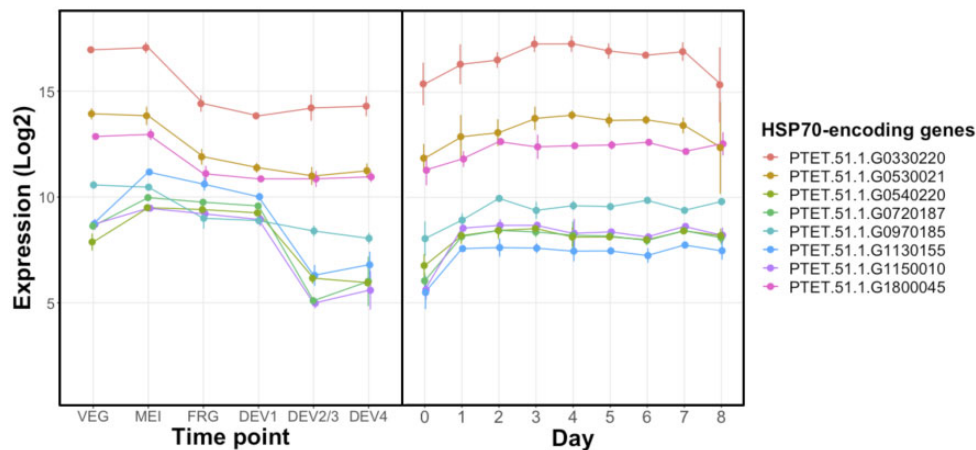


Fig. 3.—Candidate HSPs with meiotic/fertilization functions in *Paramecium tetraurelia*. HSP70 genes constitutively expressed during vegetative growth (right panel, this study) display expression levels that are significantly higher during meiosis compared with the vegetative stage (*PTET.51.1.G1130155*) or to later stages of self-fertilization (*PTET.51.1.G0330220*, *PTET.51.1.G0530021*, *PTET.51.1.G0540220*, *PTET.51.1.G0720187*, *PTET.51.1.G0970185*, *PTET.51.1.G1150010*, and *PTET.51.1.G1800045*) (left panel; Arnaiz et al. 2017); for example, the expression of Hsp70Pt01 (*PTET.51.1.G0330220*) is comparable between the vegetative (VEG) and the meiotic (MEI) stages but drops before (DEV1), the earliest stage at which a significant proportion of cells has visible macronuclear anlage. The statistical significance of the differences in gene expression levels during subsequent stages of self-fertilization was retrieved from ParameciumDB (<https://paramecium.i2bc.paris-saclay.fr/>, last accessed March 30, 2020). The right panel shows that cells take ~2 days to restore the original VEG expression levels of HSPs. The time point/stage DEV4 in Arnaiz et al.' data set (left panel) corresponds to day 0 in our study (right panel).

Tetrahymena, HSP70s may also play a role in meiosis/fertilization and respond to environmental stress at the same time.

A Trade-Off between Stress Resistance and Cell Division

When inspecting genes that are differentially expressed at consecutive time points or with extreme transcription activity at a given time point (supplementary table S1, Supplementary Material online), we uncover significantly downregulated biosynthetic activities in day 0 and day 6 (fig. 4 and supplementary figs. S4 and S5 and tables S2 and S3, Supplementary Material online). This may be unsurprising for day 0 cells, which neither feed nor divide. However, it is unexpected for day 6 cells. Not only are these cells cultivated in a nutrient-rich environment but they also divide albeit at a marginally reduced rate compared with day 5 cells (3.7 ± 0.23 vs. 4.2 ± 0.15 divisions; paired *t*-test, $P < 0.05$) (fig. 2A). When compared with day 5 cells, day 6 cells upregulate a handful of biological processes such as a response to oxidative stress, whereas they downregulate genes involved in endocytosis and components of the phagosome, among others. Additionally, our data point to a regulated withdrawal of one or more growth factors in day 6 cells (supplementary fig. S6, Supplementary Material online). These observations indicate that nutrient uptake may be hindered in day 6 cells despite the nutrient-rich conditions—a state that we dub pseudostarvation. Changes in response to oxidative stress and expression of phagosome components are also observed in the fast proliferating day 3 cells (4.3 ± 0.05 divisions), however, in the opposite direction. Overall, these findings suggest

that levels of mitotic cell division and stress-response may trade-off in *P. tetraurelia*.

