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The consequences of facultative sex in a prey adapting to predation

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Abstract

A species reproductive mode, along with its associated costs and benefits, can play a significant role in its evolution and survival. Facultative sexuality, being able to reproduce both sexually and asexually, has been deemed evolutionary favourable as the benefits of either mode may be fully realized. In fact, many studies have focused on identifying the benefits of sex and/or the forces selecting for increased rates of sex using facultative sexual species. The costs of either mode, however, can also have a profound impact on a population's evolutionary trajectory. Here, we used experimental evolution and fitness assays to investigate the consequences of facultative sexuality in prey adapting to predation. Specifically, we compared the adaptive response of algal prey populations exposed to constant rotifer predation and which had alternating cycles of asexual and sexual reproduction where sexual episodes were either facultative (sexual and asexual progeny simultaneously propagated) or obligate (only sexual progeny propagated). We found that prey populations with facultative sexual episodes reached a lower final relative fitness and suffered a greater trade-off in traits under selection, that is defence and competitive ability, as compared to prey populations with obligate sexual episodes. Our results suggest that costs associated with sexual reproduction (germination time) and asexual reproduction (selection interference) were amplified in the facultative sexual prey populations, leading to a reduction in the net advantage of sexuality. Additionally, we found evidence that the cost of sex was reduced in the obligate sexual prey populations because increased selection for sex was observed via the spontaneous production of sexual cells. These results show that certain costs associated with facultative sexuality can affect an organism's evolutionary trajectory.

Introduction

Explaining the evolutionary advantage of sexual reproduction remains a problem in evolutionary biology because as a reproductive strategy it is ubiquitous but costly (Williams, 1975; Maynard Smith, 1978; Bell, 1982; Hartfield & Keightley, 2012). Nonetheless, significant progress has been made in identifying conditions providing a benefit to sexual reproduction. Sex has been shown to be beneficial, for example, during times of adaptation to novel environments by generating fitness variance for selection to work more effectively on (Weissmann, 1889; Kaltz & Bell, 2002; Becks & Agrawal, 2012), in heterogeneous environments (Bell, 1982; Charlesworth, 1993; Becks & Agrawal, 2010), and when populations coevolve with a parasite (Jaenike, 1978; Hamilton, 1980; Morran *et al.*, 2011). Less clear are the conditions when the different costs of sexual and asexual reproduction may apply and how they may impact an organism's evolutionary trajectory. Understanding and testing these costs is, however, necessary for identifying the conditions that favour selection for sex, as selection depends on the net effect of sexual reproduction.

Perhaps the most well-known cost associated with sex is the two-fold cost of sex, which refers to the cost

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of males who do not directly contribute to offspring production (Maynard Smith, 1978) and/or the cost of meiosis, which leads to genome dilution of either parent (Williams, 1975). However, the two-fold cost only applies in [multicellular] species with separate sexes ('dioecious') and gametes of unequal size ('anisogamous') (Charlesworth, 1980; Lehtonen *et al.*, 2012). Other disadvantages of sex include recombinational load [the break-up of co-adapted gene combinations (Maynard Smith, 1978; Lewis, 1987)], increased risk of predation or sexually transmitted diseases, and increased time and energy in finding a compatible mate (or gamete) and completing the sexual cycle. The disadvantages of asexual reproduction are usually associated with some level of selection interference, for example clonal interference (Gerrish & Lenski, 1998) and the combined effect of selection and drift [Hill–Robertson effect (Hill & Robertson, 1966)]. The former refers to beneficial mutations arising in independent lineages that compete for fixation and ultimately slow the rate of adaptation, whereas the latter refers to the potential loss of beneficial mutations occurring on disadvantageous genetic backgrounds and hence the reduced efficacy of selection. The irreversible accumulation of deleterious mutations [Muller's Ratchet (Muller, 1932; Haigh, 1978)] is another cost of asexual reproduction.

In this study, we evaluate the costs of sexual and asexual reproduction indirectly by comparing the evolutionary trajectory of populations with alternating cycles of asexual reproduction ('apomixis') and either facultative or obligate sexual reproduction ('amphimixis'), and with or without strong directional selection (constant predation). Under these conditions, the benefits of sexual reproduction are expected to be the same in both environments (obligate or facultative sexual cycles). The benefit of sex arises from the increase in fitness variance which is advantageous for adaptation to the novel environment (Smith, 1988; Colegrave, 2002; Goddard *et al.*, 2005; Becks & Agrawal, 2012). The obligate or facultative sexual environments differ, however, in their costs of sexual and asexual reproduction. In the facultative sexual environment, the time needed for completing the sexual cycle and recombination load are the costs of sexual reproduction, whereas the costs of asexual reproduction include clonal interference and the Hill–Robertson effect (in conjunction, 'selection interference'). In contrast, the costs of asexual reproduction are absent or negligible in the obligate sexual environment, whereas the costs of sexual reproduction only include recombinational load. We use the term costs of sex here in a more general way to assess the evolutionary trajectories of obligate vs. facultative sexual populations and not referring to the inherent fitness loss incurred by sexuals in the absence of external selective forces.

