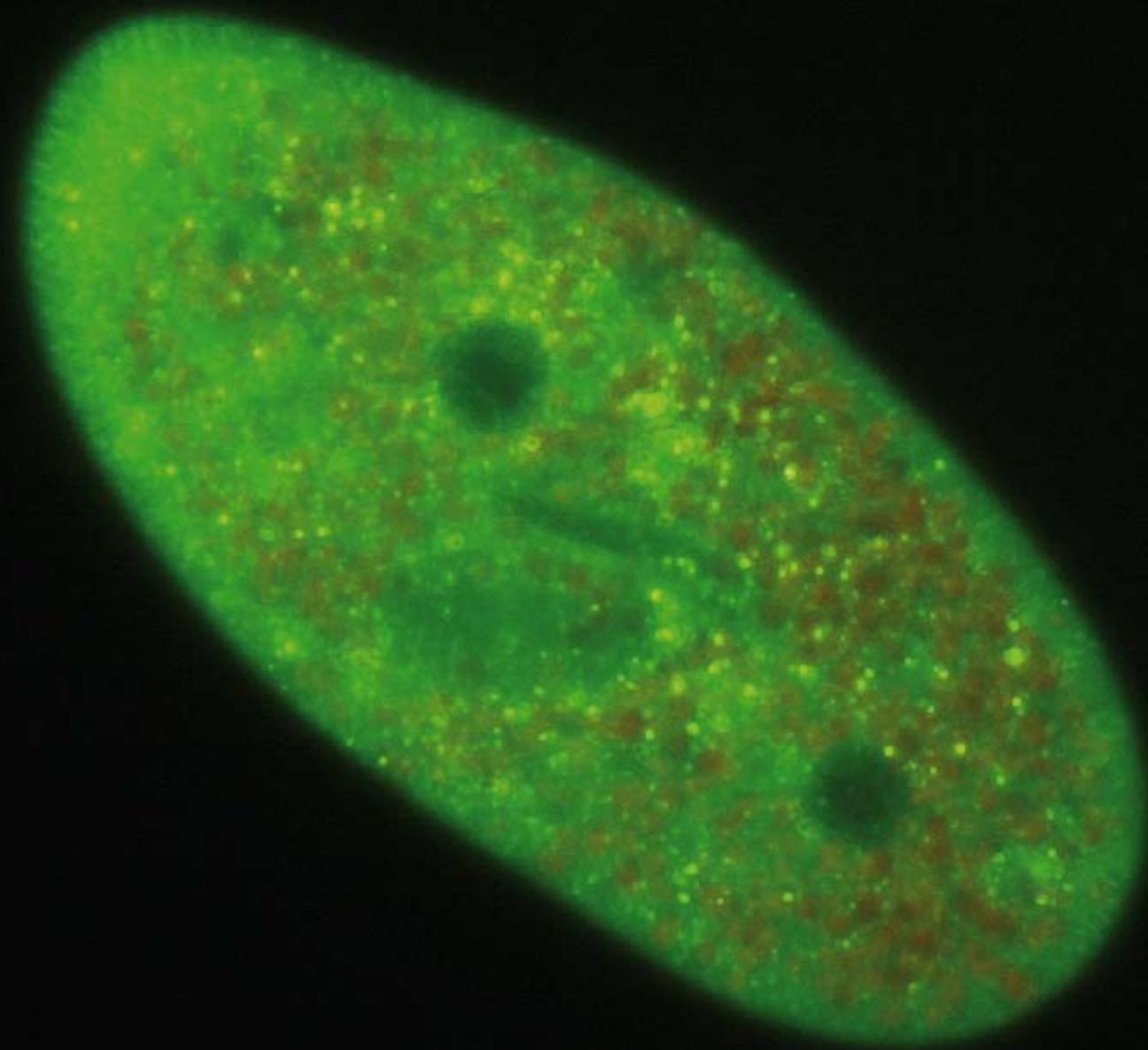


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# Photo-oxidative stress in symbiotic and aposymbiotic strains of the ciliate *Paramecium bursaria*