We also found that day 1 cells significantly upregulate an excess of HSP70 chaperones (13 observed vs. ~4 expected; $Z = 3.48$, $P < 0.001$; supplementary table S1, Supplementary Material online). Given that day 1 cells resume proliferation after the nondividing stage of self-fertilization, this observation aligns with the view of HSP70 chaperones as active regulators of mitotic cell division (Truman et al. 2012). Two of the HSP70-encoding genes that are upregulated in day 1 are also upregulated in day 7 cells. These two genes (*PTET.51.1.G0720187*, *PTET.51.1.G1150010*), alongside their two ohnologs (*PTET.51.1.G0540220* and *PTET.51.1.G1130155*), are the only HSP70s to show similarly elevated expression levels both during and between meiosis and fertilization in *P. tetraurelia* (fig. 3), indicating that these HSP70s might play a role in mitosis as well as meiosis, in line with previous reports (Maciver 2019 and references therein).

Lastly, although day 0 and day 6 cells show an elevated heat shock resistance, we detected no enrichment of upregulated stress-response genes in these cells. In fact, HSP70-encoding genes are significantly downregulated in day 0 cells more often than in day 1 cells (13 downregulated vs. 1 upregulated; supplementary table S1, Supplementary Material online). These observations may be interpreted in at least two ways. First, HSP70s are not key players in the enhanced thermotolerance of day 0 and day 6 cells. Although this will require further investigation, it is worth noting that HSP70 genes are often upregulated in response to acute exogenous stressors, whereas in our experiment, we screen only

