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Blepharismins used for chemical defense in two ciliate species of the genus *Blepharisma*, *B. stoltei* and *B. undulans* (Ciliophora: Heterotrichida)

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Abstract

It is known that the freshwater heterotrich ciliate *Blepharisma japonicum* uses five pigments called blepharismins, stored in its extrusive pigment granules, for both light perception and chemical defense against predators. In this work we focused our attention on the defensive strategies of two additional pigmented species of *Blepharisma*, *B. stoltei* and *B. undulans*. In particular: (1) we observed the predator–prey interactions of *B. stoltei* or *B. undulans* against one multicellular and two unicellular predators; (2) we clarified the nature of *B. stoltei* and *B. undulans* pigments by means of High Performance Liquid Chromatography (HPLC) and Liquid Chromatography Mass Spectrometry (LC-MS); and (3) we demonstrated and compared the toxicity of the purified pigments on a panel of ciliated protists, and against one metazoan predator. The results indicate that the chemical defense mechanism present in *B. stoltei* and *B. undulans* is mediated by the same five blepharismins previously characterized for *B. japonicum*, although produced in different proportions.

Keywords: *Extrusomes, secondary metabolites, predator–prey interaction, chemical defense, pigment granules*

Introduction

Blepharismins (BPs) are multifunctional quinone derivatives identified in the species of heterotrich ciliates belonging to the genus *Blepharisma* Perty, 1849. In *B. japonicum* Suzuki, 1954, the best-studied photophobic species of the genus, BPs are produced as a mixture of five compounds (BP-A, -B, -C, -D and -E) structurally related to hypericin, a photodynamic toxin of *Hypericum perforatum* Linnaeus, 1753, and stentorin, produced by *Stentor coeruleus* Ehrenberg, 1830, another negatively phototactic ciliate (Lobban et al. 2007; Matsuoka et al. 2010). As in other toxin-producing heterotrichs, BPs are stored in membrane-bound extrusive organelles (pigment granules) usually 0.3–0.6 µm diameter, arranged in stripes between the rows of cilia. To date, two primary functions of BPs have been clarified: light perception, and defense against predators (Miyake et al. 1990; Harumoto et al. 1998; Muto et al. 2001; Lobban et al. 2007; Uruma et al. 2007; Matsuoka et al. 2010).

With regard to light perception, *B. japonicum* shows a temporal backward swimming or rotating movement (step-up photophobic response) if exposed to a sudden increase in light intensity. The step-up photophobic response helps the cells avoid strongly illuminated regions, and lethal damage due to the photodynamic action of BPs (Kato & Matsuoka 1995). It was demonstrated that the photosensitive region is localized in the anterior end of the cell body where only BP-B is present, suggesting that this pigment may represent the photoreceptor mediating the step-up photophobic response (Matsuoka et al. 1997).

In addition to light perception, BPs were found to act as chemical weapons via their light-independent cytotoxic effect against predatory protozoans and methicillin-resistant Gram-positive bacteria (Miyake et al. 1990; Pant et al. 1997; Harumoto et al. 1998). A possible explanation for this cytotoxicity can be found in the capability of BPs to form cation-

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selective channels in planar phospholipid bilayers (Muto et al. 2001), a phenomenon also expected to occur in the cell membranes of microorganisms exposed to toxic concentrations of ciliate pigments.

From a more general point of view, the toxic secondary metabolites from ciliates have recently attracted the attention of an increasing number of researchers also for their pharmacological and medical applications (Petrelli et al. 2012; Catalani et al. 2016; Perrotta et al. 2016).

In this work: (1) we investigated whether the chemical defense mechanism known in *B. japonicum* was also present in *B. stoltei* Isquith, 1966 and *B. undulans* Stein, 1868, exposing both intact cells and pigment-deficient cells of these two species to unicellular and multicellular predators; (2) we clarified the nature of the pigments produced by *B. stoltei* and *B. undulans* using High Performance Liquid Chromatography (HPLC) and Liquid Chromatography Mass Spectrometry (LC-MS) analyses; and (3) we demonstrated and compared the toxicity of the purified pigments on a panel of free-living and raptorial ciliates, and against one microturbellarian predator.