Here, we used constant predation as the selective environment. Many studies have shown that strong (directional) selection by predators can drive rapid prey evolution in the field and laboratory (Endler, 1980; Yoshida *et al.*, 2003; Walsh & Post, 2011; Agrawal *et al.*, 2012; Koch *et al.*, 2014). Moreover, recent studies have demonstrated that predator–prey interactions can be involved in the maintenance of sexual reproduction, for example in plankton (Haafke *et al.*, 2016) and in plant–herbivore systems (Johnson *et al.*, 2015). Therefore, we predicted that there would be differences in the evolutionary trajectory of facultative and obligate sexual prey populations adapting to constant predation based on the differential costs of sexual and asexual reproduction and the net effect of sexual reproduction.

To test this prediction, we used experimental evolution in a predator–prey system to evolve replicate prey populations (unicellular alga, *Chlamydomonas reinhardtii*) with and without constant predation (rotifer predators, *Brachionus calyciflorus*), and with multiple sexual episodes where afterwards sexual offspring only ('obligate') or both sexual and asexual offspring ('facultative') were propagated to the next generation. Algal populations were set up using isogenic lines of the two mating types (+/−), and therefore all experimental populations began from the same ancestral starting point and any new genetic variation arose via *de novo* mutation and recombination. Although *C. reinhardtii* is an isogamous species, and therefore the two-fold cost of sex does not apply in this system, this species has been used extensively as a model system in a number of other studies on sexual reproduction (de Visser *et al.*, 1996; Colegrave, 2002; Colegrave *et al.*, 2002; Kaltz & Bell, 2002; Lachapelle & Bell, 2012). Overall, we found that populations with facultative sexual episodes evolved lower fitness levels and individual clones suffered an increased trade-off between competitive ability and defence as compared to prey populations with obligate sexual episodes. Unexpectedly but interestingly, we also observed selection for increased sex in the obligate sexual populations, suggesting that the cost of sex was indeed reduced in these populations.

Materials and methods

Base populations

We obtained *C. reinhardtii* strains [CC-1009, mating type − and CC-1010, mating type +] from the Chlamydomonas Resource Center at the University of Minnesota (Laudon, 2013). From these, we isolated a single mt+ and mt− clone and grew them for 2 weeks in 200 mL media containing a nitrogen concentration of 800 μм (Felpeto & Hairston, 2013). From these two base cultures, we set up four different experimental populations based on algal reproductive strategy and rotifer predation treatment: obligate and facultative sexual populations, and for each reproductive strategy treatments with and without constant predation. Each experimental population had 12 replicates, resulting in a total of 48 populations.

Experimental evolution regime

We followed standard experimental evolution techniques by constructing ancestral populations from a single clone of each mating type and tracking adaptation as occurred through *de novo* mutations. We experimentally evolved algal populations of different reproductive strategy and predation treatment over an 8-month period and lasting approximately 300 generations (Fig. S1). In general, the experimental evolution scheme involved alternating cycles of growth and sex where algal population growth (via mitotic asexual reproduction), predation and adaptation occurred during the 'growth' cycles and where one sexual generation occurred during the 'sexual' cycles. Specifically, we propagated the populations as batch cultures in 12-well plates and serially transferred them (10% volume) through two consecutive growth cycles (lasting 3 and 4 days, respectively), where all populations reproduced asexually and experienced exponential growth in 2 mL media containing a nitrogen concentration of 800 μM. During the growth cycles, well-plates were placed on an even surface, unshaken, and in indirect light. After each set of growth cycles, we induced a sexual cycle by transferring populations to nitrogen-free media (nitrogen deprivation is the sexual cycle cue), which triggered cells to undergo gametogenesis and mate. After the mating reactions sat in direct light for 2 days, we transferred a 10% aliquot of each population to an agar plate containing a nitrogen concentration of 800 μM. The agar plates were placed in direct light for 1 day and then wrapped in foil and placed in the dark for 7 days to allow zygospore maturation. Afterwards, we followed standard protocol (Harris, 2009) as described by Bell (Dasilva & Bell, 1992; Lachapelle & Bell, 2012) and exposed the obligate sexual populations to chloroform to kill unmated cells and ensure that only sexual offspring were propagated to the next growth cycle. This was done by inverting each plate over a shallow dish of chloroform for 45 s. Chloroform vapours disrupt the cellular membrane of vegetative cells, killing them, but they cannot penetrate the thick, protective wall of zygospores, leaving them intact. We did not chloroform the facultative sexual populations, thus allowing both sexual and asexual progeny to be propagated. After this, we placed all plated populations in indirect light for 1 day to allow zygospore germination, and then transferred them to direct light for 1 week. To begin the next set of growth cycles, we inoculated ~1 × 10^6 cells into each corresponding well on a new 12-well plate. Replicate populations were maintained as independent lines and never mixed. This scheme was carried out for a total of 12 growth cycles with five sexual cycles interspersed at regular intervals.