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We tested the hypothesis that photo-oxidative stress is greater in symbiotic representatives of the freshwater ciliate *Paramecium bursaria* than in aposymbiotic (*i.e.*, without *Chlorella*) ones. The level of oxidative stress was determined by assessing reactive oxygen species (ROS) with two fluorescent probes (hydroethidine and dihydrorhodamine123) by flow cytometry in exponential and stationary growth phases of both strains. Photo-oxidative stress was assessed in the laboratory after exposure of the ciliates to photosynthetically active radiation (PAR: 400–700 nm) and PAR + ultraviolet radiation (UVR: 280–400 nm). Additionally, both strains were screened for their antioxidant defenses by measuring the activity of the enzymes catalase, superoxide dismutase (SOD), and glutathione reductase. The results showed that aposymbiotic ciliates had higher levels of PAR-induced oxidative stress than symbiotic ones. Significant differences in PAR-induced oxidative stress were also found in both strains when comparing exponential and stationary growth phases with generally higher values in the former. After exposure to UVR, aposymbiotic ciliates in the stationary phase had the highest levels of ROS despite an increase in SOD activity. By contrast, exposure to UVR decreased catalase activity in both strains. Overall, our results suggest that in this ciliate symbiosis, the presence of symbionts minimizes photo-oxidative stress. This work represents the first assessment of photo-oxidative stress in an algal-ciliate mutualistic symbiosis.

## Introduction

The production of reactive oxygen species (ROS), such as superoxide radical ( $O_2^{\cdot-}$ ), singlet oxygen ( $^1O_2$ ), hydroxyl radical ( $HO^{\cdot}$ ), and hydrogen peroxide ( $H_2O_2$ ) is the natural result of cell metabolism.<sup>1,2</sup> The strong production and accumulation of ROS beyond the antioxidant capacity of an organism (*i.e.*, oxidative stress) can damage lipids, proteins, DNA, and membranes.<sup>3–5</sup> ROS are generated by chemical, physical, or photosensitized reactions inside and outside the cells.<sup>5</sup> Ultraviolet radiation (UVR, 280–400 nm) and photosynthetically active radiation (PAR, 400–700 nm) are very efficient in causing photo-oxidative stress especially in light-dependent reactions of photosynthetic active organisms.<sup>6–8</sup> In aquatic ecosystems, photo-oxidative production of ROS is known to impose a physiological burden on many organisms.<sup>9</sup> For example, inhibition of photosynthesis and increased concentrations of hydrogen peroxide and superoxide radicals take place in the marine dinoflagellate *Prorocentrum micans* after exposure to UV-B radiation (280–315 nm).<sup>7</sup> However, we know little on photo-oxidative stress in aquatic organisms, particularly in freshwater ones.

In order to minimize the harmful effects of ROS, organisms have evolved different protective strategies. Defenses against ROS include various antioxidative enzymes such as superoxide dismutase (SOD) that removes  $O_2^{\cdot-}$  producing  $H_2O_2$ ; catalase (CAT) that then removes  $H_2O_2$ , and glutathione reductase (GR) that is involved in the conversion of oxidized glutathione to its

reduced form with consumption of NADPH.<sup>10–12</sup> Furthermore, non-enzymatic antioxidants such as glutathione (GSH), ascorbic acid,  $\alpha$ -tocopherol,  $\beta$ -carotene, and uric acid can protect many cellular constituents from ROS because these substances are considered to be chain breaking antioxidants that interrupt the spreading of autocatalytic radical reactions.<sup>13</sup>

Mutualistic organisms with photosynthetic symbionts have by their very nature to cope with high levels of oxidative and photo-oxidative stress. At present, only a few symbiotic associations have been tested for the effects of ROS on hosts and symbionts. For example, in the symbiosis between anthozoans and dinoflagellates ('zooxanthellae'), raising temperatures in seawater in combination with high UVR have been found to cause the phenomenon called 'coral bleaching' that results from bursts of ROS within hosts and symbionts.<sup>14–16</sup>

