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Sex as a response to oxidative stress: the effect of antioxidants on sexual induction in a facultatively sexual lineage

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The evolution of sex is one of the long-standing unsolved problems in biology. Although in many lineages sex is an obligatory part of the life cycle and is associated with reproduction, in prokaryotes and many lower eukaryotes, sex is facultative, occurs in response to stress and often involves the formation of a stress-resistant dormant form. The proximate and ultimate causes of the connection between stress and sex in facultatively sexual lineages are unclear. Because most forms of stress result in the overproduction of cellular reactive oxygen species (ROS), we address the hypothesis that this connection involves ROS and possibly reflects the ancestral role of sex as an adaptive response to the damaging effects of stress-induced ROS (i.e. oxidative stress). Here, we report that two antioxidants inhibit sexual induction in a facultatively sexual species—the multicellular green alga, *Volvox carteri*. Furthermore, the nature of the sex response and the effect of an iron chelator on sexual induction are consistent with sex being a response to the DNA-damaging effects of ROS. In addition, we present preliminary data to suggest that sex, cell-cycle arrest and apoptosis are alternative responses to increased levels of oxidative stress.

Keywords: sex; stress; reactive oxygen species; DNA damage; evolution; *Volvox carteri*

1. INTRODUCTION

Despite over 30 years of research, the origins and evolutionary roles of sex remain a fundamental problem in biology. Although in many lineages sex is an obligatory part of the life cycle, in prokaryotes and lower eukaryotes, sex is facultative, occurs in response to stress and often involves the formation of a stress-resistant dormant form. The proximate (the mechanistic base) and ultimate (why sex is an adaptive response to stress) causes of the connection between stress and sex are unclear. Many forms of stress elevate the cellular level of reactive oxygen species (ROS) (e.g. the superoxide anion $O_2^{\bullet -}$, the hydroxyl radical $HO^{\bullet}$ and hydrogen peroxide H_2O_2). ROS function as 'double-edged swords' (Martin & Barrett 2002): while at low dose, they act as mediators in various stress responses, but at higher doses (i.e. oxidative stress) they cause damage, including DNA damage. Here, we address the hypothesis that the mechanistic connection between stress and sex in facultatively sexual lineages involves ROS and reflects the ancestral role of sex as an adaptive response to the damaging effects of stress-induced ROS.

Several adaptive advantages have been postulated to account for the origin of sex, and they fall into three major classes of hypotheses: 'variation', 'selfish DNA' and 'DNA-repair' (Maynard Smith 1978; Michod & Levin 1988; Michod 1995; Burt 2000). The DNA-repair hypothesis postulates that in eukaryotic lineages, sex (specifically, meiosis) has been selected for the recombinational repair of DNA damage during gametogenesis (Bernstein *et al.* 1985; Michod 1995). Interestingly, in haploid facultatively sexual lineages (such as most lower eukaryotes) meiosis is not involved in the formation of gametes, but takes place during the germination of the diploid dormant zygote. We think that, in these lineages, sex is the preferred way to create a stress-resistant spore (as opposed to an asexual haploid spore) and is adaptive in damaging environments, because of its contribution—in terms of both mechanism and timing—to the eventual repair, during germination, of stress-induced DNA damage. Specifically, the fusion of gametes creates the diploid state that allows for meiotic recombinational DNA repair at the end of dormancy (which often occurs after a long and unfavourable period of time) and the start of a new generation. Under this scenario, gametogenesis is triggered by increased levels of stress-induced ROS, in the anticipation of further stress and potential DNA damage.

To address this hypothesis, we are investigating the potential involvement of ROS in sexual induction, using a facultatively sexual species, the haploid multicellular green alga, *Volvox carteri*. In response to increased temperatures of the vernal waters in which these algae are found, the somatic cells of the asexual forms produce and release a 30 kDa glycoproteic sexual inducer (Kirk & Kirk 1986). The inducer acts on the asexual reproductive cells (gonidia) of both sexes and alters their developmental pathway such that sexual forms (i.e. egg- or sperm-bearing forms) are produced in the next generation. The fusion of gametes results in the formation of a dessication-resistant and over-wintering diploid zygospore whose germination involves meiosis and takes place when favourable environmental conditions return, usually next spring.