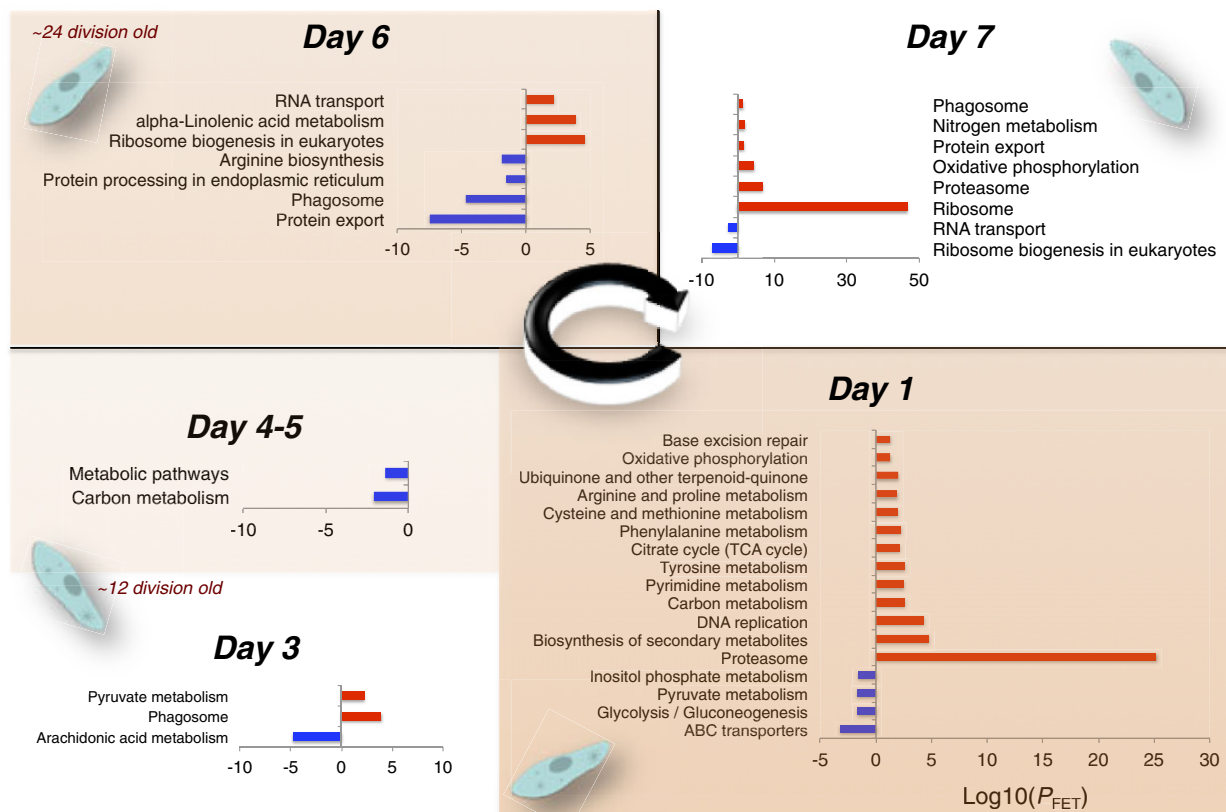


FIG. 4.—Enriched molecular pathways during the first ~33 cell divisions postself-fertilization. Reported enrichment (Fisher's exact test, $P < 0.05$) is relative to the previous day (e.g., day 1 relative to day 0) and is estimated via the functional annotation tool DAVID (Huang da et al. 2009) and the Kyoto Encyclopedia of Genes and Genomes (Kanehisa et al. 2014).

physiological levels of gene expression. Second, the expression of HSP70 genes may be decoupled from the expression/activity of their proteins, and HSP70s may already occur in day 0 (and day 6) cells. In the latter scenario, HSP that are, for example, involved in mitosis (Truman et al. 2012) might be redeployed as cytoprotectants following a developmentally regulated decrease of nutrient uptake. Consistent with this, the inhibition of cell cycle genes leads to tolerance toward environmental stress in the nematode *Caenorhabditis elegans* (Dottermusch et al. 2016). Additionally, it is established that the expression of HSP genes may be decoupled from the expression of their proteins (Kregel 2002), and that HSP may regulate negatively the transcription of their corresponding genes (Stone and Craig 1990; Amaral et al. 1993; Shi et al. 1998; Sri and Morimoto 1999; Balakrishnan and De Maio 2006).

Model: Sex Competence, Mitotic Progression, and Stress Resistance Are Mechanistically Linked in *P. tetraurelia*

The preceding time-course study indicates that HSP with meiotic/fertilization functions (HSP_{MF}) accumulate during vegetative growth and might respond to environmental stress in

concert with (or effectively acting as) HSP with stress-related functions (HSP_S). Additionally, HSP that engage in the regulation of mitotic cell division might be redeployed as cytoprotectants when cell division is downregulated. Taken together, these indications generate a prediction that recapitulates the observations depicted in figure 2A, that is, HSP_{MF} and HSP_S should be more likely to co-occur in cells that divide slowly (day 6) or not at all (day 0). In other words, reduced levels of cell proliferation—which are controlled by nutritional input—should contemporaneously hasten the acquisition of sexual maturity and enhance stress resistance. We tested these predictions.

We examined the rates of sexual maturation in *Paramecium* cells whose proliferation is sustained by two prey bacterial species, *E. aerogenes* (EA) and *A. faecalis* (KL), which promote different growth rates (Wilcoxon rank-sum test, $P < 0.001$) (fig. 5A, top panel). Consistent with our prediction, we detected a significant positive correlation between cell proliferation and the duration of autogamy immaturity ($\rho = 0.71$, $P < 0.001$), with the slow-growing KL-fed *P. tetraurelia* cells reaching sexual maturity earlier than the fast-growing EA-fed cells (Wilcoxon rank-sum test, $P < 0.01$) (fig. 5A, right panel).