In the present study, we took advantage of the ability to obtain aposymbiotic and symbiotic strains (*i.e.*, living with *Chlorella* sp.) of the freshwater ciliate *Paramecium bursaria* to first, assess any differences in PAR-induced oxidative stress and second, to assess how sensitive these strains are to UVR-induced ROS production. We hypothesized that the symbiotic *P. bursaria* strain would exhibit greater photo-oxidative stress than the aposymbiotic one. In addition, antioxidant enzymes were measured before and after exposure to UVR to test the ciliate's antioxidant defenses.

## Material and methods

### Cultivation and irradiating conditions

The endosymbiotic ciliate *Paramecium bursaria* KM2 (hereafter strain G, green) was cultivated in modified Woods Hole-MBL

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culture medium<sup>17</sup> on a 14:10 h light–dark cycle, at 25 °C. The aposymbiotic strain (strain W, white) was obtained by keeping the G strain in the darkness for several weeks in diluted lettuce–Chalkley-medium<sup>18,19</sup> at 25 °C. Afterwards, the aposymbiotic strain was grown under the same light regime as described above.

Photosynthetically active radiation (160  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) was provided by six Osram Cool White L36 W/20 lamps, which also emit very low UV-A intensities (instantaneous UV-A irradiance: 0.27  $\text{W m}^{-2}$ ). Homogenous illumination of the cultures was obtained by placing bottles tilted (*ca.* 45°) on a rack.

For the assessment of UV-induced oxidative stress, the strains were exposed to simulated solar UVR (instantaneous UV-A: 8.60  $\text{W m}^{-2}$  and UV-B irradiance: 2.47  $\text{W m}^{-2}$ ) provided by four aged (100 h) fluorescent lamps (Q-Panel UVA 340, Cleveland, OH). These lamps have a maximum emission in the UV-A range at 340 nm and produce no radiation below 280 nm. Background PAR was provided by two visible fluorescent lamps (cool white L36/W20, Osram) emitting 80  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . The spectrum emitted by the lamps used in this set-up is found elsewhere.<sup>20</sup>

### Ciliate abundance and growth rate

Population growth of the two strains was followed by estimating the abundance of the ciliates after the ‘droplet method’<sup>21</sup> at daily intervals during 17 d. Briefly, 5 × 5  $\mu\text{l}$  drops of homogenized culture were placed on a clean slide with a micropipette and the ciliates counted on each drop on a microscope (Olympus BX50) at 40× magnification. The specific growth rate ( $\mu$ ) was calculated from the change in cell numbers over time during the exponential growth phase.

### Experimental design

The assessment of ROS and antioxidant enzymes was done in the exponential (exp.) and the stationary (stat.) growth phase of both strains. PAR-induced oxidative stress was assessed in the middle of the light cycle at the same light intensity used for cultivation. To assess the effect of UVR on oxidative stress, experiments were done in a walk-in chamber at the same cultivation temperature. For this objective, 20 ml of ciliate culture was transferred into glass Petri dishes (diameter = 9 cm) and exposed without the lid cover to simulated PAR + UVR for 2 h. A control (*i.e.*, ciliates exposed only to PAR) was obtained by placing a vinyl chloride foil (CI Kasei, Co., Tokyo, Japan, 50% transmittance at 405 nm) 1 cm above the Petri dishes to exclude the UVR. Measurements were done immediately after the end of the exposure.