Here, as a first step in our investigation of the involvement of ROS in sex, we used two antioxidants (catalase, which converts H_2O_2 into water and oxygen, and copper(II)3,5-diisopropyl salicylate hydrate (CuDIPSH)—a synthetic superoxide dismutase mimetic that scavenges intracellular superoxide anions; Leuthauser *et al.* 1981) and an Fe chelator, 2,2′-dipyridyl (which removes the Fe^{2+} associated with the DNA-damaging effect of H_2O_2; Imlay *et al.* 1988; Luo *et al.* 1994). This approach has been widely employed to argue for the participation of ROS in various cellular processes (programmed cell death, the hypersensitive response in plants, ageing; Melov *et al.* 2000). In addition, we present preliminary data to suggest that sex, cell-cycle arrest and apoptosis are alternative responses to stress.

2. MATERIAL AND METHODS

Volvox carteri, female strain, was kindly provided by Dr David L. Kirk (Washington University). Synchronous cultures of asexual

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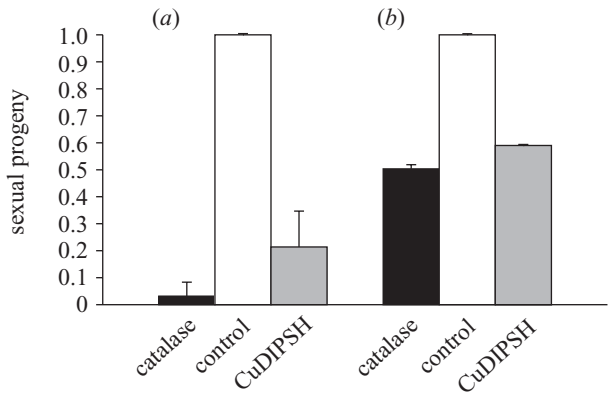

Figure 1. The inhibitory effect of the antioxidants, catalase (5 g ml^{-1}) and CuDIPSH (2 μM), on the response to (*a*) the sex-inducing heat stress and (*b*) the sexual inducer, in *Volvox carteri* female cultures. The values were normalized with the control as 1 and represent the average of up to six independent experiments with a sample size of *ca*. 500 individuals. The error bars represent the sample standard deviations using the nonbiased or '$n - 1$' method.

females were grown in standard *Volvox* medium, on a 16 L : 8 D cycle (Kirk & Kirk 1986). Cultures were subjected to a 42.5 °C-heat stress for 2 hours (Kirk & Kirk 1986). The medium in which somatic cells released the sexual inducer (i.e. the conditioned medium) was collected 18–20 hours after the heat-shock and used to induce sex in unstressed females at a density of one female per 2 ml (Kirk & Kirk 1986). The two antioxidants, catalase (Sigma; 12 800 U mg^{-1}) and CuDIPSH (Aldrich; 10 mM stock in acetone), and the Fe chelator, 2,2′-dipyridyl (Sigma; 20 mM stock in water) were added to cultures at final concentrations of 5 μg ml^{-1}, 2 μM and 20 μM, respectively.

3. RESULTS AND DISCUSSION

We heat-shocked *V. carteri* cultures of five females per ml, in the absence or presence of either catalase or CuDIPSH. Three days later, the percentage of sexual progeny relative to total progeny was calculated. If sexual induction in *V. carteri* is mediated by ROS, agents that remove ROS should diminish the sexual response. Consistent with this prediction, both antioxidants reduced (by up to 100%) the percentage of sexual progeny (figure 1*a*). As sexual development is, ultimately, triggered by the sexual inducer, we tested the effect of the antioxidants on the inducer's mode of action alone. We used conditioned media from heat-shocked cultures of five females per ml to induce sex in unstressed cultures, in the absence and presence of the antioxidants. As expected if ROS were involved in this process, the two antioxidants diminished the effectiveness of the inducer in triggering sexual development (figure 1*b*).

