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# Reciprocal effects of programmed cell death on fitness in unicellular endosymbiotic *Chlorella* and its ciliate host

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## Abstract

Programmed cell death (PCD), the genetically controlled active cellular suicide mechanism in multicellular organisms, also exists in unicellular organisms. However, explaining the evolution of PCD by natural selection in these organisms remains a challenge. PCD likely emerged during early endosymbiotic events as an initial antagonistic adaptation, enabling unicellular parasitic proto-endosymbionts to exploit their hosts, for example, by triggering host death in response to nutrient depletion or releasing offspring. Over time, during endosymbiont domestication and, as proposed, through horizontal gene transfer from endosymbionts to the host, PCD evolved in the host, providing benefits to both the host and the endosymbionts. However, the underlying assumption of this hypothesis, that PCD benefits and non-PCD (necrosis) harms the endosymbionts and/or the host, remains untested. Here, we investigated the fitness consequences of heat-shock-induced PCD in the endosymbiotic chlorophyte *Chlorella variabilis* and its facultative symbiotic ciliate host *Paramecium bursaria*, the non-symbiotic *C. sorokiniana*, and the predatory host *P. duboscqui*. Heat shock triggered PCD in *C. variabilis* and the two ciliate species, causing significant fitness consequences. The supernatant from *C. variabilis* PCD enhanced the growth of its own clones and endosymbiotic host while inhibiting the growth of the predatory host. The supernatants from necrotic *C. variabilis* reduced growth of both *Chlorella* and *Paramecium*. Similarly, PCD in the symbiotic *Paramecium* host benefited *Chlorella*, whereas PCD and necrosis in the predatory *Paramecium* host were detrimental. These results expand the understanding of unicellular PCD, highlighting its dual role in benefiting clonal populations and their specific endosymbiotic partners, thereby affecting endosymbiosis evolution.

**Keywords:** *Chlorella*, cell-death, endosymbiosis, host, necrosis, *Paramecium*

## Introduction

Programmed cell death (PCD), a genetically regulated active form of cell death once believed to be unique to multicellular organisms, has been documented in numerous single-celled organisms, including algae, bacteria, yeast, and protozoa (Ameisen, 2002; Bidle, 2015, 2016; Chen et al., 2024; Christensen et al., 1995; Cornillon et al., 1994; Deponte, 2008; Durand & Ramsey, 2019; Kerr et al., 1972; Krishnamurthy, 2000; Madeo et al., 1999). While PCD in multicellular organisms is essential for morphogenesis and differentiation—and its evolution often explained through kin selection, where the death of some cells improves the survival and reproduction of the clonal multicellular organism—the evolutionary origins and fitness benefits of PCD in unicellular organisms are unclear (Ameisen, 2002; Durand & Ramsey, 2019; Durand et al., 2016; Foster et al., 2006; Hamilton, 1964; Nedelcu et al., 2011). PCD in unicellular organisms, described here as an intrinsic, energy- and time-dependent process requiring de novo gene expression, differs from extrinsic, passive incidental cell death (referred to as necrosis; for definitions and forms, see Durand & Ramsey, 2019). It also shares important hallmark features with multicellular PCD. These include cell shrinkage, chromosomal condensation, genomic DNA fragmentation, phosphatidylserine (PS) externalization, reactive oxygen species (ROS) production, caspase-like protease activation, and mitochondrial membrane permeabilization (Bidle, 2016; Durand et al., 2011; Kaczanowski et al., 2011; Moharikar et al., 2006; Sathe et al., 2019). PCD in unicellular organisms can be induced in response to a variety of abiotic and biotic stresses (e.g., nutrient deprivation, oxidative stress, temperature and light fluctuations, viral infections [Bidle, 2015, 2016]) and has been shown to contribute to morphogenesis and differentiation during transient multicellularity (e.g., biofilm formation in bacteria and stalk cell differentiation in the single-celled social amoeba *Dictyostelium discoideum* [Cornillon et al., 1994; Lewis, 2000]), or serves as a mechanism for regulating population dynamics (e.g., the collapse of algal blooms [Berman-Frank et al., 2004]), and as a mediator to facilitate interactions within microbial communities (Durand & Ramsey, 2019; Durand et al., 2011; Orellana et al., 2013). While these benefits may partially explain why unicellular PCD is maintained, the ecological and evolutionary drivers underlying its origin remain largely unknown.

It has been hypothesized that PCD originated in unicellular ancestors much before the transition to multicellularity, likely emerging during the evolution of ancient endosymbiotic events in which a bacterial prey was integrated into a eukaryotic-like host via endosymbiosis (a phenomenon in which one organism resides within the cells of another), ultimately leading to the formation of extant mitochondria. This hypothesis is supported by multiple lines of evidence, includ-

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ing phylogenetic analyses and the recognition that mitochondria are central regulators of PCD processes in both animals and plants (Ameisen, 1996, 2002; Chibucos et al., 2009; Frade & Michaelidis, 1997; Kaczanowski, 2016, 2020a, 2020b; Koonin & Aravind, 2002; Kroemer, 1997). As a crucial step, primitive PCD-like cell death may have allowed proto-endosymbionts (the hypothetical ancestral unicellular organism that either lived freely or engaged in parasitic facultative endosymbiosis) to exploit their hosts more efficiently, either by triggering cell death in response to nutrient depletion to release host resources or by enabling host lysis to transfer proto-endosymbiont progeny to new hosts (Ameisen, 2002; Bruchhaus et al., 2007; Frade & Michaelidis, 1997; Klim et al., 2018). For the host, this interaction may have represented a strategy for survival in an increasingly oxygen-rich environment (Margulis, 1975). In addition to the predator–prey hypothesis for the origin of PCD, several alternatives and potentially synergistic explanations have been proposed (Ameisen, 1996, 2002; Kaczanowski, 2020a; Klim et al., 2018; Nedelcu et al., 2011; Zielenkiewicz et al., 2025). One such explanation is the "addiction" hypothesis, which suggests that PCD genes originated from bacterial toxin-antidote systems. Another is the "original sin" hypothesis, which posits that genes initially essential to early cellular life forms were later co-opted for PCD due to their pleiotropic and deleterious effects (Ameisen, 1996, 2002; La et al., 2022).

Independent from the underlying hypotheses for the origin of PCD and endosymbiosis, the primitive PCD-like cell death was likely a more advantageous form of cell death, as the alternative, uncontrolled necrosis, causes harm to the neighbouring surviving host and proto-endosymbiont cells due to the haphazard release of cellular contents (Chen et al., 2024; Durand et al., 2011; Samuilov et al., 2000). As hosts and proto-endosymbionts co-evolved, the initially antagonistic effects of PCD-like cell death likely shifted to mutually beneficial roles, where cell death provided advantages to both the endosymbionts and the host clones. This shift may have been driven by selection for greater interdependencies, partly facilitated by horizontal transfer of cell death-related genes from proto-endosymbionts to the host, endosymbiont genome reduction, and the evolution of vertical mode of symbiont transmission (Ameisen, 1996; Brockhurst et al., 2024; Fisher et al., 2017; Koonin & Aravind, 2002; Kroemer, 1997; Moran & Bennett, 2014). Thus, the evolution of PCD is thought to have played a key role during the integration of proto-endosymbionts.

A key, yet experimentally untested, hypothesis underlying this idea is that PCD provides benefits to both the host and its endosymbionts, creating a cooperative loop that enhances the stability and success of their endosymbiotic relationship (Ameisen, 1996, 2002; Frade & Michaelidis, 1997). Although potentially providing important insights into the general role of PCD in unicellular organisms, this hypothesis cannot be experimentally tested in obligate host-endosymbiont associations (e.g., endosymbiotic mitochondria within their host cells) because the host and endosymbionts have been fully integrated, they have completely lost their independence and reproduce as a single unit. Moreover, these systems have likely undergone extensive genetic changes unrelated to PCD or endosymbiosis, which could influence the effects of PCD on both partners and complicate the interpretation of experimental outcomes. To overcome these limitations and test our central hypothesis, that PCD provides mutual benefits to both the host and its endosymbionts, creating a cooperative loop that enhances the stability and success of their endosymbiotic relationship, we used organisms that form facultative endosymbiosis. Such relationships may resemble the relevant characteristics of early interactions between primitive hosts and proto-endosymbionts during the evolutionary transition to stable endosymbiosis.

We used the unicellular chlorophyte *Chlorella variabilis*, which forms a facultative endosymbiosis with the unicellular ciliate *Paramecium bursaria*, along with the non-symbiotic *C. sorokiniana* and the obligate predatory ciliate *P. duboscqui* (Figure 1A–D) as an experimental model to examine whether PCD in the endosymbiotic partners influences each other's fitness and, consequently, likely contributes to the evolution of endosymbiosis (Fujishima, 2009; Fujishima & Kodama, 2012). The *Paramecium* and *Chlorella* model system is appropriate as the outcome of their endosymbiosis varies from predator–prey-like interactions to mutually beneficial relationships where both the host and symbionts have net benefits (Fujishima, 2009; Fujishima & Kodama, 2012; Holland et al., 2002; Horas et al., 2022; Lowe et al., 2016). Both *P. bursaria* and *C. variabilis* can live independently but readily establish endosymbiosis when co-cultured (Fujishima & Kodama, 2012). In endosymbiosis, hundreds of *C. variabilis* cells inhabit the cytoplasm of the *P. bursaria* host, where they can replicate controlled by the host and algal cells. Although the ciliate host can digest its endosymbiont upon starvation or in the absence of light, the interaction is under most conditions mutualistic, with the ciliate providing nitrogen in exchange for photosynthetic sugars produced by *C. variabilis* (Albers et al., 1982; Brown & Nielsen, 1974). Our study also included an obligate predatory ciliate host, *P. duboscqui*, which rapidly digests *C. variabilis*, and a free-living (non-symbiotic) *Chlorella* species, *C. sorokiniana,* which is preyed upon by both ciliate species. Using these unicellular algae and ciliates, we first investigated their ability to undergo PCD upon heat shock. The heat shock was used because of its ecological relevance as a stressor and is also known to induce PCD in chlorophyte algae (Durand et al., 2011; Zuppini et al., 2007). We used Evans blue and Sytox staining to confirm cell death, and the cell death phenotype was assessed by commonly used PCD markers, including caspase-like proteases activity, PS externalization, ultrastructural changes using transmission electron microscopy (TEM), ROS accumulation, and the genomic DNA fragmentation (Durand et al., 2011; Moharikar et al., 2006; Sathe et al., 2019; Zuppini et al., 2007). To mimic necrosis, we subjected these organisms to sonication (Durand et al., 2011).