Next, we tested whether reduced levels of cell proliferation enhance stress resistance. We compared the response of

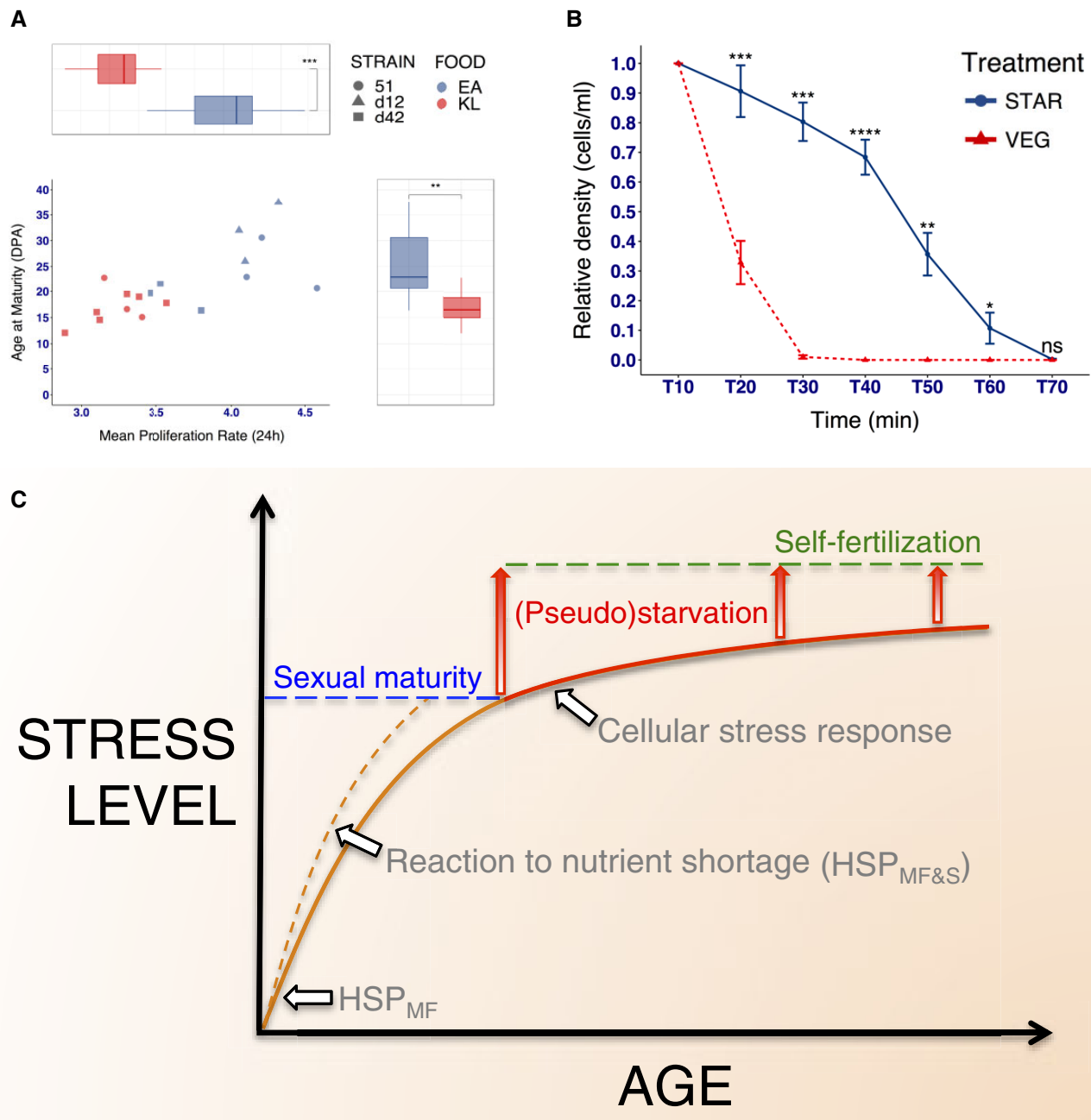


FIG. 5.—Stress response, sexual maturation, self-fertilization, cell proliferation, and nutritional input are mechanistically linked. (A) Low-quality food accelerates sexual maturation. The box plot at the top compares the main proliferation rate of cells fed with EA and KL. The box plot to the right compares the age at maturity between the two food-quality groups. DPA, divisions postautogamy. (B) Dietary-restricted cells resist heat shock. Nutritionally deprived cells show prolonged survival during uninterrupted exposure to 40 °C, whereas actively proliferating cells expire quickly. Plot shows the mean culture density ± the SEM. Mean differences were tested with a Wilcoxon signed-rank test. ns: $P > 0.05$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$. (C) Model for the effect of nutritional deprivation on sexual maturation, stress response, and self-fertilization in *Paramecium tetraurelia*. HSP_{MF} with meiotic/fertilization functions (HSP_{MF}) accumulate physiologically during development (solid orange curve) and favor sexual maturation. Nutrient shortage in the early postautogamy period may enhance the expression of HSP_{MF} and HSP with stress-related functions HSP_S (dashed orange curve), thereby reducing the HSP_{MF} shortfall in sexually immature *P. tetraurelia* cells. This is predicted to concurrently increase the ability to cope with stress and to speed up sexual maturation (lowering the age of sexual maturity). Nutrient shortage is also required to initiate self-fertilization. A state of pseudostarvation in sexually mature cells (arrows with red gradient, lower portion) is a first (reversible) step in the process of self-fertilization. This cellular state of regulated reduced nutrient uptake is coupled with (and possibly prompted by) a stress reaction to cell-intrinsic events. Consequently, pseudostarving cells are particularly resistant to environmental

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young, dietary restricted, cells and exponentially growing cells of comparable age to prolonged heat shock (fig. 5B). Studying young *Paramecium* cells with no or little competence for self-fertilization helps decouple the effects of dietary restriction from those of the sexual process. We detect significant prosurvival effects of nutritional deprivation (fig. 5B). During the heat shock bout, dietary-restricted cells survived on an average twice as long (long rank test, $P < 0.001$), with ~10% of the culture still alive beyond 1 h of exposure (exponentially growing cells did not survive past 30 min).