Interestingly, the efficiency of the antioxidants in reducing the sex response during the heat-shock experiments is higher than that observed when sexual development was induced via conditioned media (figure 1). The difference suggests that in the former case, in addition to inhibiting the inducer's mode of action (i.e. the 50% inhibition observed when sex was induced using conditioned media), the antioxidants have also diminished the production of the sexual inducer. This argues for the participation of ROS in both the production (by the somatic cells) and the activity of the inducer in triggering sexual development in gonidia.

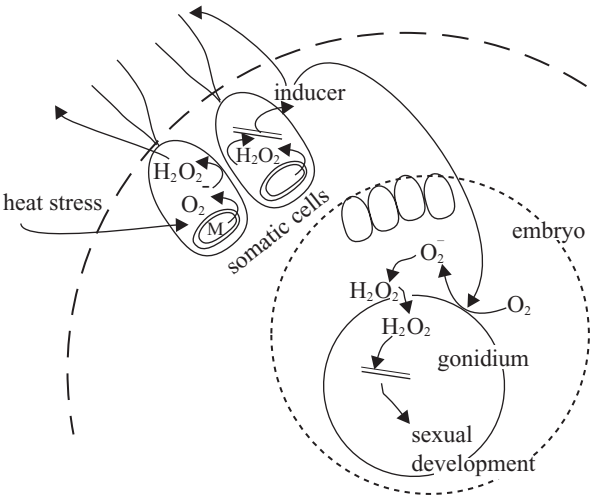

Figure 2. Model for the involvement of ROS in sexual induction in *Volvox carteri*. The heat-shock induced H_2O_2 (via mitochondria, M) triggers the expression of the sex inducer genes in somatic cells. The sexual inducer activates membrane-bound NADPH oxidases and the H_2O_2 formed via the dismutation of O_2^- triggers a switch in the developmental programme towards the sexual pathway.

In plants, abiotic stresses (including heat stress) are known to enhance ROS production by chloroplasts and mitochondria, whereas biotic stresses trigger the production of O_2^- via enhancing the enzymatic activity of plasma-membrane-bound NADPH oxidases (the H_2O_2 formed from the superoxide's dismutation can act as a diffusible signal inducing stress-response and defence genes in adjacent cells; Mittler 2002). Based on these pathways and the inhibitory effect of both the superoxide and the H_2O_2 scavengers that we used (figure 1), in figure 2 we suggest a model for the involvement of ROS in sexual induction in *V. carteri*. Consistent with this model are two additional facts (A. M. Nedelcu, O. Marcu and R. E. Michod, unpublished data). First, the sex-inducing heat-shock in *V. carteri* increases the production of H_2O_2 in somatic cells. Second, catalase inhibits the expression of the sexual inducer gene and of a gene that is triggered by the sexual inducer. (The latter belongs to a set of genes that are also induced by mechanical stress and, in higher plants, are implicated in defence mechanisms against pathogens (Amon *et al.* 1998).)

As the effect of H_2O_2 is dependent on its dose, we have investigated the sex response as a function of algal density, inducer dose and stress intensity. We have heat-stressed cultures of increasing density and noted that the sex response is strongly affected by the density of the culture, in a nonlinear fashion: the sex response is high at low algal densities, then drops as much as 60–70%, after which the response increases again with increasing density (figure 3*a*). A nonlinear relationship was also observed when

(i) conditioned media collected from heat-shocked cultures of increasing algal density and
(ii) 1 : 1 serial dilutions of conditioned media collected from a culture of 20 females per ml were used to induce sex in unstressed cultures (figure 3*b*,*c*).

Interestingly, the relationship between inducer dose and sex response (figure 3*c*) is very similar to the pattern