Predation

To expose algal populations to predation, we placed five rotifers (*B. calyciflorus*) into each well. To ensure constant grazing pressure, we checked the well-plates every day during the growth cycles to either add to or subtract from each well to attain exactly five rotifers per algal population. Rotifers did not evolve within this experiment as they were replaced at every transfer from a monoclonal asexual stock population. We fed rotifer stocks with the unicellular alga, *Chlorella vulgaris*. Twenty-four hours prior to their use, we washed and starved the rotifers in nitrogen-free media to prevent *Chlorella* contamination. Before each algal population was transferred to nitrogen-free media to induce the sexual cycle, we removed all rotifers by filtration using Falcon 40-μm nylon cell strainers. For reasons most likely related to stock culture maintenance, rotifers died in all replicate populations during growth cycle 6, and thus data from these timepoints were omitted.

Populations: relative fitness

To compare adaptive responses, we tracked the relative fitness of algal populations over time. We did this by calculating population growth rates from optical density measurements (absorption wavelength of 680 nm) taken for every population every second growth cycle, using a Tecan Infinite 200 PRO plate reader. From these data, we calculated relative fitness of each population as the population growth rate normalized to the ancestral growth rate of growth cycle 1 for that line. Population sizes were 2 × 10^6–10^8 cells. All statistical analyses were conducted using R (R Core Team 2014), version 3.1.3. To test the effects of predation treatment, reproductive strategy, growth cycle (time) and interactions on the relative fitness of algal populations, we used a linear mixed-effects model (LMM) with each factor as a fixed effect and growth cycle nested within replicate population nested within reproductive strategy as random effects to account for the temporal correlation of the measurements within one replicate. We used linear models (LM) to test the effect of predation on relative fitness within each reproductive strategy at every growth cycle as well as for testing the effect of reproductive strategy on final relative fitness (growth cycle 12) of the prey populations.

Clones: fitness

Clones were isolated by subsampling each population at the end of every second growth cycle and streaking them out onto an agar plates. All plates were then placed in dim light for long-term storage. To confirm population-level results and the existence of differently evolved genotypes, we conducted growth rate and defence assays testing evolved clones from each treatment in common-garden conditions that matched the conditions populations evolved under during the selection experiment. To do this, we picked 10 random clones from each of the 12 replicate populations for each treatment from growth cycle 10 ($n = 480$). We had to test clones from growth cycle 10 instead of 12 because a few of the stored agar plates had become contaminated with fungus and were therefore unusable. Clones were placed in 10 mL media containing a nitrogen concentration of 800 μM and grew in direct light on a linear shaker for 3 days; thus, the observed differences in the subsequent fitness assays are predicted to be genetic. Growth rate and defence assays were conducted in 24-well plates, with three replicate measurements per clone. We measured the fitness of each clone as the growth rate over 4 days in their evolved environment (based on optical density measurements as previously described). Each day, rotifers were added to or subtracted from each well to attain exactly five rotifers and ensure constant predation. To estimate clone defence, we added five rotifers to each well and then counted the final number of rotifers after a 3-day period where plates sat stationary in indirect light. The less rotifers present at the end of the assay indicates increased prey defence and vice versa. Phenotypes that grow in colonies or lose motility ('defended') increase handling time of the predator and lead to reduced predator growth rates (Becks *et al.*, 2010) (H. Koch, S. Wagner, L. Becks. under review). Alternatively, motile single-celled phenotypes (wild type) are considered undefended and more competitive (grow faster) because they do not suffer a trade-off which can occur with evolved defensiveness (Yoshida *et al.*, 2004; Becks *et al.*, 2010). We obtained asexual rotifers from the same monoclonal laboratory stock as previously described. To plot the defence data in a meaningful way, we took the reciprocal of each value so that defence increased with the *x*-axis but added 1 to each value before doing so to prevent division by 0. We used LM to test the effects of reproductive strategy, predation treatment and interaction on mean growth rate and mean defence of clones and performed a *post hoc* Tukey multiple comparisons of means test with a 95% family-wise confidence level independently for both traits.

Spontaneous sex

Spontaneous zygote aggregations were observed in half of the replicates of the obligate sexual predation treatment throughout growth cycle 6, which was recorded. We then tracked the occurrence of spontaneous zygote aggregations in the remaining populations, recording the percentage (%) of replicate populations with spontaneous sex over time. During growth cycle 9 (the first growth cycle that every replicate obligate sexual population had spontaneous sex), we took random samples of spontaneous zygotes ('sexual') and vegetative ('asexual') cells from each replicate population of the obligate sexual predation treatment ($n = 12$). Zygotes were obtained from surface pellicles or bottom aggregations, whereas asexual cells with the wild type phenotype (i.e. single, motile cells) were obtained from within the water column. We streaked out asexual samples onto agar plates and placed them in dim light to reduce further rounds of asexual reproduction, whereas the sexual samples were allowed to complete the sexual cycle. We plated the sexual samples and carried out the sexual cycle as previously described, including the chloroform step. After germination, we streaked out the sexual samples onto fresh agar plates to isolate individual clones and let them grow for 5 days in bright light. Asexual samples were placed in bright light for the same duration. We also isolated ancestral clones of both mating types on agar plates at this time.