We evaluated the fitness effects of endosymbiont PCD on their clones and host cells, as well as the fitness effects of host PCD on their clones and endosymbiont cells. Building on previous findings that PCD is beneficial and necrosis is detrimental in multi- and unicellular organisms (Durand et al., 2011; Lambert et al., 2023), we formulated and tested the following specific predictions: (a) PCD in unicellular endosymbiotic *C. variabilis* benefits its clones and the cells of its endosymbiotic host, *P. bursaria*, but has non-beneficial (i.e., either harmful or neutral) effects on the cells of non-symbiotic *C. sorokiniana* or the predatory host, *P. duboscqui*. (b) PCD in *P. bursaria* benefits its clones and the cells of its endosymbiont, *C. variabilis*, but is not beneficial to *P. duboscqui* or *C. sorokiniana*. (c) PCD in the predatory host, *P. duboscqui*, benefits its clones but is not beneficial to *P. bursaria* and both the endosymbiotic and non-symbiotic *Chlorella* species. (d) PCD in non-symbiotic *C. sorokiniana* benefits its clones but is not beneficial to both the

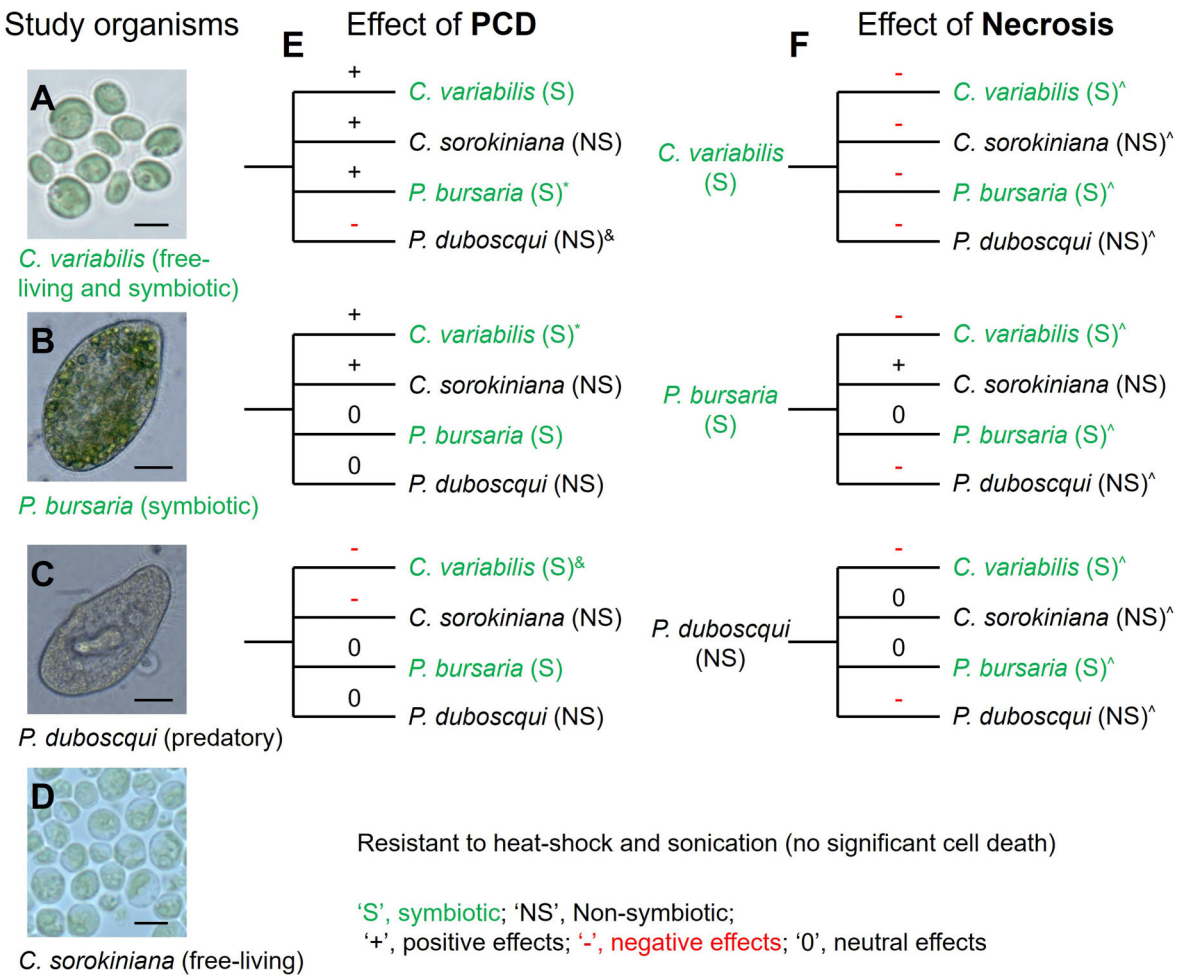


**Figure 1.** Symbiotic and non-symbiotic organisms used in the study and the impact of their PCD or necrosis on each other's fitness. (A) The *C. variabilis* strain used in this study can survive independently (free-living) but is frequently found intracellularly within its ciliate host, *P. bursaria* (B). (C) *Paramecium duboscqui* is a predatory ciliate that preys on *Chlorella* without forming an endosymbiotic relationship. (D) *Chlorella sorokiniana* does not form endosymbioses and typically exists in a free-living (non-symbiotic) state. The key findings of this study, which explore how PCD and necrosis influence the fitness of unicellular endosymbiotic partners, are summarized in (E) and (F). To assess these fitness effects, both symbiotic (S) and non-symbiotic (NS) *Chlorella*, along with their ciliate hosts, were grown in supernatants collected from *Chlorella* and *Paramecium* following either PCD or non-PCD (necrosis) induction. The effects of these supernatants were classified as positive (+) if they stimulated growth, negative (−) if they inhibited growth, and neutral (0) if their effects were comparable to those of the control supernatants. Heat shock or sonication did not induce significant cell death in the non-symbiotic *C. sorokiniana*; therefore, this species was excluded from the supernatant effects assay. *Supports our central hypothesis that PCD in the unicellular endosymbiont *C. variabilis* and its ciliate host *P. bursaria* confer mutual fitness benefits. &Supports the hypothesis that the mutual fitness benefits of PCD observed in endosymbiotic partners are absent—or even detrimental—in non-endosymbiotic partners. ^Supports the hypothesis that PCD is beneficial, whereas necrosis provides no benefits or is detrimental. The scale bar indicates 5 μm in (A, D) and 25 μm in (B, C).

ciliate species and the cells of *C. variabilis*. (e) Lastly, necrosis in all species is generally not beneficial and likely harmful to their clones and to the cells of the endosymbiotic host and/or endosymbionts.

## Materials and methods

### Study organisms and culture conditions

We used two unicellular chlorophytes (*C. variabilis* and *C. sorokiniana* UTEX 1230) and two ciliates (*P. bursaria*; Culture Collection of Algae and Protozoa, 1660/21 and *P. duboscqui*; National BioResource Project, PD000001A). The endosymbiotic *Chlorella variabilis* was isolated from the *P. bursaria* strain mentioned above (Horas et al., 2022). The *Chlorella* species are photosynthetic autotrophs. *Paramecium* species are heterotrophic as they prey on a wide range of microorganisms such as bacteria, algae, and yeast. However, some *Paramecium* species, such as *P. bursaria*, can harbour *C. variabilis* cells intracellularly as stable endosymbionts (Figure 1B). *Paramecium bursaria* feeds on *C. sorokiniana* but does not form an endosymbiosis with them (our observations). Similarly, *P. duboscqui* feeds on *C. sorokiniana* and *C. variabilis* without developing endosymbiosis. We grew *Chlorella* in a modified Bold's basal medium (BBM; Frickel et al., 2016) and *Paramecium* in diluted wheatgrass medium (2.5 gm wheatgrass powder [Azafran, Germany] boiled for 15 min in 1 L of MilliQ water, filtered and autoclaved). The cultures were incubated at 20 °C while shaken (120 RPM) under continuous white light (45 $\mu$mol photons $m^{-2}$ $s^{-1}$) and subcultured weekly with fresh growth media. The initial *Paramecium* cultures were seeded with the cultures of the bacterium *Serratia marcescens*. Since bacteria grew in the wheatgrass medium, the subsequent subcultures received bacteria from the subculture inoculum, and no fresh bacterial inoculum was required.

## Heat treatment

We harvested exponentially growing *Chlorella* cells by centrifugation (3,000 RPM, 5 min) and resuspended them in fresh BBM. We heat-shocked the *Chlorella* cells by incubating the *Chlorella* cultures for 2 hr in a water bath set at 45°C. The untreated cultures (controls) were kept at 20 °C. We chose 45 °C based on our initial observations that the *C. variabilis* cells tolerated heat exposure up to 40 °C, but the exposure to severe heat stress (50 °C and above) killed them. After the heat shock, we transferred the cultures to shakers maintained at 20 °C in a standard algal growth chamber and monitored various parameters (see below) at regular time intervals. To heat-shock the ciliates, the cultures were filtered through a 10 μm filter to remove any free-living *C. variabilis* cells (from *P. bursaria* cultures) and bacteria (from *P. duboscqui* cultures). The concentrated ciliates were then resuspended in fresh BBM, aliquoted into culture flasks, and heat-shocked at 40 °C for 60 min (*P. bursaria* cultures) and 35 °C for 30 min (*P. duboscqui* cultures). Control cultures were maintained at 20 °C. Since the temperature sensitivity of *P. bursaria* and *P. duboscqui* was previously uncharacterized, a preliminary temperature sensitivity assay was conducted to identify conditions that would induce cell death without immediate lysis. As anticipated, due to differences in their symbiotic and non-symbiotic states, *P. duboscqui* exhibited greater heat sensitivity than *P. bursaria* (see the *Results* section), and thus, a lower temperature was used for *P. duboscqui*.

## Total chlorophyll, photosynthetic efficiency, and growth

The total chlorophyll and the photosynthetic efficiency (yield) are reliable indicators of the relative health of algal cells. We measured these two parameters every 24 hr in heat-shocked and controlled *Chlorella* cells using a fluorometer (AquaFlash Handheld Active Fluorometer, Turner Designs, USA). A 3 ml culture was transferred to a transparent plastic cuvette of the fluorometer, and the above parameters were measured at room temperature. The yield ($F_V/F_M$; see details in Emerson, 1958) was calculated automatically by the fluorometer based on two measurements ($F_0$ = fluorescence of dark-adapted samples; $F_M$ = maximum fluorescence when all the photon-accepting reaction centers are open) and using the formula ($F_M - F_0/F_M$). We monitored the growth of *Chlorella* and *Paramecium* by counting cells using a haemocytometer and a light microscope. To assess whether heat shock impacted motility in ciliates, we collected ciliates from control and heat-shocked culture flasks, transferred them into a 24-well plate, and observed them under a stereomicroscope (Zeiss, Germany) to count both motile and stationary cells.