### ROS detection

Reactive oxygen species were detected by flow cytometry using the fluorescent probes dihydrorhodamine123 (DHR123) and hydroethidine (HE) after Lesser.<sup>7</sup> Whereas DHR123 is an indicator of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) levels, HE was used for a more general detection of ROS because this fluorescent probe reacts with intracellular superoxide ( $\text{O}_2^{\cdot-}$ ) but also with  $\text{H}_2\text{O}_2$ . Briefly, 4  $\mu\text{l}$  (DHR 123) or 25  $\mu\text{l}$  (HE) of a 1 mM solution (in DMSO) was added to 1 ml of culture and incubated for 15 min in the dark at room temperature. Flow cytometric analysis was performed on a MoFlo (Dako Cytomation, Glostrup, Denmark) equipped with a water cooled argon-ion laser (Innova 90 C+, Coherent Inc.,

Santa Clara, CA) tuned to 488 nm (200 mW) and a 200  $\mu\text{m}$  diameter nozzle. Fluorescent microspheres (1  $\mu\text{m}$  TransFluoSpheres 488/560, Molecular Probes, Eugene, OR, USA) were added as an internal fluorescence reference. About 200 cells per strain and replicate ( $n = 3$ ) cultures were measured. The possible interference of the autofluorescence of symbiotic algae with signals of the fluorescent probes was previously checked by comparing both signals, however, it was irrelevant.

### Enzymatic assay of antioxidants

The antioxidant enzymes catalase (CAT; EC:1.11.1.6), superoxide dismutase (SOD; EC:1.15.1.1), and glutathione reductase (GR; EC:1.6.4.2) were assayed spectrophotometrically as described by Bergmeyer,<sup>22</sup> Greenwald,<sup>23</sup> and Lesser,<sup>7</sup> respectively. Measurements were done for both strains and growth phases using the same experimental design as for testing the UV-induced oxidative stress. Briefly, after exposure, 10000 ciliates of each strain were concentrated by centrifugation (2000  $g$  for 5 min). The supernatant was removed and the ciliates were washed twice with autoclaved 0.2  $\mu\text{m}$  filtered tap water before being resuspended in 500  $\mu\text{l}$  of 60 mM phosphate buffer (pH = 7.0) for CAT and GR analyses, and 50 mM phosphate buffer for SOD analysis. Afterwards, the resuspended sample was first sonicated on ice for 1 min at 42 W (Bandelin, Sonoplus, HD 2070) to ensure the rupture of the organisms and again centrifuged for 10 min at 4 °C and 13000  $g$ . The retained supernatant was assessed for CAT, SOD, and GR in a UV/visible spectrophotometer (Ultrospec II LKB Biochrom NR4050) at 25 °C using a 1 cm quartz (Suprasil) cuvette. CAT activity was measured at 240 nm using 200  $\mu\text{l}$  supernatant dissolved in 2 ml  $\text{H}_2\text{O}_2$  [35%] and CAT–phosphate buffer solution. SOD activity (850  $\mu\text{l}$  SOD phosphate buffer and 100  $\mu\text{l}$  epinephrine to 50  $\mu\text{l}$  supernatant) was detected at 485 nm, and GR activity (2 ml GR phosphate buffer, 40  $\mu\text{l}$  reduced nicotinamide adenine dinucleotide phosphate (NADPH) and 40  $\mu\text{l}$  oxidized glutathione (GSSG)) was measured at 340 nm. Results were expressed as units (U) of enzyme activity per cell, where 1 U of catalase is defined as the amount of enzyme that halves its substrate in 100 s during the linear response.<sup>24</sup> One unit of superoxide dismutase activity is defined as the quantity of SOD required to cause 50% inhibition of the oxidation of epinephrine under the specified conditions.<sup>23</sup>

### Statistics

Statistical tests were carried out using Sigma Stat (Systat, Software Inc., San Jose, CA). Significant differences among treatments were tested using a non-parametric Kruskal–Wallis one-way ANOVA. The pertinent post-hoc comparisons were made by a Tukey test with a significance level of 0.05.