From each of the 12 replicate sexual and asexual samples, we randomly picked 10 individual clones ($n = 120$ each) and grew them up in 15-mL falcon tubes containing 4 mL media with a nitrogen concentration of 800 μM. For the ancestors, we picked 10 clones for each mating type ($n = 20$). All tubes were placed on a linear shaker in bright light for 14 days. To measure the growth rate and defence of clones, we conducted fitness assays as previously described. To test the effect of offspring type on growth rate and defence independently, we used LMM with offspring type as the fixed effect and clone replicated within population nested within treatment as the random effect. To test for differences in mean growth rate and mean defence across offspring type, we performed a *post hoc* Tukey multiple comparisons of means test with a 95% family-wise confidence level independently for both traits. To test for differences in variance in defence between treatments (asexually and sexually-produced offspring), we first assessed among-clone variation by comparing treatment-specific models (GLMM) with and without the clone term, using maximum-likelihood ratio tests. Then, we applied the same test in comparing among-population variation. The among-clone and among-population tests were performed separately for asexual and sexual treatments, as well as for growth rate and defence traits. For testing for differences in variance in growth rate between treatments, we applied a similar test as just described but used LMM. Then, to test for significant differences in genetic variation between sexual and asexual treatments and for both traits, we performed a bootstrap analysis with a 95% confidence interval on the variance statistic of each sample, resampled 5000 times. We used a Wilcoxon rank sum test with a continuity correction to test for significant differences in growth rate and defence between the top 20% fittest asexually and sexually-produced clones. Plotting of the defence data was done in the same manner as for the clones from growth cycle 10.

Results and discussion

Reduced adaptation in facultative sexual populations

Supporting our expectation of a difference in the evolutionary trajectory of facultative and obligate sexual prey populations adapting to predation, we found that the costs of reproduction were more apparent in the prey populations with facultative sexual episodes. In general, reproductive strategy, predation treatment, time (growth cycle), and each interaction had a significant effect on the relative fitness of algal populations (Fig. 1A, statistics in Table S1). Even though both of the sexual (facultative and obligate) predation treatments eventually reached a fitness level not significantly different than that of its corresponding control (LM: relative fitness at growth cycle 12; obligate sexual predation vs. no predation, $F_{1,16} = 1.15$, $P = 0.30$; facultative sexual predation vs. no predation, $F_{1,22} = 0.96$, $P = 0.34$; for comparisons at all other growth cycles, see Table S2), the facultative sexual predation treatment had a significantly lower final relative fitness compared to the obligate sexual predation treatment (LM: relative fitness at growth cycle 12; obligate vs. facultative, $F_{1,16} = 52.07$, $P < 0.0001$). Additionally, evolved clones isolated (from growth cycle 10) from the prey populations with facultative sexual episodes suffered a greater trade-off in traits under strong directional selection, i.e. competitive ability and defence (Fig. 2). Although clones from both sexual predation treatments evolved increased defence, it was at the cost of reduced growth rates (Tukey's HSD: defence: facultative no predation vs. facultative predation, P adj = 0.0008; obligate no predation vs. obligate predation, P adj < 0.0001; facultative no predation vs. obligate predation, P adj < 0.0001; obligate no predation vs. facultative predation, P adj = 0.0005; growth rate: facultative no predation vs. facultative predation, P adj = 0.00; obligate no