## Cell death

### Evans blue staining

We assessed cell death using Evans blue (Sigma, USA, catalog no.: E2129), a dye that can penetrate the compromised cell membrane of dead cells and, therefore, stain them blue. A 0.9 ml cell culture was mixed with 0.1 ml 1% Evans blue (w/v in distilled water), and the mixture was incubated in the dark at room temperature for 20 min. The cells were rinsed of excess dye and observed under the oil immersion lens (100×) of a light microscope (Axioskop 40, Carl Zeiss, Germany). A total of ~150 cells in each sample were counted, and the percentage of dead cells was computed. The phenotype of cell death (i.e., PCD vs. necrosis) was determined using the following assays.

### Cell membrane permeability

The cell membrane of dead cells is typically compromised and therefore permeable to several dyes. We assessed the cell membrane permeability of heat-shocked *Chlorella* cells using the fluorescent dye SYTOX™ blue (Thermo Fisher Scientific, Invitrogen, USA; catalog no.: S34857), which can penetrate the compromised cell membrane, bind to the DNA, and emit blue fluorescence upon excitation with 405 nm wavelength of the violet laser light. Although Sytox staining alone is not sufficient to confirm PCD, it can indicate the phenotype of dead cells when used in combination with other markers (see below). *Chlorella* cells were collected from the heat-shocked and control cultures every 24 hr and stained with 1μM SYTOX™ blue for 15 min in the dark, and the samples were analysed using a CytoFLEX LX (Beckman Coulter) cytometer with 405 nm excitation and a 450/45 BP emission filter.

### Transmission electron microscopy

We followed the heat-shock-induced ultrastructural changes in *Chlorella* cells using TEM. The cells from heat-shocked and control cultures were harvested and fixed with 2.5% glutaraldehyde in 0.02 M potassium phosphate buffer (pH 7) at room temperature for 1 hr. Fixed cells were washed, post-fixed with 2% osmium tetroxide (for 1 hr on ice), and dehydrated using an ethanol series (25%, 50%, 70%, 80%, 90%, 95%, and 100% for 10 min each) and acetone (20 min). Dehydrated cells were suspended in modified Spurr's resin series (25%, 50%, and 75% resin in acetone), incubated on a specimen rotator for 20 min each, and finally in 100% resin for 30 min. The infiltrated cells were mixed with fresh Spurr (100%) and cured at 65 °C for 24 hr. The resin blocks were further allowed to harden at room temperature for 48 hr and then sectioned (50 nm thickness) using an ultramicrotome UC7 (Leica Microsystems, Wetzlar, Germany) and an ultra 45° diamond knife (Drukker, Netherlands). The sections were collected on Pioloform-coated copper grids, stained with UAZero (Agar Scientific Ltd., Stansted, Essex, UK) and lead citrate, and examined with a Zeiss EM912 Omega Transmission Electron Microscope (Carl Zeiss, Oberkochen, Germany).

### Caspase-like protease activity

We used a commercially available CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit (Thermo Fisher Scientific, Invitrogen, USA; catalog no.: C10427) to determine if caspase-3- and caspase-7-like proteases activity is observed in heat-shocked *Chlorella* and *Paramecium* cells. The kit contains a short peptide with the sequence DEVD (aspartic acid–glutamic acid–valine–aspartic acid) conjugated to a fluorogenic dye. The peptide can be hydrolyzed by caspases 3/7-like proteases (DEVDase) activated specifically in PCD positive cells. The assay was performed according to the manufacturer's guidelines. Briefly, the heat-shocked and control cells were harvested, washed to remove the growth medium, resuspended in PBS at $5 \times 10^5$ cells $ml^{-1}$, and loaded with 500 nM CellEvent™ caspase-3/7 dye. The cells were incubated in the dark at room temperature for 1 hr and analysed using a CytoFLEX LX (Beckman Coulter) cytometer with 488 nm excitation and a 530/45 BP emission filter.

### Reactive oxygen species

We used 2′,7′-dichlorodihydrofluorescein diacetate (H2DC FDA; Thermo Fisher Scientific, Invitrogen, USA; catalog no.: D399) to detect intracellular accumulation of ROS. Control and heat-shocked *Chlorella* and *Paramecium* cells were collected after 24 hr of treatment and stained with 10 μM H2DCFDA for 10 min at room temperature in the dark. Upon oxidation by ROS and removal of acetate group by intracellular esterases, the nonfluorescent H2DCFDA is converted into a brightly fluorescent 2′,7′-dichlorofluorescein (DCF). The intracellularly trapped DCF made cells highly fluorescent, which were visualized using a fluorescence microscope (Axioskop 40, Carl Zeiss, Germany; Ex/Em: ∼490/520 nm).

### Alkaline comet assay

The single-cell alkaline comet assay is widely used to detect the single and double-stranded nucleases-mediated DNA breaks characteristic of cells undergoing PCD in algae and is a more sensitive method than the routinely used population-level DNA agarose gel electrophoresis (Sathe et al., 2019; Yordanova et al., 2013). An electric field is applied to single cells embedded in a low-melting point agarose gel so that the nicked genomic DNA in PCD-positive cells can migrate out of the nucleus and form a typical comet tail. The DNA in healthy cells is intact and remains confined to the cell interior. We used an alkaline comet assay kit (Trevigen; catalog no.: 4250-050-K) and performed the assay using previously optimized protocol (Sathe et al., 2019). *Chlorella* cells from heat-shocked and control cultures were collected, adjusted to a cell density of $1 \times 10^5$ cells $ml^{-1}$, and mixed in a 1:10 ratio with low melting point agarose, which was boiled and kept molten at 37 °C. A 50 μl of this cell suspension in molten agarose was spread on unique cavity slides (Trevigen) and allowed to form a thin agarose layer by incubating the slides at 4 °C in the dark for 30 min. The slides were immersed in a cold lysis buffer (Trevigen) for 18 hr at 4 °C and then incubated in an alkaline DNA unwinding solution (Trevigen) at 4 °C for 1 hr. The slides were placed in a long gel tray (30 cm) containing cold alkaline electrophoresis buffer and electrophoresed at 30 volts for 25 min. The slides were gently rinsed with distilled water, followed by 70% ethanol, and dried at 37 °C for 10 min. The electrophoresed cells were stained with SYBR green I (30 min at room temperature in the dark) and observed using a 100x oil immersion lens of a fluorescence microscope (Axioskop 40, Carl Zeiss, Germany).

### Cell surface exposure of PS

PS is a component of the eukaryotic cell membrane that is present on the inner (cytoplasmic) side of healthy cells. However, in cells undergoing PCD, the PS is externalized and displayed on the cell surface. We tested whether the PS was exposed on the cell surface in heat-shocked *Chlorella* and *Paramecium* cells using a commercially available PS detection kit (Thermo Fisher Scientific, Invitrogen, USA; catalog no.: 88-8005-74). The kit contains the PS-binding protein Annexin V conjugated to the fluorescent molecule FITC. *Chlorella* and *Paramecium* cells were collected after 24 hr of heat shock, washed with phosphate-buffered saline, and stained with Annexin V–FITC according to the manufacturer's protocol. The cells were incubated in the dark at room temperature for 10 min, and whether the cells were stained (i.e., PS positive) was analysed microscopically using a 100× oil immersion lens of a fluorescence microscope (Axioskop 40, Carl Zeiss, Germany).

## Necrosis

Sonication induces necrotic cell death in the green alga *Chlamydomonas reinhardtii* (Durand et al., 2011). *Chlorella* and ciliate cultures were prepared as described above, and cells were sonicated using a metal probe attached to an ultrasonic homogenizer (UW 70, Bandelin Sonoplus, Germany). The probe was inserted directly into the cultures, and the sonication was performed at maximum intensity for 2 min to ensure necrotic cell death. The sonicated cells were incubated at 20 °C incubator, and the effect of sonication was monitored after 24 hr.

## Supernatant assays

We generated cell-free supernatants from the heat-shocked (PCD), sonicated (necrosis), and control *C. variabilis* cultures and checked whether these supernatants affected the reproductive fitness (RF; increase or decrease in the cell number) of *Chlorella* and their *Paramecium* host (Figure 6A; Figure S8). The *Chlorella* cultures were heat-shocked or sonicated (as mentioned above) and transferred to 20 °C shakers; the untreated control cultures were maintained at 20 °C. We allowed the cells to undergo PCD/necrosis for 48 hr, after which the cultures were centrifuged at 5,000 RPM for 3 min. The supernatants were then filtered through 0.2 μm filters. In an independent experiment, the supernatants were collected immediately after the heat shock (insufficient time for cells to undergo PCD). To study the effect of supernatants on the RF of *Chlorella*, we first prepared a 1:1 mixture of supernatants and sterile BBM and inoculated freshly harvested *Chlorella* cells in them. The *Chlorella* cells were allowed to grow in this mixture for 48 hr and then counted using a hemocytometer. Similarly, the effect of *Chlorella* supernatants on the RF of *Paramecium* was assessed by inoculating freshly harvested *Paramecium* cells in a 1:1 mixture of supernatants and a sterile BBM medium. The cultures were incubated at 20 °C for 5 days, and the ciliate numbers were recorded. All the assays were performed in six-well plates with 10 ml final volumes and under shaken conditions. We used a full factorial design (Figure 6A; Figure S8) such that the heat-shocked (PCD), sonicated (necrosis) and control supernatants collected from *C. variabilis* were applied to *C. variabilis* (effect on self-clones), *C. sorokiniana* (effect of *C. variabilis* supernatants on the cells of other *Chlorella* species), *P. bursaria* (effect on the symbiotic host), and *P. duboscqui* (effect on predatory *Paramecium* species). The cell numbers of the test species were compared in the heat-shocked (PCD), sonicated (necrosis), and control supernatants, and the effects of the PCD supernatants were evaluated by calculating the relative RF. The RF was calculated using the formula $RF = \ln(Hs/Cc)$, where Hs and Cc represent the total cell density of the test species in cultures supplemented with either heat-shocked (Hs) or control (Cc) supernatants. We used the same formula ($RF = \ln(Ss/Cc)$) to check the effect of necrosis culture supernatants; here, Ss represents the cell density of the test species in cultures supplemented with sonicated culture supernatants. The $RF = 0$ indicates that the control and heat-shocked supernatants had no differential impact on RF (the cells of the test species grew equally well in the two types of supernatants). However, $RF > 0$ indicates that the heat-shocked supernatants stimulated (benefited) the growth of the test species, whereas $RF < 0$ indicates that the heat-shocked supernatants had an inhibitory effect. We used a one-sample *t*-test to check if the relative RF was significantly