Overall, these observations are compatible with a model where stress resistance, autogamy maturation, and cell proliferation are mechanistically linked in *P. tetraurelia* and call for additional investigations.

Discussion

The persistence and pervasiveness of sexual reproduction, despite its substantial time and resources requirements, continue to intrigue biologists. The classical explanation for the success of sex is that it creates genetic variation that enhances the offspring's chances of survival in stressful environments, or that it purges deleterious recessive alleles.

Here, we capitalize on knowledge offered by previous molecular and cell biology studies and leverage the properties of the ciliate *Paramecium* to provide integrative answers to three questions. How can sex—self-fertilization, in particular—persist in eukaryotic species where it generates no genetic variation? Why does stress induce sex? And last, why do individuals bear the costs of sex even when, typically, they do not immediately or directly benefit from its putative benefits? Our observations suggest that the molecular mechanisms that underlie sex and stress-response overlap in *Paramecium*. As a consequence, not only can stress facilitate sex in this ciliate but sex also takes place in a molecular environment that promotes stress-resistance. These observations extend previous suggestions that sex is beneficial for *Paramecium* as it allows the cell to rejuvenate (Bell 1988; Maupas 1889). Our observations suggest that the intimate coupling between stress response and self-fertilization provides a protective environment, where the replacement of the old macronucleus may take place undisturbed even during exposure to environmental insults. We propose that the coupling between stress-related and sex molecular machineries contributes to the persistence of self-fertilization in *P. tetraurelia*, a ciliate whose level of intraspecific genetic diversity (Catania et al. 2009)

and *per* generation base-substitution mutation rate (Sung et al. 2012) are exceedingly low.

Our observations further suggest that stress response, the onset of sexual competence, cell proliferation, and nutritional input are mechanistically linked in *P. tetraurelia* (fig. 5C). We propose a model where a reduced nutrient uptake may dually speed up the acquisition of the capacity for sex and increase the ability to cope with stressors. When sufficiently prolonged, nutrient shortage can favor self-fertilization while protecting the cell against environmental stressors. These dynamics raise the possibility that even young, sexually immature, *P. tetraurelia* cells may develop the capacity for sex when they are exposed to a sufficiently prolonged nutrient shortage. This expectation is consistent with previous observations (Berger and Rahemtullah 1990).