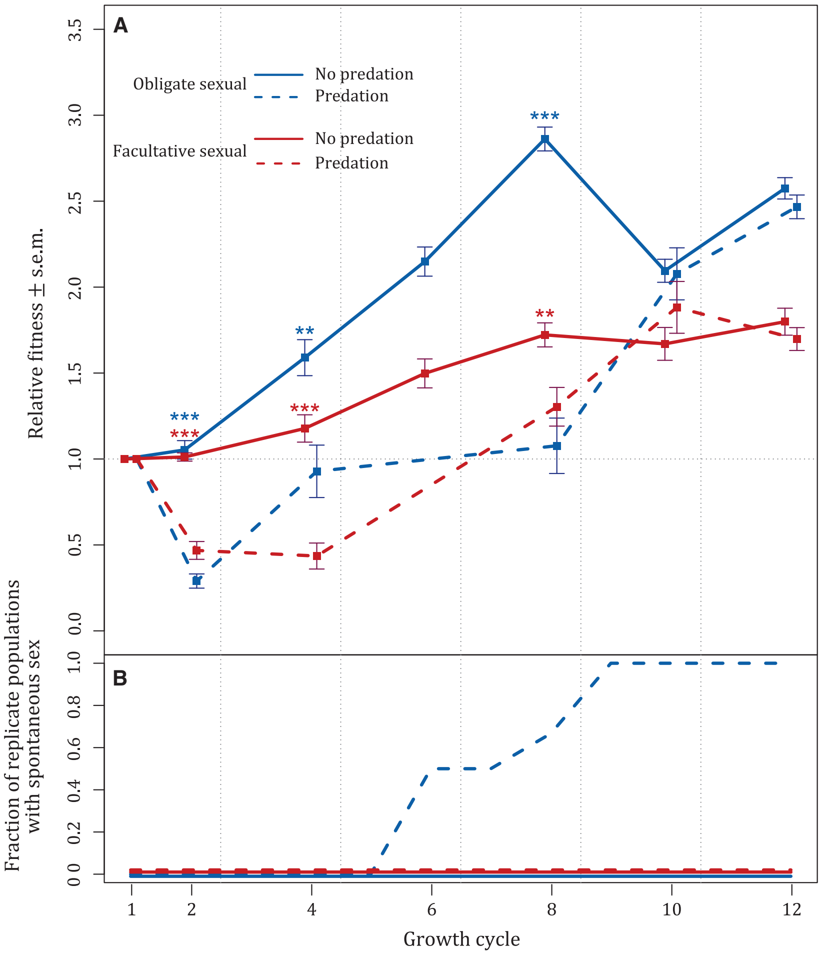

Fig. 1 Relative fitness and occurrence of spontaneous sex over time of experimental populations. (A) The relative fitness (*y*-axis) of 12 replicate populations (mean ± SEM, population growth rates normalized to ancestral starting point at growth cycle 1 for that line) over 12 growth cycles (*x*-axis) with sexual episodes (dashed vertical lines) interspersed at regular intervals. Obligate sexual populations (blue) with (dashed line) and without predation (solid line). Facultative sexual populations (red) with (dashed line) and without predation (solid line). Asterisks (**$P < 0.01$; ***$P < 0.0001$) indicate a significant difference in relative fitness between predation and no predation treatments within each reproductive strategy (coloured accordingly) at indicated growth cycle (statistics in Table S2). (B) Fraction of replicate populations with occurrence of spontaneous sex over time. Spontaneous sex only occurred in the obligate sexual predation treatment (blue, dashed line) and went to 100% by the end of the experiment, whereas in all other experimental populations the occurrence was '0'.

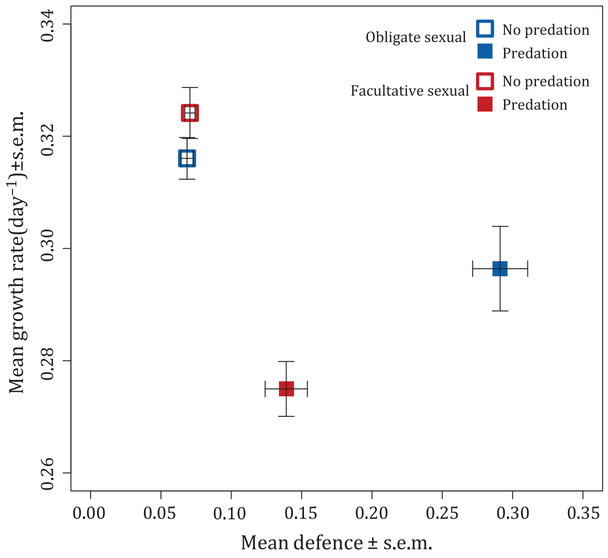

Fig. 2 Fitness of evolved clones varies between reproductive strategy and predation treatment. Defence (*x*-axis, mean ± SEM) and growth rate (*y*-axis, mean ± SEM) of a subset of evolved clones ($n = 10$) from each replicate population ($n = 12$) of each treatment ($n = 4$) from growth cycle 10. Clones from prey populations with obligate sexual episodes (blue) and either with (solid square) or without predation (open square). Clones from populations with facultative sexual episodes (red) and either with (solid square) or without predation (open square). The mean growth rate and mean defence of evolved clones depended on reproductive strategy and predation treatment (LM: defence, $F_{3,476} = 68.3$, $P < 0.0001$; growth rate, $F_{3,476} = 16.8$, $P < 0.0001$). Clones from facultative sexual predation (solid red square) and obligate sexual predation (solid blue square) treatments evolved increased defence but at the cost of reduced growth rates; however, clones from the former treatment suffered a costlier trade-off (statistics in text). For a description of how the defence data were plotted, see Methods section, Clones: Fitness.

predation vs. obligate predation, P adj = 0.048; facultative no predation vs. obligate predation, P adj = 0.0016; obligate no predation vs. facultative predation, P adj < 0.0001), and clones from the facultative sexual prey populations were significantly less defended and less competitive as compared to clones isolated from the obligate sexual prey populations (Tukey's HSD: facultative predation vs. obligate predation: defence, P adj < 0.0001; growth rate, P adj = 0.025). As expected, there was no significant difference in defence or growth rate of clones isolated from the controls (Tukey's HSD: facultative no predation vs. obligate no predation: defence, P adj = 0.99; growth rate, P adj = 0.71).

Although the effect of sex was quite pronounced in the obligate sexual predation treatment, its effect was reduced in the facultative sexual predation treatment (Figs 1A and 2) where the cost of sex, with respect to the time necessary for completing the sexual cycle, was more apparent. The last stage in the sexual cycle, zygospore germination, takes time which may result in a dilution of sexual offspring by the vegetative growth of unmated cells. Any gametes that did not successfully mate would revert back to being vegetative cells once plated on agar plates containing nitrogen (Fig. S1: sexual cycle, stage: drying period) and remained an asexual lineage (Harris, 2009). Hence, the net advantage of sexuality would be lowered in the facultative sexual populations due to the time necessary for germination and increased competition between sexual and asexual lineages. This would not have occurred in the obligate sexual populations where germinating zygospores did not have to compete with unmated cells because they were killed by chloroform exposure. A similar result was observed for facultative and obligate sexual populations of *C. reinhardtii* when adapting to increased salinity (Lachapelle & Bell, 2012). Additionally, in a study testing mutation load and rapid adaptation in outcrossing vs. self-fertilizing populations of *Caenorhabditis elegans*, natural (wild type) outcrossing rates also yielded a reduced response compared with obligate outcrossing (Morran *et al.*, 2009).