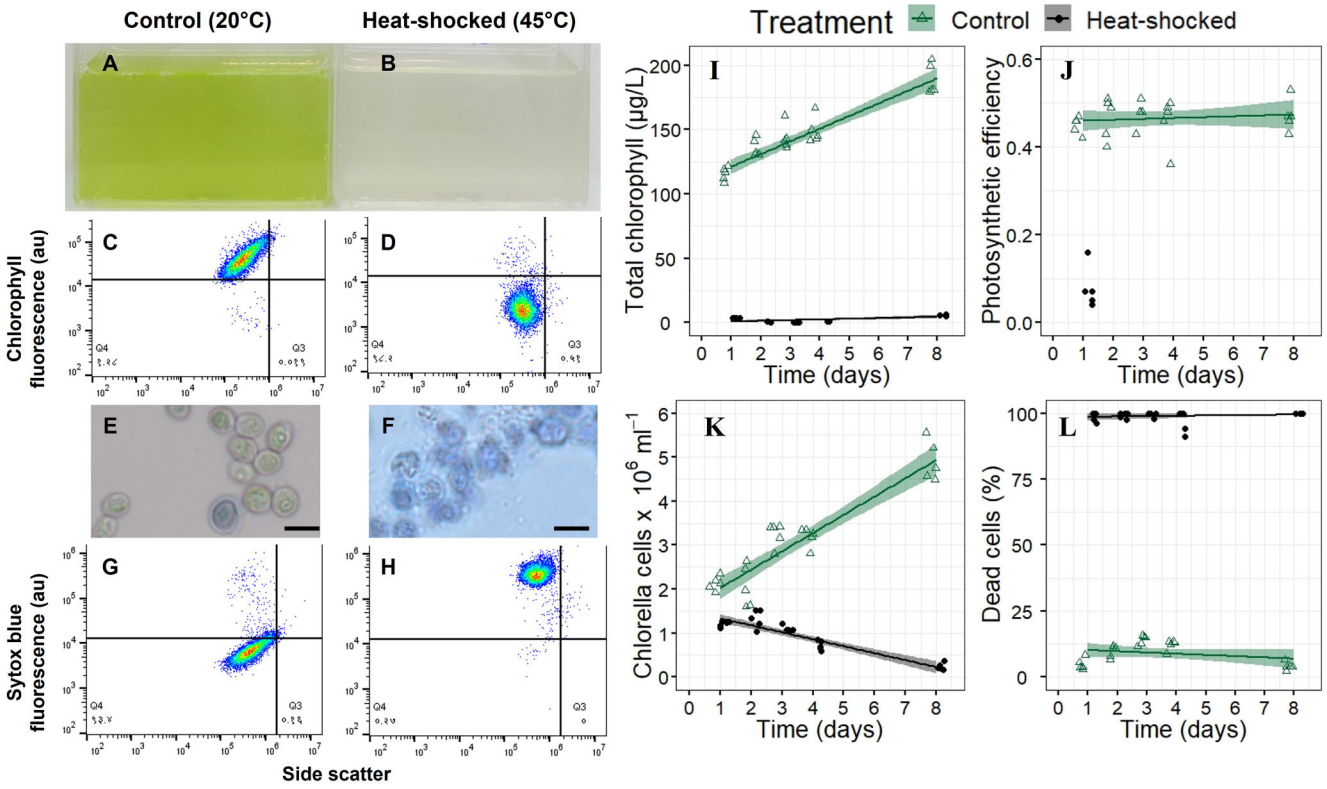


**Figure 2.** Heat shock is detrimental to symbiotic *C. variabilis*. Compared to the control (A, C, I), the heat-shocked *C. variabilis* cultures (B, D, I) had significantly lower chlorophyll (A, B: visual appearance of culture flasks; C, D: chlorophyll autofluorescence; I: total chlorophyll). The heat-shocked cells were photosynthetically inactive (J), the cell number in the heat-shocked cultures decreased (K), and most of the cells in these cultures were dead (L). Evans blue (E, F; dead cells are blue) and Sytox blue (G, H; dead cells have relatively higher Sytox fluorescence) were used to differentiate and quantify dead cells (see the *Materials and methods* section). Measurements of photosynthetic efficiency in heat-shocked cells (J) were stopped after 24 hr because the dead cells (with disintegrated chlorophyll and proteins) are not suitable for photosynthetic efficiency measurements with a laser-based fluorometer. Scale bars are 5 μm.

different than 0. We used a similar experimental setup to evaluate how PCD in the endosymbiotic host *P. bursaria* influenced the RF of its own clones, the ciliate species *P. duboscqui*, and, importantly, the endosymbiotic algae *C. variabilis* as well as the non-symbiotic *C. sorokiniana* (Figure S8A). Likewise, we assessed the impact of PCD and necrosis supernatants from *P. duboscqui* on these organisms (Figure S8B).

### Statistical analysis

We used the linear model to assess the overall effect of heat shock on growth (cell counts), total chlorophyll, and photosynthetic efficiency. The effect of the supernatant was assessed by one-way ANOVA. In cases where the analysis involved multiple comparisons, the *p*-values were corrected by performing post hoc tests using Tukey's honest significance difference method. Statistical analysis was performed using R 4.3.2 (http://www.r-project.org). All the experiments were repeated at least five times.

## Results

### The symbiotic *C. variabilis* is more sensitive to heat shock than the non-symbiotic *C. sorokiniana*

We first determined whether *Chlorella* cells undergo PCD by subjecting them to heat shock at 45 °C for 120 min. While most of the heat-shocked symbiotic *C. variabilis* cells died quickly (Figure 2), the non-symbiotic *C. sorokiniana* cells were largely unaffected by the heat shock (Figure S1). Compared to untreated controls (20 °C), the heat-shocked *C. variabilis* cultures had significantly reduced chlorophyll concentrations (Figures 2A–D, I; Analysis of Variance (ANOVA): $F_{3,92} = 73.16$; $p < .0001$) and photosynthetic efficiency (Figure 2J; ANOVA: $F_{3,92} = 20.94$; $p < .0001$). Furthermore, in the cultures of heat-shocked *C. variabilis*, the population size decreased continuously over 8 days, and the final population size was significantly lower compared to the control cultures of both *C. variabilis* and *C. sorokiniana* (Figures 2K, S1K; ANOVA: $F_{3,93} = 34.86$; $p < .0001$). Almost all the cells within the heat-shocked *C. variabilis* cultures were dead (Figures 2F, H, L). The control *C. variabilis* populations grew and had significantly fewer dead cells (Figures 2E, G, L; ANOVA: $F_{3,96} = 907.6$; $p < .0001$).

### The symbiotic *P. bursaria* host tolerates heat stress better than the predatory *P. duboscqui*

The *Paramecium* host cells that we routinely cultivate at 20 °C were instantly lysed upon exposure to 45 °C for 120 min (data not shown). Subsequently, we systematically decreased both the temperature and exposure duration (temperature ranging between 45 and 30 °C and duration from 120 to 30 min) to identify the temperature threshold at which ciliates experienced heat shock without instant cell lysis. The predatory ciliate species *P. duboscqui* underwent lysis at temperatures exceeding 35 °C, while the symbiotic ciliate species *P. bursaria*

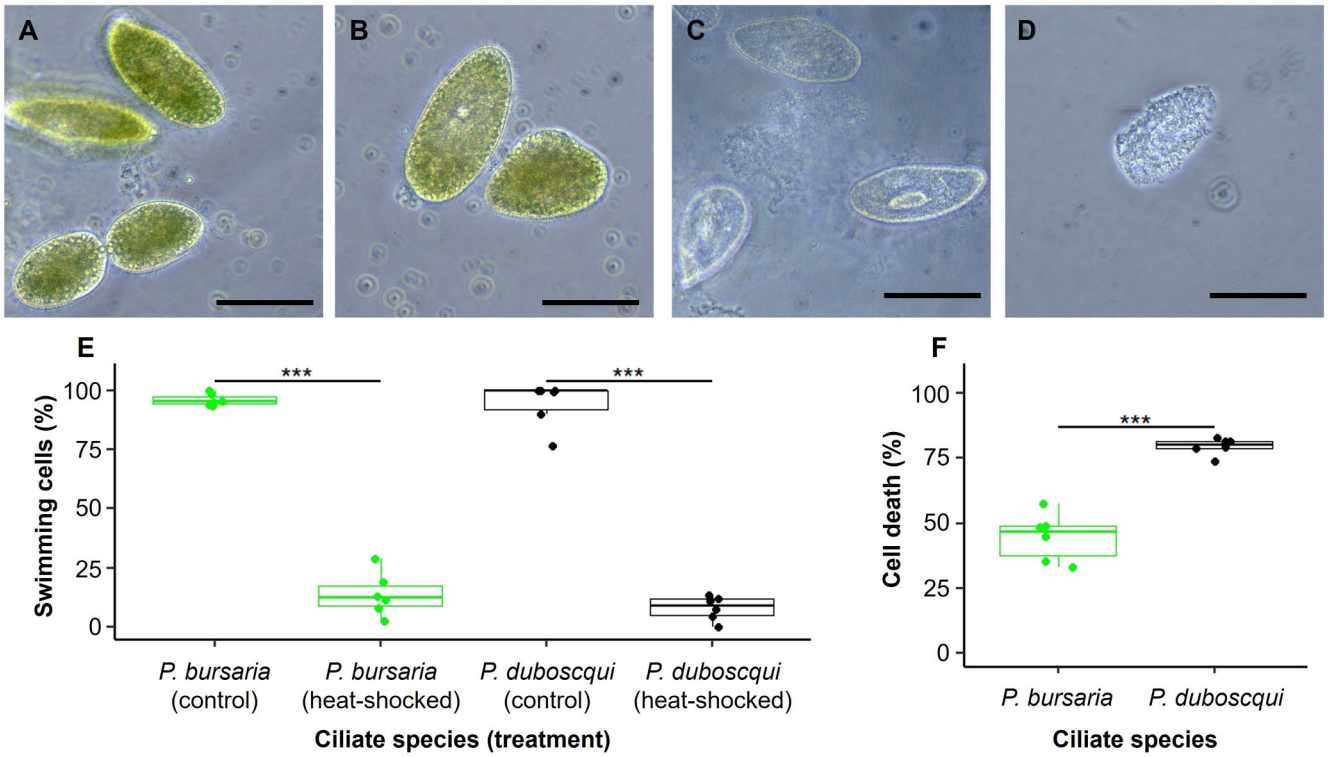


**Figure 3.** Heat shock is detrimental to both the symbiotic and predatory ciliates. Symbiotic *P. bursaria* cells (A) subjected to heat shock (B) exhibited slight rounding, cessation of swimming (E), and eventually, approximately 50% cell mortality (F). The impact of heat shock was more severe on predatory *P. duboscqui* (C), resulting in the disintegration of cell membranes (D), cessation of swimming in most cells (E), and eventual cell death (F). The scale bar represents 100 μm.

exhibited cell lysis only above 40 °C. Consequently, all subsequent heat shock experiments were conducted at 35 °C for 30 min for *P. duboscqui* and 40 °C for 60 min for *P. bursaria*. Following the heat-shock treatment, we immediately conducted microscopic observations of both heat-shocked and control ciliate populations and found notable differences between the two groups (Figure 3A–D). In untreated *P. bursaria* cells, 95.9% ± 2.6% cells exhibited vigorous swimming behaviour (mean ± *SD*; $n = 6$; total ciliates counted = 497 ciliates), indicating robust health (Figure 3E). Conversely, among heat-shocked *P. bursaria* cells, only 12.8% ± 9.6% cells displayed vigorous swimming (mean ± *SD*; $n = 6$; 527 ciliates), with the remaining 87.2% cells remaining stationary and potentially dead (Figure 3E). The impact of heat shock was even more pronounced in predatory *P. duboscqui* as the swimming cells decreased drastically from 94.2% ± 9.6% (mean ± *SD*; $n = 6$; 688 ciliates) in untreated cultures to a mere 8.1% ± 5.1% (mean ± *SD*; $n = 6$; 269 ciliates) in heat-shocked cultures (Figure 3E). After a 24-hr period, we assessed total cell mortality and found that 79.4% ± 3.3% of *P. duboscqui* cells succumbed to the heat shock, whereas 44.6% ± 9.2% of *P. bursaria* cells died because of the heat shock (Figure 3F; the results are mean ± *SD*; $n = 6$ in both cases). This difference suggests that symbiotic species such as *P. bursaria* exhibit greater heat resistance compared to predatory *P. duboscqui* (ANOVA: $F_{3,20} = 271.4$; $p < .05$; Figure 3).