## Results

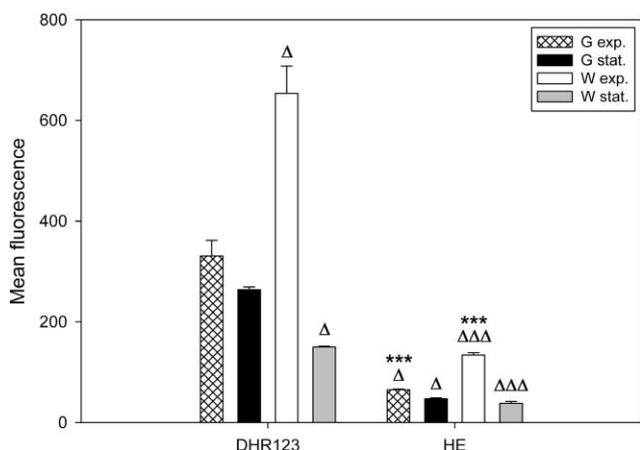
### Growth rates

The exponential growth phase of strain G started after one day and lasted for 8 d. Therefore, measurements in the mid-exponential and stationary phase were done on day 4 ( $\pm 1$ ) and day 10, respectively. Mean specific growth rate of strain G was 0.22  $\text{d}^{-1}$ . Strain W had a longer lag phase (4 d) and reached the stationary growth phase

after 10 d (mean specific growth rate = 0.17 d<sup>-1</sup>). Thus, cultures on day 7 (±1) and on day 10 were chosen for the experiments.

### PAR-induced oxidative stress

In the exponential growth phase, strain W showed a significantly higher mean fluorescence than strain G when tested for HE ( $p < 0.001$ ,  $q = 20.7$ ) (Fig. 1). When comparing growth phases of the respective strains, W exp. had a higher mean fluorescence as compared to the stationary phase as assessed with both fluorescence probes (DHR123:  $p < 0.05$ ,  $q = 4.3$  and HE:  $p < 0.001$ ,  $q = 28.9$ ). Similarly, strain G showed higher PAR-induced photo-oxidative stress in the exponential growth phase than in the stationary one, although it was significantly different only when assessed with HE ( $p < 0.05$ ,  $q = 5.6$ ) (Fig. 1).

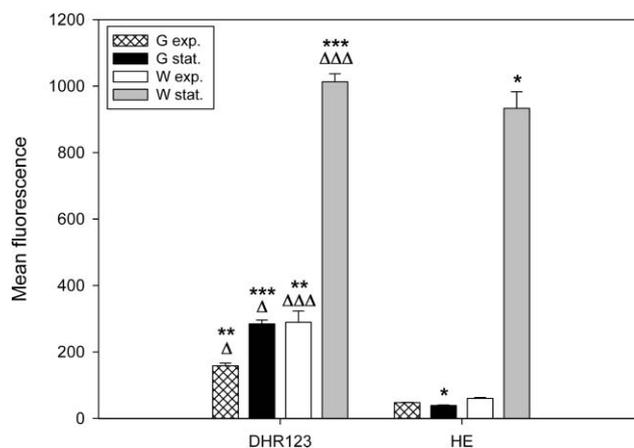


**Fig. 1** PAR-induced oxidative stress in aposymbiotic and endosymbiotic strains of *P. bursaria* expressed as mean fluorescence ( $n = 3 \pm \text{SE}$ ) using two ROS detection methods. Asterisks (\*) denote significant differences between endo- (G) and aposymbiotic (W) strains, whereas triangles ( $\Delta$ ) indicate significant differences between exponential (exp.) and stationary (stat.) growth phases.  $^{*/\Delta} p < 0.05$ ,  $^{**/\Delta\Delta} p < 0.01$ ,  $^{***/\Delta\Delta\Delta} p < 0.001$ .