We might imagine a similar response in nature where the effect of sex is lesser or takes longer to be realized in facultatively sexual populations composed of both asexual and sexual lineages, but also where 'obligate' sexuals may occur if sexual cells are protected from predation due to their protective spore wall (Harris, 2009) and/or as part of an inedible zygospore aggregation. A study about natural yeast populations (*Saccharomyces cerevisiae*) and their predators (*Drosophila*) revealed that after differentiation into stress-resistant spores these protected cell types were able to successfully survive passage through the gut of predatory flies (Coluccio *et al.*, 2008). Even though the frequency of sex in natural populations of *C. reinhardtii* is unknown, we do know that both mating types of *C. reinhardtii* are found in nature (Harris, 2009) and there may be an adaptive ecological consequence of sex for predation (protective zygospore wall) as well as evolutionary.

It is also likely that selection interference was stronger in the facultative sexual prey populations. Although both sexual treatments (facultative and obligate) experienced asexual reproduction during the growth cycles, only sexual offspring were passaged to the next generation in the obligate sexual prey populations. Any lineages in the facultative sexual prey populations that remained asexual and were propagated would suffer greater selection interference and selection inefficiency as a result of competition between independently arising beneficial mutations and genetic drift (Hill & Robertson, 1966; Gerrish & Lenski, 1998). This could explain why facultative sexual prey populations had lower final relative fitness (Fig. 1A), and the presence of more suboptimal genotypes (Fig. 2).

Spontaneous sex in the obligate sexual predation treatment

During the evolution experiment, we observed spontaneous sex in the obligate sexual predation treatment but not the facultative sexual predation treatment or controls (Fig. 1B). On different days during growth cycle 6, large aggregations appeared in half of the replicate populations, some clinging to the surface as a pellicle and others sinking to the bottom. *Chlamydomonas reinhardtii* is known to evolve colonies in response to predation (Becks *et al.*, 2010), but the aggregations observed in this experiment were not composed of haploid, vegetative cells. Instead, they were very large zygote aggregations and the result of spontaneous sex (termed 'spontaneous' as conditions necessary to trigger the sexual cycle, i.e. nitrogen deprivation, were not induced). The difference between haploid colonies and zygote aggregations was readily distinguishable as haploid colonies do not form surface pellicles or such large irregular aggregations as we observed here; aggregations were on the order of hundreds of zygotes and similar or greater in size than a single rotifer. We took this as evidence of selection for sex because by growth cycle 9 spontaneous zygote aggregations occurred independently, and at different times, in all replicate populations. This phenotype was predicted to confer defence, but only indirectly since a zygote is the diploid sexual stage and growth only occurs during the haploid, vegetative stage.

To determine the haploid-stage phenotype and the effect of spontaneous sex on offspring fitness, we compared the fitness of offspring from the spontaneous sexuals and co-occurring wild type phenotype asexuals in each replicate population of the obligate sexual predation treatment for growth cycle 9 (Fig. 3, see Methods: Spontaneous sex). Results from this comparison revealed a significant effect of offspring type on both traits (LMM: growth rate, $\chi^2 = 9.99$, d.f. = 2, $P = 0.007$; defence, $\chi^2 = 11.96$, d.f. = 2, $P = 0.003$) and that spontaneous sexuals produced significantly superior offspring, in terms of increased growth rate (Fig. 3A) and defence (Fig. 3B), as compared to the asexuals (statistics in Fig. 3 legend). Additionally, a higher mean growth rate and defence of the spontaneous sexual offspring indicates a direct benefit of sex, arising from the production of significantly more high-fitness genotypes (Fig. 3C,D, statistics in figure legend). Despite significant among-clone and among-replicate population variation in fitness for both spontaneous sexual and asexual offspring, there was no significant difference in variance between offspring type (statistics in Table S3). Ultimately, we found that most of the offspring from the spontaneous sexuals had a nonmotile phenotype, whereas all asexual offspring maintained the wild type phenotype. The nonmotile phenotype was impaired flagella that resulted in cells sinking to the bottom of the well. Consequently, cells of this phenotype ('defended') found refuge in the bottom-occurring layer and thus escaped predation by the filter-feeding rotifers. We showed in another study that predator fitness was significantly reduced when given an equal number of algal cells with the defended phenotype vs. the wild type phenotype and the fraction of total cells remaining at the end of the test was significantly higher for the defended algae (Koch, Wagner, & Becks in review 2016). Importantly, the nonmotile phenotype also evolved within the facultative sexual predation treatment in this study and was present in half of the replicate populations by growth cycle 12, but spontaneous sex was never observed.