## Heat-shock-induced PCD in symbiotic *C. variabilis*

To further characterize the death phenotype of heat-shocked *Chlorella* cells, we measured phenotypes frequently associated with PCD in microalgae (Durand et al., 2011; Moharikar et al., 2006; Sathe et al., 2019; Yordanova et al., 2013; Zuppini et al., 2007). We used TEM to look for ultrastructural changes associated with PCD positive cells such as membrane blebbing, chromatin condensation, plasma membrane detachment from the cell wall, and vacuole formation (Durand et al., 2011; Moharikar et al., 2006; Sathe et al., 2019; Yordanova et al., 2013; Zuppini et al., 2007). In contrast to *C. variabilis* cells collected from control cultures (Figure 4A, C), the membrane of heat-shocked *C. variabilis* cells had detached from the cell wall, their chromatin was condensed, and the organelles in these cells (e.g., mitochondria and chloroplasts) had lost their typical shape (Figure 4B, D; Figure S2). The dead cells had a large electron transparent region indicating substantial physical damage, vacuolization, and degradation of intracellular organelles. None of these phenotypes were observed in the heat-shocked *C. sorokiniana* cells (Figure S2).

We then investigated whether the heat-shock-induced oxidative stress by monitoring the levels of ROS and tested whether caspase-like proteases were activated in the heat shocked cells. ROS is a potent PCD inducer, and the caspase-like proteases play vital roles in both the induction and execution of PCD in plants and algae (Durand et al., 2011; Jiménez et al., 2009; Moharikar et al., 2006; Petrov et al., 2015; Sathe et al., 2019; Yordanova et al., 2013; Zuppini et al., 2007). We detected ROS using the non-fluorescent H2DCFDA, which enters cells and, upon oxidation by intracellular ROS, is converted into a brightly fluorescent DCF. Unlike *C. variabilis* control cells, most of the heat-shocked *C. variabilis* cells were brightly fluorescent, indicating ROS accumulation (Figure 4E, F). In most heat-shocked *C. sorokiniana* cells, ROS accumu-

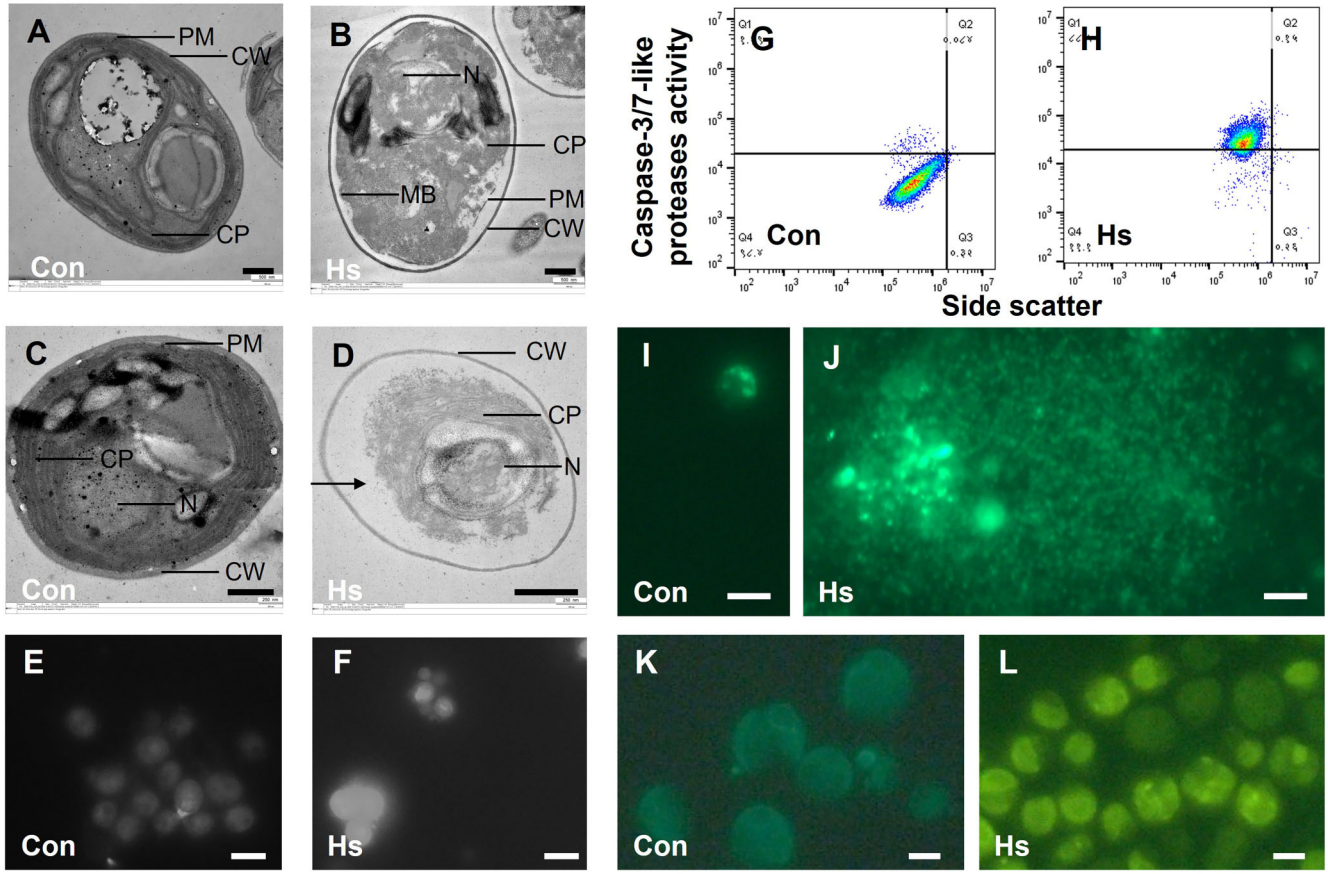


**Figure 4.** Heat-shock-induced PCD in the symbiotic *C. variabilis*. The most prominent PCD-like ultrastructural deformations observed in the TEM images of the heat shocked cells (B, D; see also Figure S2) were the detachment of the plasma membrane (PM) from the cell wall (CW), the chromatin condensation (see nucleus, N), chloroplast degradation (CP), membrane blebbing (MB), and the presence of large electron-transparent regions, indicated by the arrow in panel D (A, C: control cells (Con); B, D: cells after 24 and 96 hr of heat shock (Hs)). In contrast to the cells from control cultures (E, G), heat-shocked cells accumulated ROS (F), and caspase-like proteases were activated in these cells (H). While the DNA of control cells was intact (I), the DNA of the heat-shocked cells showed single- and double-stranded nicks (comet positive nuclei in J; see Figure S4 for additional images). In contrast to control cells (K), the phosphatidylserine was translocated from the inner to the outer membrane in heat shocked cells (see FITC-Annexin V positive cells in L). The scale bar indicates 500 nm in A–D; 5 μm in E–F and I–L.

lation was not observed (Figure S3C–F). To detect caspase-like protease activity, the heat-shocked and the control cells were incubated with a caspase substrate (DEVD peptide attached to a synthetic dye) that can be cleaved by the activated caspase-3/7-like proteases. The fluorescence emitted upon the substrate hydrolysis indicates the caspase-like protease (DEVDase) activity. Heat-shocked *C. variabilis* cells had higher fluorescence than the control *C. variabilis* cells, indicating that the caspase-3/7-like proteases were activated in these cells (Figure 4G, H). Caspase-like proteases were not activated in the heat-shocked *C. sorokiniana* cells (Figure S3A, B).

Finally, we examined two more PCD-associated phenotypes, namely, the nucleases-mediated single and/or double-stranded DNA nicks and the cell surface exposure of PS (Moharikar et al., 2006; Yordanova et al., 2013). We used a highly sensitive alkaline comet assay (Trivegn, catalog # 4250-050-K) and a previously standardized protocol (Sathe et al., 2019) to detect DNA nicks in single cells and found that the heat-shock-induced nucleases-mediated DNA fragmentation typically observed in algal cells undergoing PCD was only observed in the symbiotic *C. variabilis* cells (see comet nuclei in Figure 4J). Most of the heat-shocked *C. variabilis* cells showed severe DNA fragmentation, as the length of the comet tails (indicating the severity of the DNA fragmentation) varied insignificantly (Figure S4). The DNA fragmentation and the comet-positive nuclei were not seen in the heat-shocked non-symbiotic *C. sorokiniana* cells (not shown). Finally, we found that the heat shock resulted in cell-surface exposure of PS in symbiotic *C. variabilis* cells. In healthy cells, the lipid PS is located on the inner (cytoplasmic) side of the cell membrane. However, in cells undergoing PCD, the PS is translocated and exposed on the cell surface. We used a PS-binding reagent (FITC conjugated to the Annexin V protein) to detect the cell surface exposed PS. In the heat-shocked symbiotic *C. variabilis* cells, the PS was translocated to the outer side of the cell membrane (when these cells were stained with FITC-Annexin V, they appeared brightly fluorescent (Figure 4K, L; also Figure S5H). The cell surface-exposed PS was not detected in the untreated control *C. variabilis* and in the non-symbiotic *C. sorokiniana* cells (Figure S5).