The degree to which the sex-coupled survival advantage observed for *Paramecium* extends to other organisms warrants further investigation. Much can also be gained from examining the discrepancies that might emerge between *Paramecium* and other species. For example, the heat shock response (HSR) collapses with the onset of reproductive maturity in *C. elegans*, leaving adult individuals more vulnerable to stress (Labbadia and Morimoto 2015). This HSR collapse is maintained when the entire gonad is absent, whereas it disappears, and in fact the HSR is enhanced at the organism level, when germline stem cells (GSCs) are removed (Labbadia and Morimoto 2015). This suggests that *C. elegans*' somatic gonadal cells, which play important roles in GSCs maintenance as well as in germ cell proliferation and differentiation (Spradling et al. 2011), produce pro-HSR signals that counterbalance the GSCs signals (Hsin and Kenyon 1999; Arantes-Oliveira et al. 2002). Although one has to draw cautious parallels, the somatic nucleus of *Paramecium* could be viewed as the somatic gonadal cells in *C. elegans*. In this light, the enhanced stress resistance that we observe for day 0 and day 6 cells could reflect a transiently unbalanced signaling that favors the somatic nucleus. Based on our findings, *Paramecium* could achieve this unbalanced signaling by downregulating the nutrient-sense signaling. Similar mechanisms could shape similar responses in *C. elegans* (Kenyon 2010; Dottermusch et al. 2016). At any rate, because of the deep evolutionary conservation of the stress response and sex molecular machineries, our study urges caution over swiftly invoking genetic diversity to explain increased stress tolerance in sexually produced offspring. Indeed, because HSP70-mediated stress tolerance may be passed on to successive

Fig. 5.—Continued

challenges. In nutrient-limiting conditions, the pseudostarvation state can transition into a state of full starvation, with a consequent intensification of cellular stress reactions (arrows with red gradient, upper portion). In contrast, the pseudostarvation state is rapidly lost in nutrient-rich conditions, where normal feeding resumes. It is predicted that cells undergo a new state of pseudostarvation after some unspecified number of divisions. As cells grow older, the cellular stress response increases and the level of starvation that is required for initiating self-fertilization decreases. It is predicted that in sufficiently (clonally) old *P. tetraurelia* cells self-fertilization is possible even in nonnutrient-limited conditions.

sexual generations even in the absence of the parental stressor (Norouzitallab et al. 2014), enhanced stress tolerance in sexually produced offspring might reflect an environmentally induced phenotype that is trans-generationally inherited (Suter and Widmer 2013), rather than a genetically controlled trait.

Last, the model that we propose for the maintenance of sex in *P. tetraurelia* might help shed light on the evolutionary origin(s) of sex in ciliates and perhaps other eukaryotes. Given that heat shock factors have established roles in meiosis and fertilization and that the stress response is ancient and ubiquitous—HSP expression is a universal response to a variety of stresses (e.g., physical, physiological)—whereas sex is not necessary for reproduction, it is tempting to speculate that the sex machinery might have evolved from, and be partly modulated by, the heat shock signaling pathway or, more in general, the integrated stress response (Wek 2018). If so, the evolutionary success of sex could partly rely on the strong and pervasive selective forces that preserve the cellular defense mechanisms.

In conclusion, we update current theories on sex benefits by illustrating the possibility that the molecular environment where meiosis unfolds can confer a survival advantage in the absence of new genetic variation. Our observations in *Paramecium* extend the dominant paradigm. They suggest the existence of molecular connections between sex and stress response through which sex-performing unicellular organisms may gain direct fitness benefits.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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Author Contributions

A.S.T. and V.V. performed experiments and measurements, analyzed the data, and wrote the article. M.R.G. helped

supervise the project, provided expertise and feedback. F.C. conceived and designed the project, analyzed the data, supervised, wrote the article, and secured funding.

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