The cost of sex was reduced in the obligate sexual prey populations, which most likely explains the observation of selection for increased sex. Moreover, the ability to rapidly adapt to stressful conditions (e.g. predation) may be strong enough to overcome the costs of sex and lead to selection for greater levels of genetic mixing (Morran *et al.*, 2009; Becks & Agrawal, 2012). We believe that this occurred in the obligate sexual predation treatment where spontaneous sex occurred independently in all replicate populations and without the cue for sexuality induced. Population genetic theory suggests that sex or more specifically, rates of genetic mixing, can increase in a sexual population during adaptation because modifier genes that promote recombination increase in frequency by hitchhiking with alleles under positive selection; that is, through linkage they become associated with the high-fitness genotypes they created (Williams, 1975; Maynard Smith, 1978; Bell, 1982; Hartfield & Keightley, 2012). By comparing the fitness of offspring from spontaneous sexuals and asexuals from the same populations, we were able to see that the spontaneous sexuals produced significantly superior offspring in terms of growth rate and defence, which indicates the presence of these high-fitness genotypes (Fig. 3). Additionally, increased rates of genetic mixing may evolve if there is epistatic interaction between loci combined with directional selection (Otto & Barton, 2001; Hartfield & Keightley, 2012). A theoretical study by Iles *et al.* (2003) demonstrated that increased selection for recombination can occur in very large, finite populations if individuals contain more loci experiencing directional selection (Iles *et al.*, 2003). In this case, increased recombination (and sex) may have been selected for because the breakdown of selection interference between beneficial and deleterious alleles at different loci can increase the response to selection (Hill & Robertson, 1966). These ideas underline the importance of testing multiple traits under selection, as we have done here with defence and competitive ability. Alternatively, we did not observe spontaneous sex in the facultative sexual predation treatment, most likely due to the amplified costs of sexual and asexual reproduction. Had chloroform selection been

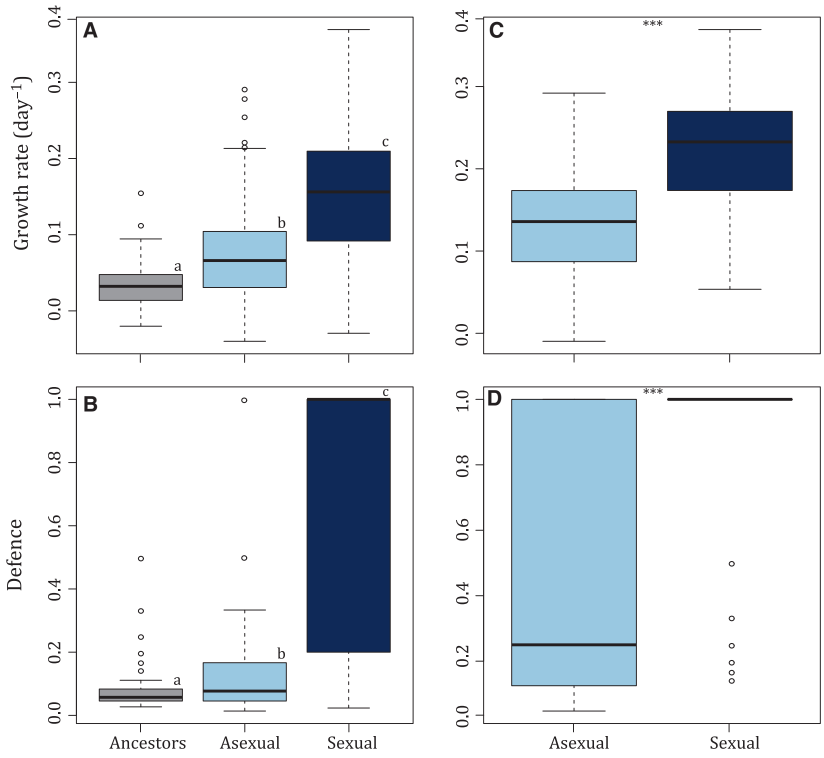

Fig. 3 Effect of spontaneous sex on offspring fitness. Comparison of growth rate (A, C) and defence (B, D) between asexually and spontaneously sexually-produced offspring ('sexual') isolated from the obligate sexual predation treatment during growth cycle 9. (A, B) Letters denote significantly different mean trait values (Tukey's HSD: growth rate: ancestors (a) < asexual (b) < sexual (c), a–b: $P < 0.0001$, a–c: $P = 0.0$, b–c: $P = 0.0$; defence: (a) ancestors < (b) asexual < (c) sexual, a–b: $P = 0.0143$, a–c: $P = 0.0$; b–c: $P = 0.0$; statistics in text). A higher mean growth rate and defence of sexual offspring indicates a direct benefit of sex, which arises from the production of significantly more (asterisks) high-fitness genotypes (C, D: top 20% fittest clones for each trait and offspring type; Wilcoxon: growth rate, W = 793, $P < 0.0001$; defence, W = 2344, $P < 0.0001$). For test of variances and bootstrap analyses, see statistics in Table S3. For a description of how the defence data were plotted, see Methods section, Spontaneous sex.

responsible for increased levels of sex in the obligate sexual predation treatment, we would have expected to see a similar result in the obligate sexual control populations, which were also exposed to chloroform, but we did not.