## Heat-shock-induced PCD in both symbiotic and predatory ciliate host species

We evaluated the death phenotype of heat-shocked ciliates using the cell death markers described above. Due to the high sensitivity of the *Paramecium* host cells to heat shock and subsequent cell lysis, we were unable to monitor all of the cell

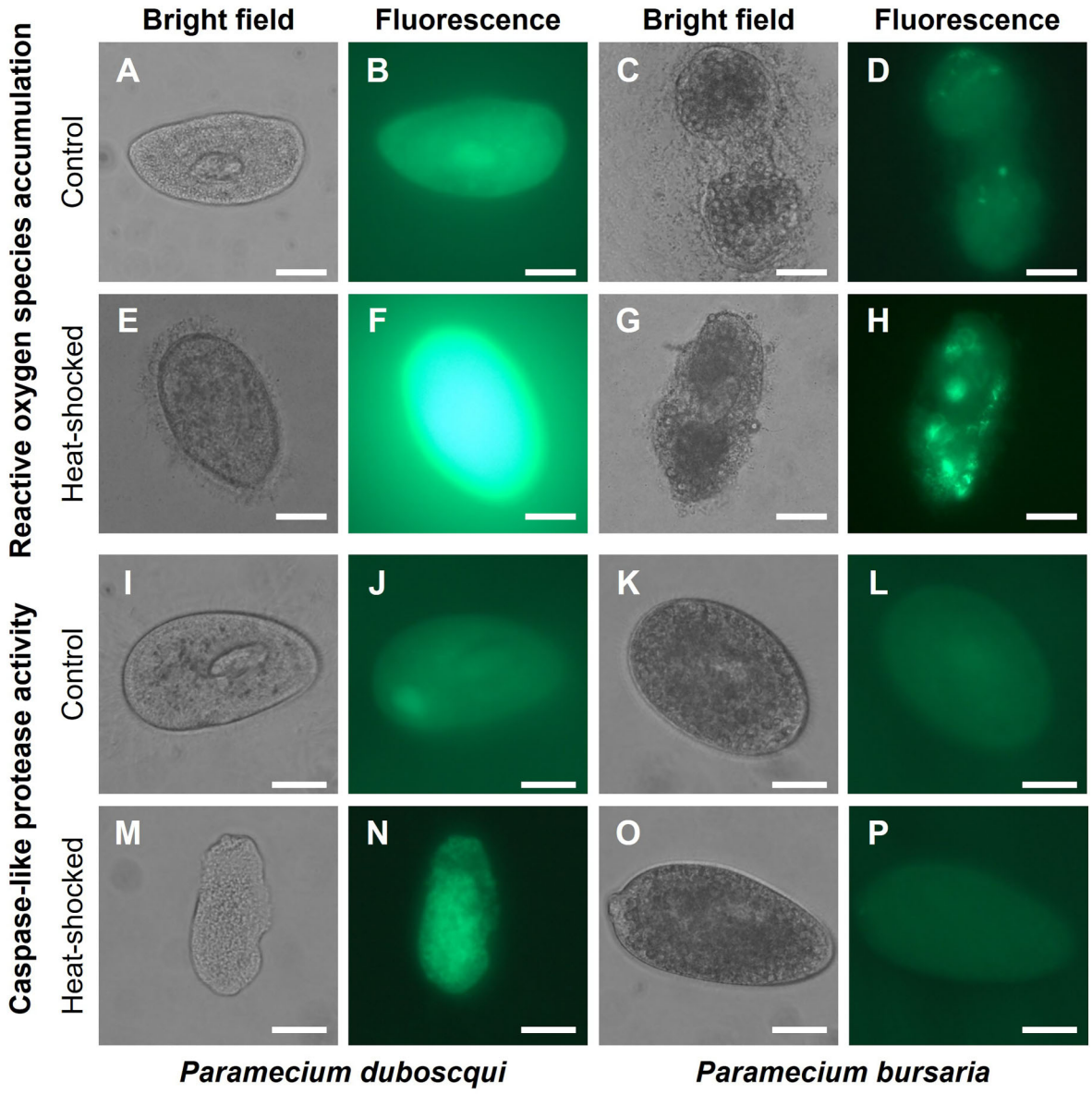


**Figure 5.** Heat shock triggered PCD in both the predatory *P. duboscqui* and the symbiotic *P. bursaria*. Accumulation of reactive oxygen species (A–H) and the caspase-like protease activity (I–P) was monitored in *P. duboscqui* and *P. bursaria* cells collected from the untreated control and the heat-shocked cultures. Compared to control (A, B), the heat-shocked *P. duboscqui* cells (E, F) had much higher ROS accumulation. The ROS accumulation in *P. bursaria* cells (control: C, D; heat-shocked: G, H) was localized. Unlike untreated *P. duboscqui* cells (I, J), the heat shocked cells (M, N) had higher caspase-like proteases specific fluorescence. The fluorescence comparison in untreated *P. bursaria* cells (K, L) and heat-shocked cells (O, P) suggests no caspase-like protease activity in heat-shocked cells. The scale bar indicates 25 μm. The original-coloured background in bright field images has been converted to black and white using Microsoft PowerPoint; the unchanged figure is shown as Figure S9.

death phenotypes that were investigated in *Chlorella* studies. We observed that both the symbiotic *P. bursaria* and the predatory *P. duboscqui* exhibited PCD-like characteristics, though with notable species-specific variations (Figure 5; Figure S6). Following heat shock, a significant proportion of *P. duboscqui* cells exhibited accumulation of ROS, while in the symbiotic *P. bursaria*, ROS accumulation was primarily localized, indicative of sequestration within intracellular symbiotic *C. variabilis* cells (Figure 5A–H). It is noteworthy that even in *P. bursaria* control populations, ROS accumulation was observed to a lesser extent, likely because of the photosynthetic activities within intracellular *C. variabilis* symbionts (Figure 5C, D). Subsequently, we assessed the presence of caspase-like proteases (DEVDase) using the fluorescent dye mentioned earlier (Figure 4G, H). Results revealed caspase-like protease activity in heat-shocked *P. duboscqui* (Figure 5M, N), contrasting with the absence of such activity in heat-shocked *P. bursaria* (Figure 5O, P). Furthermore, we investigated PS externalization using the PS-binding reagent mentioned earlier and found that PS was exposed on the cell surface in ciliates collected from heat-shocked cultures of both *P. bursaria* and *P. duboscqui* (Figure S6). From the outcomes of these three PCD markers and other heat-shock effects (Figure 3), we inferred that both predatory *P. duboscqui* and endosymbiotic *P. bursaria* exhibited PCD-like cell death, but *P. duboscqui* was significantly more impacted by heat shock.

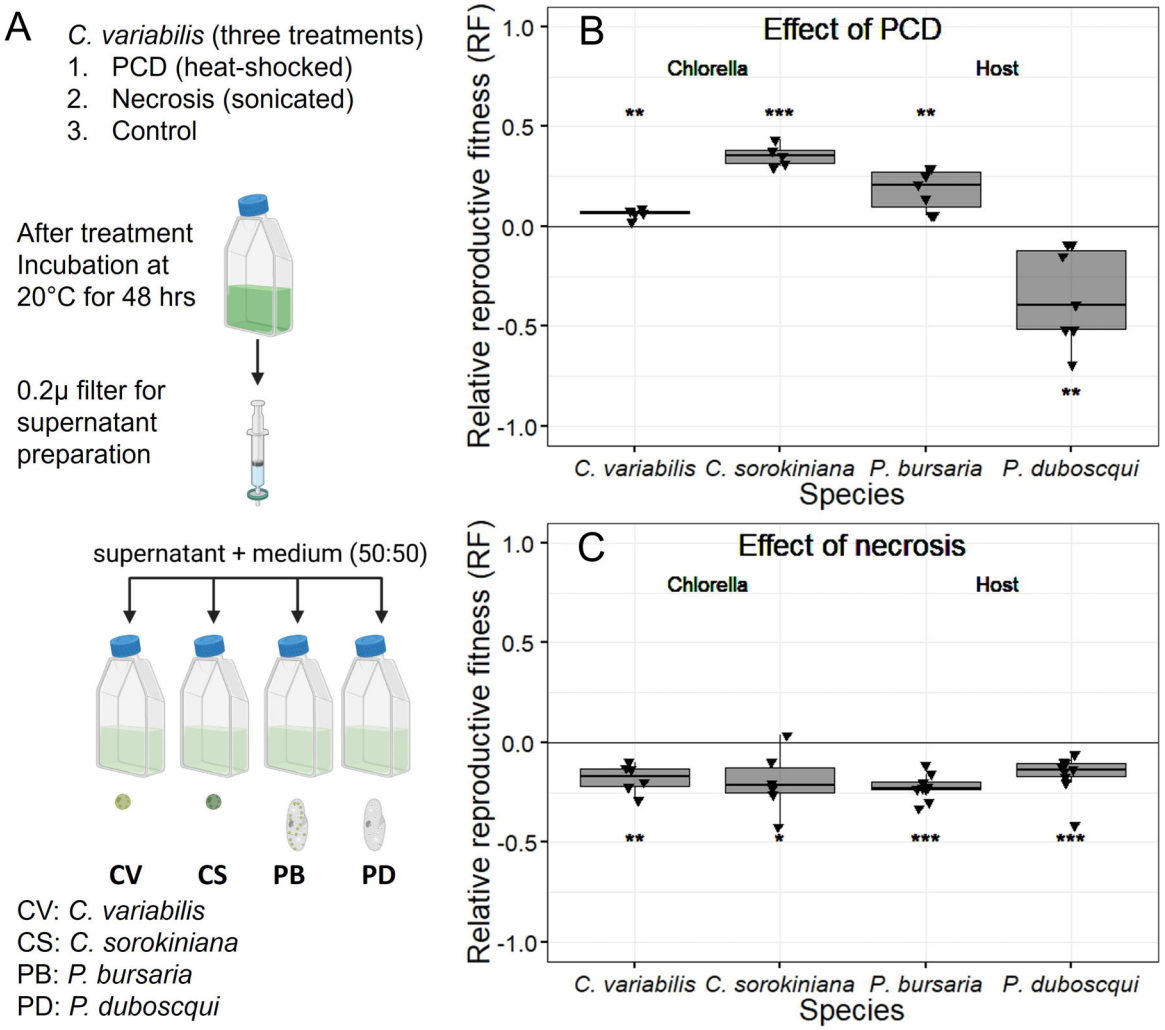


**Figure 6.** Supernatant assays indicate that the endosymbiotic *C. variabilis* affects the reproductive fitness of clones and their host via PCD. *Chlorella variabilis* cells were subjected to either heat shock (to induce PCD) or sonication (to induce necrosis), while untreated cultures served as controls and were maintained at 20 °C. Following the treatment, all three culture types were incubated at 20 °C for 48 hr. The cell-free supernatants were prepared by filtering these cultures through 0.2 μm sterile filters. The effect of PCD, necrosis, and control supernatants was tested on the cells of the symbiotic *C. variabilis*, the non-symbiotic *C. sorokiniana*, the endosymbiotic ciliate host *P. bursaria*, and the predatory host *P. duboscqui* (panel A and method details). The relative reproductive fitness (RF) of *Chlorella* and *Paramecium* species was calculated based on their growth in PCD or necrosis and control supernatants. The horizontal line (RF = 0) in the panels (B) and (C) indicates that the cells of the test strain grew equally well in control supernatants and in PCD or necrotic supernatants; the positive RF values suggest that the test strains grew better in PCD or necrotic supernatants than in control supernatants, while the negative RF values indicate that the test strains grew worse in PCD or necrotic supernatants than in control supernatants (see the *Materials and methods* section for RF calculations). The *C. variabilis* PCD supernatants (B) were beneficial (RF > 0) for *C. variabilis* (clones), *C. sorokiniana* (cells of the other *Chlorella* species), and *P. bursaria* (the endosymbiotic host). However, the PCD supernatants were detrimental (RF < 0) to the predatory host *P. duboscqui*. In contrast, the necrosis supernatants produced by sonication of the symbiotic *C. variabilis* cells were harmful to both *Chlorella* and its *Paramecium* host (C). Significance levels are denoted as follows: ∗ indicates $p < .05$; ∗∗ indicates $p < .01$; ∗∗∗ indicates $p < .001$, and "ns" indicates results that are not significant.