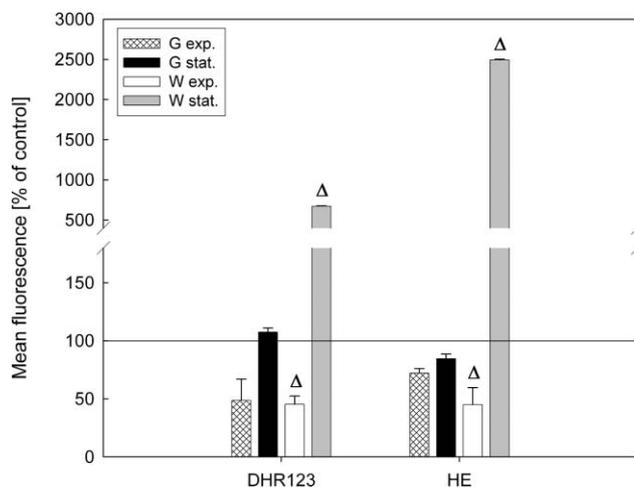
### UV-induced oxidative stress

After 2 h exposure to simulated solar UVR, DHR123 mean fluorescence was higher in aposymbiotic ciliates in both phases than in endosymbiotic ones (Fig. 2, W exp. > G exp.  $p < 0.01$ ,  $q = 6.3$ ; W stat. > G stat.  $p < 0.001$ ,  $q = 33.1$ ). Similarly, in the stationary growth phase, the aposymbiotic strain tested with HE showed higher mean fluorescence than the endosymbiotic one ( $p < 0.05$ ,  $q = 4.32$ , Fig. 2). For both strains, ciliates in the stationary growth phase showed a higher content of H<sub>2</sub>O<sub>2</sub> compared to exponential phase when tested with DHR123 (W stat.  $p < 0.001$ ,  $q = 32.4$  and G stat.  $p < 0.05$ ,  $q = 5.7$ , Fig. 2).

When comparing the ratio of PAR- to UVR-induced photo-oxidative stress, only the strain W in the stationary growth phase showed a significant increase in ROS after UV exposure. Thus, the mean fluorescence increased 6.7 times and 24.9 times for DHR123 and HE, respectively (Fig. 3). After 2 h exposure to UVR, an important percentage of the aposymbiotic ciliates showed erratic swimming movements, however, this was not observed in the PAR control or in endosymbiotic ciliates.



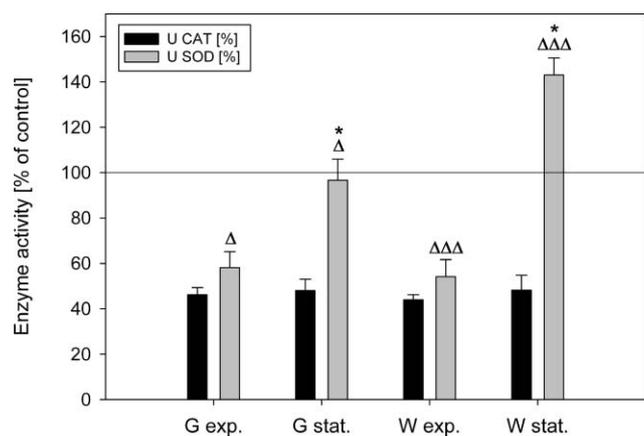
**Fig. 2** UV-induced oxidative stress in endosymbiotic and aposymbiotic *P. bursaria* strains expressed as mean fluorescence ( $n = 3 \pm \text{SE}$ ) for two different ROS detection methods. Asterisks (\*) denote significant differences between endo- (G) and aposymbiotic (W) strains, whereas triangles ( $\Delta$ ) indicate significant differences between exponential (exp.) and stationary (stat.) growth phases of the ciliates from strain G or W.  $^{*/\Delta} p < 0.05$ ,  $^{**/\Delta\Delta} p < 0.01$ ,  $^{***/\Delta\Delta\Delta} p < 0.001$ .