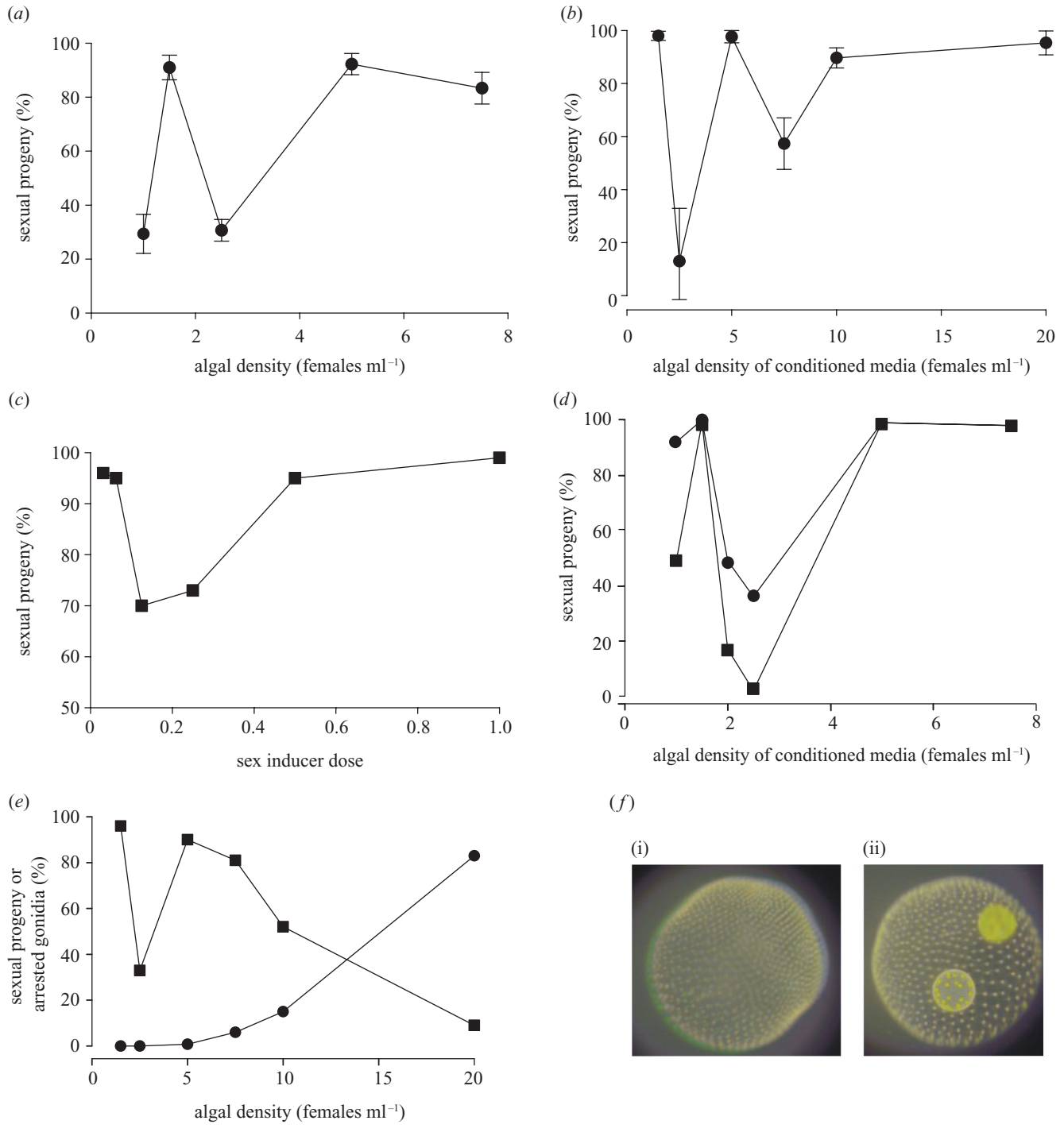

Figure 3. Sex response as a function of algal density, inducer dose and level of stress. The error bars represent sample standard deviations using the nonbiased or '$n - 1$' method; each point represents the average of three independent experiments. When only one experiment is shown, it indicates the trend observed in more than three independent experiments. (*a*) Sex response as a function of algal density; the percentage of sexual progeny was calculated relative to total progeny (*ca*. 500 individuals). (*b*) Sex response to conditioned media collected from heat-shocked cultures of increasing algal density; conditioned media were used to induce sex in unstressed females at a density of one female per 2 ml; the percentage of sexual progeny was calculated relative to the total progeny (*ca*. 200 individuals). (*c*) Sex response as a function of inducer dose; conditioned medium from a heat-shocked culture of 20 females per ml was 1 : 1 serially diluted and used to induce asexual females at a density of one female per 2 ml. (*d*) The effect of the Fe^{2+} chelator, 2,2′-dipyridyl, on the sex response to conditioned media from heat-shocked cultures of increasing algal density; the circles and squares denote the sex response in untreated and treated media, respectively. (*e*) Heat-induced sex response (squares) and cell-cycle arrest of gonidia (circles) as a function of algal density; the percentage of arrested gonidia was calculated as the percentage of females in which more than half of the gonidia have not developed into embryos by the second day after exposure to heat-shock. (*f*) Heat-induced (i.e. 43.5 °C for 120 min) apoptosis in gonidia; (i) complete dissolution of the gonidia; (ii) surviving gonidia develop into sexual embryos.