## PCD in endosymbiotic *C. variabilis* benefits their clones and *Paramecium* host

To gain insights into the potential role of PCD in the evolution of endosymbiosis, we quantified how PCD in each partner affects the fitness of the other. We collected cell-free supernatants from the endosymbiotic *C. variabilis* cultures subjected to three distinct treatments: those subjected to heat shock to induce PCD, those treated with sonication to rapidly induce necrosis (Durand et al., 2011), and untreated controls maintained at 20 °C (Figure 6A). We cultured *C. variabilis*, *C. sorokiniana*, the endosymbiotic host *P. bursaria*, and the predatory ciliate *P. duboscqui* in these supernatants to evaluate their influence on the fitness of each organism (Figure 6A).

We found that *C. variabilis* cells grew significantly better in their own PCD supernatants than in their control supernatants (relative fitness > 0; *t*-test, $t_4 = 5.59$, $p = .005$; Figure 6B). The *C. variabilis* PCD supernatants were also beneficial to *C. sorokiniana* (relative fitness > 0; $t_4 = 14.58$, $p = .0001$; Figure 6B). These results indicate that the PCD in *C. variabilis* is beneficial to its clones and other *Chlorella* species (Figure 1E). Following this, we investigated how PCD in the endosymbiotic *C. variabilis* influenced the fitness of the endosymbiotic and predatory ciliate. We observed that the PCD supernatants generated from *C. variabilis* were also beneficial to its endosymbiotic host *P. bursaria* (relative fitness > 0; $t_6 = 4.77$, $p = .003$; Figure 6B), but harmful to

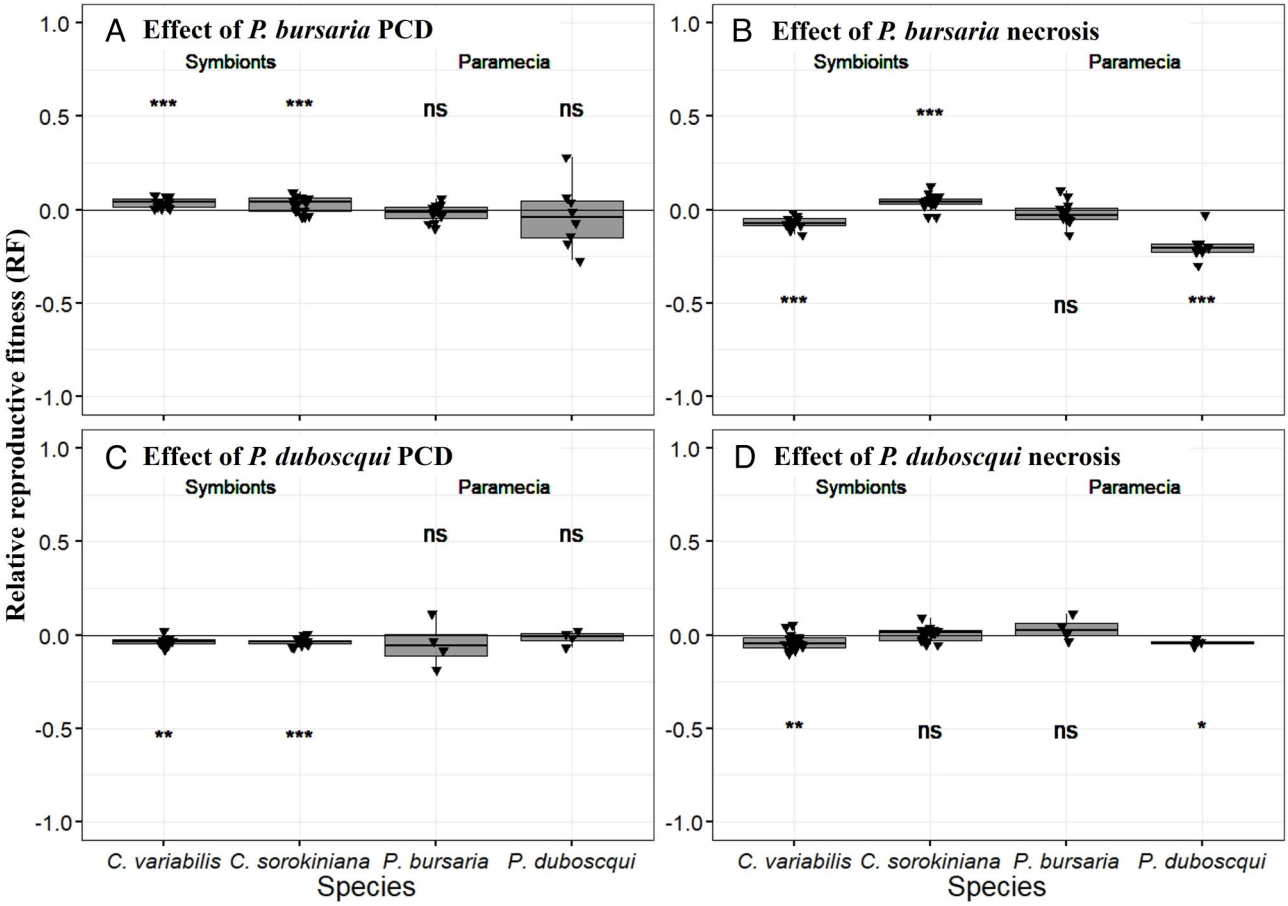


**Figure 7.** PCD in the endosymbiotic *P. bursaria*, but not in the predatory *P. duboscqui*, benefits the endosymbiotic *C. variabilis*. We used the same protocol mentioned earlier (see Figure 5, Figure S8, and the *Materials and methods* section) to prepare control, PCD, and necrosis supernatants from the ciliate species *P. bursaria* (A, B) and *P. duboscqui* (C, D). We tested the effects of these supernatants on the growth of the algal species (*C. variabilis*, *C. sorokiniana*) and ciliate species (*P. bursaria*, *P. duboscqui*), and calculated the relative reproductive fitness (RF) of each species to assess whether the supernatants had beneficial (RF > 0) or harmful (RF < 0) effects (see Figure 5 and the *Materials and methods* section for RF calculations). PCD in the endosymbiotic *P. bursaria* had positive effects on its endosymbiont *C. variabilis*, while necrosis proved harmful (A, B). In contrast, both PCD and necrosis in the predatory *P. duboscqui* were detrimental to *C. variabilis* (C, D). The heat shock resistant *C. sorokiniana* cells benefited from both PCD and necrosis in *P. bursaria*, but experienced negative effects from *P. duboscqui* PCD. Host PCD did not significantly affect their own clones or those of other ciliate species, with the exception of *P. duboscqui*, which was negatively affected by necrotic supernatants from both itself and *P. bursaria* (B, D). Significance levels are denoted as follows: ∗ indicates $p < .05$; ∗∗ indicates $p < .01$; ∗∗∗ indicates $p < .001$, and "ns" indicates results that are not significant.

its predator *P. duboscqui* (relative fitness < 0; $t_6 = -3.84$, $p = .008$; Figure 6B; Figure 1E). The PCD supernatants for these assays were collected 48 hr post-heat-shock treatment (Figure 6A), as PCD requires time for fitness-affecting molecules to be actively generated during the process (Durand et al., 2011). Consistent with this, *C. variabilis* culture supernatants collected immediately after the heat shock did not exhibit beneficial fitness effects; here, the *C. variabilis* cells grew equally well in control and heat-shocked supernatants (relative fitness = 0.006 was insignificantly different from 0; $t_2 = 1.61$, $p = .24$; Figure S7).

Next, we investigated the fitness effects of necrosis (non-PCD) in the endosymbiotic *C. variabilis* (Figure 6A). Unlike PCD supernatants, culture supernatants collected from sonicated *C. variabilis* cultures negatively affected the fitness of *C. variabilis* (Figure 6C; relative fitness = −0.179; $t_5 = -6.10$, $p = .0017$) and *C. sorokiniana* (Figure 6C; relative fitness = −0.196; $t_5 = -3.11$, $p = .026$). The necrotic culture supernatants generated from sonicated *C. variabilis* were also harmful to both the ciliate species (*P. bursaria* relative fitness < 0; $t_8 = -10.33$, $p < .0001$; *P. duboscqui* relative fitness < 0; $t_8 = -4.65$, $p = .001$; Figure 6C). Although the nature of the harmful molecules present in the necrotic supernatants was not investigated, it is possible that the photo-oxidation of the released chlorophyll leads to oxidative damage or that cellular contents such as proteases and other molecules at higher concentrations are lethal to the cells. *Chlorella sorokiniana* demonstrated significant resistance to both heat shock and sonication, with no observed cell death, requiring the exclusion of its supernatants from the fitness assays.