**Fig. 3** Comparison of photo-oxidative (*i.e.*, PAR and UV) stress in endo- (G) and aposymbiotic (W) strains of *P. bursaria*. Mean fluorescence values ( $\pm \text{SE}$ ) for the two ROS probes in the PAR + UV treatment are expressed as percentage of the PAR control (horizontal line reference). Triangles ( $\Delta$ ) indicate significant differences between exponential (exp.) and stationary (stat.) growth phases.  $^{\Delta} p < 0.05$ .

### Antioxidant enzymes

A significant decrease of the CAT activity was observed after exposure to UVR in both strains (mean reduction: 46.6% of the PAR control, Fig. 4). By contrast, a decrease in SOD activity was only found in the exponential growth phase of strain G (58.2% of control) and strain W (54.1% of control). While SOD activity did not significantly change in strain G stat., the aposymbiotic strain in the stationary growth phase showed a significant increase in SOD activity (143.0% of control, Fig. 4). The activity of glutathione reductase (GR) was in all cases undetectable.



**Fig. 4** Relative activity of the antioxidant scavenging enzymes catalase (CAT) and superoxide dismutase (SOD) in endo- (G) and aposymbiotic (W) strains of *P. bursaria* exposed for 2 h to PAR + UVR. Enzyme activity ( $n = 3 \pm \text{SE}$ ) is expressed as percentage of the PAR control (horizontal line reference) for the respective strains and growth phases. Asterisks (\*) denote significant differences between endo- (G) and aposymbiotic (W) strains, whereas triangles ( $\Delta$ ) indicate significant differences between exponential (exp.) and stationary (stat.) growth phases of the ciliates from strain G or W. \*/ $\Delta$   $p < 0.05$ , \*\*\*/ $\Delta\Delta\Delta$   $p < 0.001$ .

## Discussion

Few studies have assessed photo-oxidative stress in aquatic organisms, particularly in freshwater ones. Our study aimed to compare photo-oxidative stress in aposymbiotic and symbiotic strains of *P. bursaria* in order to assess whether endosymbiotic *Chlorella* burden its host. Different symbiotic organisms have been screened for ROS production hitherto,<sup>14,25,26</sup> however, this is the first time the photo-oxidative stress in an algal-ciliate mutualistic symbiosis is assessed. Our hypothesis that PAR-induced oxidative stress in *P. bursaria* is generally higher in endosymbiotic than in aposymbiotic ciliates was disproved by the observation that exponentially growing aposymbiotic ciliates showed the highest ROS content (Fig. 1). This finding, however, is in contrast to observations in the sea anemone *Anthopleura elegantissima* and its endosymbiotic algae, where production of the hydroxyl radical is higher in endosymbiotic individuals than aposymbiotic ones.<sup>14</sup>

During exponential growth, high cell division rates are accompanied by an increase in metabolic activity. For most organisms, this growth phase usually results also in an increase in levels of oxidative stress. For example, in the dinoflagellate *Chattonella antiqua*, production of superoxide is maximum during exponential growth and minimum in the stationary growth phase.<sup>27</sup> Our results on higher PAR-induced oxidative stress observed in exponentially growing *P. bursaria* (Fig. 1) are in agreement with those results. However, no general pattern is found among aquatic protists or for different ROS within a species. Thus, for example, no significant difference in H<sub>2</sub>DCF-DA detectable oxidative stress is found within growth phases of free-living *Chlorella vulgaris*.<sup>28</sup> Moreover, in *C. antiqua*, the maximum production of hydrogen peroxide as detected by the *p*-hydroxyphenylacetate assay occurs in the early stationary growth phase.<sup>27</sup>

Several studies have shown that UVR stimulates the generation of ROS in heterotrophic<sup>29–31</sup> and photo-autotrophic organisms.<sup>29,32</sup>