This may help to explain why sex increased in frequency but not necessarily how it became 'spontaneous' without the sexual cycle cue induced. We contend that spontaneous sex was an adaptive and indirect response to predation that involved a change in the trigger for sexuality and not a density-dependent effect where populations grew so quickly, depleted all available nitrogen and subsequently turned sexual. Otherwise, we would expect to also see spontaneous sex in the obligate sexual populations without predation where population growth rates were higher and population sizes larger as compared over a single growth cycle (Fig. 1). Additionally, we do not believe that spontaneous sex was a stress-induced response by rotifers as it was not observed in any facultative sexual population under predation. A previous study on sexual selection in *C. reinhardtii* showed that the developmental switch for differentiating as a zoospore or gamete could be altered by selection; under intense and long sexual selection, the signal was lost or reset and gamete fusion occurred spontaneously and early in the culture growth phase when nitrogen was replete (Bell, 2005). As a result, the gender system evolved from heterothallic to homothallic as an indirect response to sexual selection for spontaneous mating. A similar event may have occurred in our experiment but after only a few growth cycles and not 3 years as previously reported. Additionally, in a separate study using the same predator–prey system but in closed continuous cultures (chemostats), selection for sex occurred in all replicate populations of the predation treatment where nitrogen was also present (Koch, Wagner, & Becks in review 2016). Therefore, additional work is needed to determine the underlying mechanism of spontaneous sex in this study.

Potential limitations of the study should be discussed. Had we run the experiment for longer, we may have observed a reduction in the rate of spontaneous sex and in population mean fitness within the obligate sexual predation treatment. This would occur if once the populations became fully adapted to predation, sex would be too costly to maintain and thus decrease in frequency (Becks & Agrawal, 2012; Lively & Morran, 2014). If sex was no longer beneficial for adaptation but sexual episodes were still induced, recombinational load could break up co-adapted gene combinations and reduce population mean fitness (Colegrave *et al.*, 2002; Kaltz & Bell, 2002; Becks & Agrawal, 2011). Additionally, while we suggest that the net benefit of sex was greater in the obligate sexual predation treatment because the costs of sex were reduced in these populations, we cannot exclude the potential benefits of additional rounds of sexual reproduction, as a result of spontaneous sex, which may have contributed to the higher degree of adaptation in these populations. Even though the study was designed to have the same number of sexual episodes in both facultative and obligate sexual treatments, we could not predict the occurrence of spontaneous sex nor control for it once it happened. Finally, we cannot completely exclude additional selection imposed by chloroform exposure in the obligate sexual populations. There is, however, currently no evidence that chloroforming selects for survival of zygospores as these are already resistant to chloroforming. We also render an effect on the fitness measurements as unlikely as we used only vegetative cells and fitness was measured under the experimental conditions (+/− predation). We cannot, however, further disentangle the effects of sex and potential chloroform exposure to adaptation here.

Conclusion

Facultative sexual species, including algae, fungi and zooplankton, alternate between asexual and sexual reproduction, with the former lasting for extended periods of time and ensuring rapid population growth (Grishkan *et al.*, 2003; Snell *et al.*, 2006; Harris, 2009; Allen & Lynch, 2012). Therefore, at any given time a population could be composed of both asexual and sexual individuals (or vegetative and gametic cells for unicellular species), which can ultimately affect the evolutionary trajectory of that species based, in part, on the costs associated with either mode (Williams, 1975; Maynard Smith, 1978; Hartfield, 2016). Evolutionary models for sex which include interactions between beneficial mutations (Peck, 1994; Green & Noakes, 1995) and mutational load (Kondrashov, 1985) have shown facultative sex to be evolutionary favourable (Peck *et al.*, 1997; Dacks & Roger, 1999). This is, in part, because sex can generate new genotypes beneficial for rapid adaptation to novel and stressful environments (Colegrave *et al.*, 2002; Kaltz & Bell, 2002; Becks & Agrawal, 2010, 2012; Allen & Lynch, 2012; Lachapelle & Bell, 2012). Even though previous work has shown that a little bit of sex has the same advantage as obligate sex with regard to breaking apart selection interference and aiding fixation of multiple beneficial alleles (Peck, 1994; Green & Noakes, 1995; Roze & Barton, 2006), our results show that the costs of reproduction may still play a role; slowing down adaptation due to the costs of sex (time) or lack of sex (selection interference) in facultative sexual populations. This, however, needs to be explored more systematically. Finally, *C. reinhardtii* has extensively been used as a model system to investigate various evolutionary biology questions about the advantages and disadvantages of asexual and sexual reproduction, including sexual selection (Bell, 2005), clonal interference (Colegrave, 2002), the effect of sex on the mean and variance in fitness (DaSilva & Bell, 1996), deleterious mutation clearance (de Visser *et al.*, 1996), evolutionary rescue (Lachapelle & Bell, 2012), adaptation to novel environments and various abiotic selective pressures (Colegrave *et al.*, 2002; Kaltz & Bell, 2002), and the cost of sex in benign environments (Renaut *et al.*, 2006). By incorporating predator–prey interaction and biotic stress, we believe our study adds to the experimental work on *C. reinhardtii*.

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Supporting information

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

Figure S1 Experimental evolution scheme.

Table S1 Effect of predation, reproductive strategy, growth cycle, and interactions on relative fitness of algal populations.

Table S2 Effect of predation on relative fitness of algal populations per growth cycle.

Table S3 Comparison of variances in growth rate and defence between offspring type.

Data deposited at Dryad: doi: 10.5061/dryad.sn622