## PCD in endosymbiotic *P. bursaria* benefits endosymbiotic *Chlorella*

We then examined how PCD and necrosis in ciliate hosts impact the fitness of both their own clones and the algal species. To do this, we collected cell-free culture supernatants from the endosymbiotic ciliate *P. bursaria* and the predatory ciliate *P. duboscqui* following three treatments: control, PCD, and necrosis (Figure S8). These supernatants were then used to grow two algal species, endosymbiotic *C. variabilis* and

non-endosymbiotic *C. sorokiniana*, as well as the two ciliate species themselves, *P. bursaria* and *P. duboscqui*. PCD in the endosymbiotic *P. bursaria* had positive effects on its endosymbiont *C. variabilis* (relative fitness > 0; $t_{14} = 5.49$, $p < .0001$; Figure 7A, Figure 1E), while the supernatants from necrotic *P. bursaria* were detrimental to their endosymbionts (relative fitness < 0; $t_{11} = -6.81$, $p < .0001$; Figure 7B). In contrast, both the PCD and necrosis supernatants from the predatory *P. duboscqui* were harmful to *C. variabilis* (PCD: $t_{12} = -5.10$, $p < .0001$; necrosis: $t_{17} = -3.88$, $p = .0011$; Figure 7C, D). The non-symbiotic and heat shock- and sonication-resistant *C. sorokiniana* benefited from both PCD and necrosis supernatants derived from *P. bursaria* (PCD: $t_{19} = 2.94$, $p = .0083$; necrosis: $t_{19} = 5.30$, $p < .0001$; Figure 7A, B). However, it experienced adverse effects when grown in PCD supernatants from *P. duboscqui* (PCD: $t_{12} = -5.70$, $p < .0001$; necrosis: $t_{17} = 0.48$, $p = .632$; Figure 7A, B). PCD supernatants from *P. bursaria* had no significant effect on its own clones or on *P. duboscqui* cells, and similarly, PCD supernatants from *P. duboscqui* showed no significant impact on either its own clones or those of *P. bursaria* (Figure 7A, C). However, *P. duboscqui* cells were negatively affected by necrotic supernatants from both itself ($t_3 = -4.39$, $p = .0218$; Figure 7B) and *P. bursaria* ($t_6 = -6.09$, $p = .0008$; Figure 7D).

## Discussion

We tested for the fitness impact of PCD in the partners of unicellular facultative endosymbiotic organisms in comparison to non-symbiotic relatives to gain novel insights into the relationship between the evolution of unicellular PCD and endosymbiosis. We found that the unicellular endosymbiotic partners *C. variabilis* and its host *P. bursaria*, as well as the predatory ciliate *P. duboscqui*, undergo PCD, whereas the non-symbiotic *C. sorokiniana* exhibited significant heat resistance. The PCD phenotypes observed in *C. variabilis* include chromatin condensation, genomic DNA fragmentation, ROS accumulation, caspase-like protease activation, and PS externalization, as well as characteristic ultrastructural changes seen in TEM images (Figure 4, Figures S2–S5), and are consistent with prior reports on unicellular microalgal PCD (Bidle, 2016; Durand et al., 2011; Moharikar et al., 2006; Sathe et al., 2019; Zuppini et al., 2007). The demonstration of PCD in *P. bursaria* and *P. duboscqui* (Figure 5, Figures S6 and S9) represents a novel addition to the growing list of unicellular organisms exhibiting PCD (Bidle, 2016; Deponte, 2008; Durand & Ramsey, 2019; Kaczanowski et al., 2011). By comparing the fitness impact of PCD and necrotic death in symbiotic and non-symbiotic algae and ciliates, we found that endosymbiotic partners can significantly enhance each other's fitness through PCD, but not through necrotic death (Figure 1E, F). This expands the role for unicellular PCD extending its benefits not only to clonal populations of the same species but also to their endosymbiotic partners in a specific way.

In agreement with our first prediction (that "PCD in unicellular endosymbiotic *C. variabilis* benefits its clones and the cells of its endosymbiotic host, *P. bursaria*, but has non-beneficial (i.e., either harmful or neutral) effects on the cells of non-symbiotic *C. sorokiniana* or the predatory host, *P. duboscqui*"), we found that PCD in the endosymbiotic *C. variabilis* was beneficial to their clones and to their endosymbiotic host, *P. bursaria*, and harmful to the predatory ciliate *P. duboscqui*. An exception was *C. sorokiniana* (see below), which also benefited from *C. variabilis* PCD supernatants (Figure 6B, Figure 1E). Similarly, PCD in the endosymbiotic host *P. bursaria* proved beneficial for its endosymbionts and had neutral effects on their own clones (Figure 7A, Figure 1E). This finding aligns with the core premise of the second prediction that "PCD in *P. bursaria* benefits its clones and the cells of its endosymbiont, *C. variabilis*, but is not beneficial to *P. duboscqui* or *C. sorokiniana*." As *C. variabilis* PCD was induced at 45 °C and *P. bursaria* were exposed to 40 °C to trigger PCD, the benefits of PCD discussed here are likely attributed to host PCD. The observed neutral effects of *P. bursaria* PCD on the predatory ciliate *P. duboscqui* were as predicted, whereas the positive effects on *C. sorokiniana* were unpredicted (Figure 7A, Figure 1E). These findings indicate that *C. variabilis* PCD can provide both direct benefits by aiding clones and endosymbiotic hosts, as well as indirect benefits by harming predatory hosts, which indirectly benefits the endosymbiotic host. This is similar to observations in other unicellular systems, which showed direct fitness benefits of PCD, such as providing released resources to kin, and indirect benefits, such as harming non-kin (Chao & Levin, 1981; Durand et al., 2011; Vostinar et al., 2019). These harmful effects of *C. variabilis* PCD on *P. duboscqui* may benefit both *P. bursaria*, by reducing interspecies competition, and *C. variabilis*, by lowering predation pressure on free-living *C. variabilis* clones (Figures 6 and 1E).

In contrast to the positive PCD fitness effects observed in the endosymbiotic partners *P. bursaria* and *C. variabilis*, PCD in the exclusively predatory host *P. duboscqui* was harmful to the endosymbiotic *C. variabilis* (Figure 7C; Figure 1E). While the predicted positive effect of *P. duboscqui* PCD on their clones was not observed, the predicted non-beneficial effects on *P. bursaria* and *C. sorokiniana* cells were observed (Figure 7C; Figure 1E). This aligns with the third prediction that "PCD in the predatory host, *P. duboscqui*, benefits its clones but is not beneficial to *P. bursaria* and both the endosymbiotic and non-symbiotic *Chlorella* species." Consistent with the general prediction that necrosis is typically harmful, necrosis in *C. variabilis* reduced the fitness of both *Chlorella* species and the ciliate species (Figure 6C, Figure 1F). Necrosis in the ciliate host was either detrimental to *Chlorella* and ciliates, or had no significant effect compared to the control supernatants (Figure 7B, D, Figure 1F). An exception was observed with *C. sorokiniana*, whose growth was enhanced by *P. bursaria* necrosis but reduced by *P. duboscqui* necrosis. *Chlorella sorokiniana* cells showed exceptional resilience, as heat shock and sonication treatments that were lethal to other algal and ciliate species had little to no effect on them (Figures S1 and S2). However, it remains unclear whether this general resilience to abiotic stresses also contributes to their resistance to necrotic supernatants. The absence of cell death in *C. sorokiniana* meant that our fourth prediction that "PCD in non-symbiotic *C. sorokiniana* benefits its clones but is not beneficial to both the ciliate species and the cells of *C. variabilis*" could not be tested.

The facultative endosymbiotic relationship between *C. variabilis* and its host *P. bursaria* presents an intriguing case for kind selection (Queller, 2011), potentially operating via greenbeard genes (Gardner, 2019; Sathe, 2024). In this context, the benefits of PCD are directed towards "kind-related" endosymbiotic partners, where individuals share gene copies not because of recent common ancestry but as a result of horizontal

gene transfer. Although the frequency of horizontal gene transfer in the ciliate–algal system has not been directly studied, gene transfer from endosymbionts to hosts is widespread in both recently evolved endosymbiotic systems (e.g., *Buchnera*-aphid associations) and ancient ones, such as mitochondria and plastids (Ameisen, 1996; Boore, 1999; Fisher et al., 2017; Koonin & Aravind, 2002; Moran & Bennett, 2014).

In general, endosymbiosis can evolve from predator–prey interactions if the number of births is on average greater than the number of deaths in the symbiosis and the number of deaths is on average greater than the number of births in the free-living state (Law & Dieckmann, 1998), which is the case for *P. bursaria* and *C. variabilis* (Horas et al., 2022). While the positive effects of PCD observed on the fitness of endosymbiotic partners and the harmful effects on the predatory ciliate can shift this balance in favour of both partners, additional mechanisms are likely involved in the long-term stability of the endosymbiosis as costs, benefits and the net effect of the interaction can be context dependent (Horas et al., 2022; Iwai et al., 2019; Lowe et al., 2016), and because potential cheaters may evolve or infect a host through horizontal transmission, where the endosymbionts are recruited from the environment.

The observation that PCD has negative population effects on the predatory ciliate (*P. duboscqui*) but positive effects on the host (*P. bursaria*) may have implications for population and community dynamics. If both species grow together and compete for the free-living *C. variabilis* as prey, PCD of *C. variabilis* would shift the competition in favour of *P. bursaria* and indirectly benefit *C. variabilis*, since one of the main benefits of the endosymbiotic interaction for *C. variabilis* is escape from predation (Horas et al., 2022). This prediction is consistent with other studies showing that PCD can impact microbial communities in complex ways, not only via nutrients and resources released upon cell death and could further contribute to the stabilization of the endosymbiosis (Durand et al., 2016).

While explaining the evolution of PCD in unicellular organisms is challenging (Durand & Ramsey, 2019; Nedelcu et al., 2011), the unicellular PCD has been shown to contribute to facultative multicellular group formation (Durand & Ramsey, 2019; Ratcliff et al., 2012; Sathe & Durand, 2016) and has been suggested to benefit the clones or the individuals of other species by releasing and sharing resources (Bar-Zeev et al., 2013; Durand et al., 2011, 2014; Orellana et al., 2013), communicating information about the environment (Durand et al., 2016; Vardi et al., 2007), or coordinating cooperative functions such as the division of labour and/or mutual dependencies (Cornillon et al., 1994; Durand & Ramsey, 2019; Lewis, 2000). Our findings reveal an expanded role for unicellular PCD as a mechanism to influence the fitness of endosymbiotic partners. The evolution of endosymbiosis, where two independent organisms form an intimate, interdependent association and reproduce as an individual unit, represents a major evolutionary transition. Early endosymbiotic events likely originated as predator–prey interactions, but PCD may have been pivotal in shifting these antagonistic relationships towards cooperative interactions, forming the foundation for the evolution of endosymbiosis.

## Supplementary material

Supplementary material is available at *Journal of Evolutionary Biology* online.

## Data availability

The data underlying this article are deposited in Zenodo: https://zenodo.org/records/15854730.

## Author contributions

Santosh Sathe (Conceptualization [lead], Data curation [lead], Formal Analysis [lead], Funding acquisition [supporting], Investigation [lead], Methodology [lead], Project administration [lead], Resources [supporting], Supervision [lead], Validation [lead], Visualization [lead], Writing – original draft [lead], Writing – review & editing [lead]), and Lutz Becks (Conceptualization [equal], Data curation [supporting], Formal Analysis [supporting], Funding acquisition [lead], Investigation [supporting], Methodology [supporting], Project administration [equal], Resources [lead], Supervision [supporting], Validation [supporting], Visualization [supporting], Writing – original draft [supporting], Writing – review & editing [supporting])

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## Conflicts of interest

The authors declare no competing interests.

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