We expected to find higher UV-induced oxidative stress in the endosymbiotic strain of *P. bursaria*. However, the UV-induced oxidative stress estimated by DHR123 in *P. bursaria* was higher in both exponentially and stationary growing aposymbiotic ciliates than in endosymbiotic ones (Fig. 2). In addition, the aposymbiotic strain in stationary phase showed significantly higher HE levels than the endosymbiotic one. In support of this finding, the comparison of PAR- and UV-induced oxidative stress clearly showed an increase of ROS solely in aposymbiotic *P. bursaria* during stationary phase (Fig. 3). One potential mechanism to explain the lower UV-induced oxidative stress in the endosymbiotic strain of *P. bursaria* could be the UVR screening provided by *Chlorella*. Estimations based on an optical model using data on *Chlorella* distribution within *P. bursaria* obtained by electron microscopy show that the algal symbionts substantially minimize intracellular UV exposure (M. Summerer *et al.* unpublished results).

The presence of ROS within an organism initiates a series of responses that provide increased protection against the damaging effect of these species such as the synthesis of the antioxidative enzymes catalase and SOD.<sup>5</sup> For example, this adaptive response is known in bacteria such as *E. coli*<sup>33</sup> and in the planktonic diatom *Thalassiosira pseudonana*.<sup>34</sup> In symbiotic cnidarians, the presence of ‘zooxanthellae’ is also known to increase the activity and even influence the regulation of SOD.<sup>35</sup> On the other hand, an increased content of ROS<sup>3</sup> or exposure to UVR<sup>36</sup> can seriously damage enzymes and proteins in aquatic organisms. For example, Lesser<sup>37</sup> showed that UVR damages the primary carboxylating enzyme Rubisco in symbiotic dinoflagellates. In our experiments, there was a distinct trend for catalase and SOD in that the activity of the former was strongly reduced in the exponential and stationary growth phases of both strains (Fig. 4). This finding is in agreement with the results from studies with Arctic amphipods<sup>38</sup> and marine microalgae,<sup>39</sup> where exposure to UVR causes a strong decrease in catalase activity. In particular, UVR is known to cause structural and functional changes on catalase in solution and also in living cells.<sup>40</sup> However, a UV reduction of catalase activity has not been found in the freshwater cladoceran *Daphnia longispina*<sup>41</sup> and the free-living *Chlorella vulgaris*.<sup>28</sup>

Our observation that SOD activity was significantly increased under UVR exposure in the aposymbiotic strain during the stationary growth phase (Fig. 4) was coincident with the increase in ROS (Fig. 3). However, the increase in SOD activity was apparently not enough to counteract the direct and indirect (*i.e.*, ROS) damaging effects of UVR on aposymbiotic ciliates as suggested by our observation of an erratic swimming behavior after 2 h exposure.

Dykens and Shick<sup>42</sup> argued that oxygen production by endosymbiotic zooxanthellae controls SOD activity in the sea anemone *Anthopleura elegantissima*. In the demosponge *Petrosia ficiformis*, antioxidant defenses are higher in symbiotic than aposymbiotic individuals.<sup>43</sup> Taken into account that free-living *Chlorella* have high SOD activity,<sup>28</sup> and that photosynthetic organisms have other natural quenching agents of ROS such as carotenoids, we suggest that the lower photo-oxidative stress in the endosymbiotic strain of *P. bursaria* can be also attributed to the antioxidant defenses from the algae.

In conclusion, our results demonstrated that photo-oxidative stress was different between endo- and aposymbiotic *P. bursaria* and was also affected by the growth phase of the ciliate. A

significant increase in ROS after UVR exposure was only found in the aposymbiotic strain during the stationary growth phase. Changes in UV sensitivity during different growth phases have been observed in different unicellular organisms. For example, in the heterotrophic ciliate *P. tetraurelia*, cells in the late exponential and stationary phases have higher UV sensitivity than in the early exponential phase.<sup>44</sup> Although the mechanism for this higher sensitivity is not completely understood, our results suggest that ROS production is involved. Overall, our findings indicate that symbiosis with *Chlorella* does not enhance the levels of photo-oxidative stress in *P. bursaria*.

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