observed for cellular responses to increasing doses of DNA-damaging H_2O_2: the responses are high at low H_2O_2 doses, then drop off, to increase again and become roughly independent of concentration at higher H_2O_2 doses (Imlay *et al.* 1988). The damaging effect of H_2O_2 is attributed to hydroxyl radicals (and other unknown antioxidants) formed during the Fenton reaction ($Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + H_2O + HO^{\bullet}$) and to the fact that at distinct H_2O_2 doses, Fe^{2+} produces oxidants with different affinities for DNA and different DNA-damaging effects (Imlay *et al.* 1988; Luo *et al.* 1994). As Fe^{2+} chelators can only remove Fe^{2+} ions that are loosely associated with DNA (and which are responsible for the DNA-damaging effect of low H_2O_2 doses), we tested the effect of the Fe^{2+} chelator, 2,2′-dipyridyl, on the sex response to conditioned media collected from heat-stressed cultures of increasing algal densities. The chelator was effective in inhibiting sexual induction, but its effectiveness was dependent on the density of the culture from which the media was collected (figure 3*d*), in a fashion that opens up the possibility of an Fe^{2+}-mediated H_2O_2–DNA interaction in the inducer's mode of action. A direct correlation between the sexual inducer and DNA damage in *V. carteri* would not be without precedent: at high concentrations, the yeast sex inducer increases the production of intracellular ROS in its target cells, inducing DNA damage and apoptosis (Severin & Hyman 2002).

It is noteworthy that the unusual H_2O_2 dose-dependent response discussed here has been shown to be associated with preferential DNA nicking; at low doses of H_2O_2, purine–thymine–guanine–purine (RTGR) sites are preferentially nicked, whereas at higher concentrations, purine–guanine–guanine–guanine (RGGG) sites are preferred (Rai *et al.* 2001). Furthermore, RTGR sequences are known to be parts of required motifs in regulatory elements of genes involved in responses to oxidative stress. Thus, it is tempting to speculate that in *V. carteri*, sex might be induced by DNA nicking of sequences present in regulatory regions of genes associated with sexual induction.

Various cellular processes are known to be responses to increasing levels of ROS (and often DNA damage), including cell-cycle arrest and apoptosis. Our observation that, in *V. carteri*, sex is part of a series of cellular responses to increasing levels of stress and ROS argues further for the involvement of ROS in sexual induction. At densities of higher than five females per ml, the usual sex-inducing heat-shock triggers a transitory cell-cycle arrest in most or all gonidia; this is followed by a drastic decrease in the percentage of sexual progeny (figure 3*e*) as these arrested gonidia develop into asexual rather than sexual embryos. Furthermore, a slight increase in the heat-stress duration or intensity (e.g. 42.5 °C for 3 hours, or 2 hours at 43.5 °C) results in many or all gonidia undergoing apoptosis (as evidenced by the complete dissolution of gonidia within several hours (figure 3*f*) and by the DNA laddering effect indicative of apoptosis (A. M. Nedelcu and R. E. Michod, unpublished data)), while the surviving gonidia develop into sexual embryos (figure 3*f*). These results suggest that sex, cell-cycle arrest and apoptosis are alternative responses to increased levels of stress.

The evolution of sex remains one of the great, unsolved problems in biology. If the participation of ROS in sex proves to extend to other types of stress and other lineages (A. M. Nedelcu and R. E. Michod, work in progress), these findings will impact greatly on our understanding of the ecological and adaptive significance of sex.

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