Materials and methods

Organisms and culture methods

Blepharisma japonicum strain R1072 (Harumoto et al. 1998), *Blepharisma stoltei* strain HT-IV and *Blepharisma undulans* strain K78 (Kobayashi et al. 2015) were cultured in Synthetic Medium for *Blepharisma* (SMB) medium (Miyake 1981) and fed with the flagellate *Chlorogonium elongatum* Dangeard, 1899, cultivated as described in Buonanno et al. (2005), or in Jaworski's Medium (JM) solution. Light microscopy observations were carried out *in vivo*, in SMB or in a solution of methyl cellulose in SMB (2%) to slow cilia motility. *Coleps hirtus* Müller, 1786 (clone PC-4) (Buonanno et al. 2014), *Euplotes aediculatus* Pierson, 1943 (Buonanno et al. 2017), *Paramecium multimicronucleatum* Powers & Mitchell, 1910 (clone TL-2, collected in Lake Trasimeno, Perugia, Italy) and *Stentor roeseli* Ehrenberg, 1835 (clone TL-4) (Buonanno et al. 2017) were also cultured in SMB and fed with *C. elongatum*. *Stenostomum sphagnetorum* Luther, 1960 (Platyhelminthes: Turbellaria), a common freshwater ciliate predator (Buonanno 2005), was cultured as described in Buonanno et al. (2013).

Removal of pigment granules and toxicity tests of BPs

In this work, the cold shock-based method (Buonanno & Ortenzi 2016) was applied to *B.*

japonicum, *B. stoltei* or *B. undulans* in order to obtain the pigment granule-deficient (PGD) cells. These cells appear partially or totally bleached, and are substantially healthy as control cells (data not shown). The same procedure allows us also to collect the toxin-enriched supernatant (TES) containing the discharge from the pigment granules.

To evaluate the toxicity of the purified BPs, triplicate samples of 10 ciliate cells or metazoan specimens were placed in depression slides containing 250 μ L SMB and increasing concentrations of each toxin (from 1 to 100 μ g/mL). The number of surviving organisms (normal morphology and locomotion) was counted, after 24 h, and the median lethal concentrations (LC₅₀) were estimated on the basis of a concentration–survival curve, essentially according to the procedure described by Buonanno (2009). The data were evaluated by nonlinear regression analysis using GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA) with the 95% level of confidence.

Predator–prey experiments

Samples of five or 10 cells of either untreated or PGD cells of *B. japonicum*, *B. stoltei* or *B. undulans* were mixed with samples of 500 cells of *C. hirtus*, 20 cells of *S. roeseli* or 10 specimens of *S. sphagnetorum* in 250 μ L of SMB. The mixtures involving *C. hirtus* as predator are here termed 500C-5J, 500C-5S, 500C-5U and 500C-10J, 500C-10S, 500C-10U, respectively, for those involving five or 10 cells of *B. japonicum*, *B. stoltei* or *B. undulans*. Each mixture was prepared in six replicates, for both untreated and PGD cells of the three *Blepharisma* species, and observed under a stereomicroscope after 2 or 24 h. The data represent the means \pm standard error (SE) of six independent determinations, and the significance of the differences between the mean values was examined by Student's *t*-test with the significance threshold set at $p < 0.005$.

Chemical characterization of BPs by LC-MS

Lyophilized ethanol extracts of *Blepharisma* cell cultures or aliquots of lyophilized TES were resuspended in ethanol, sonicated in an ultrasonic bath for 15 min (Sonorex Super, Bandelin electronics, Berlin, Germany), and centrifuged at 3000 \times *g* for 10 min at room temperature. The procedure was repeated 3 times, and the supernatant was dried down in a rotatory evaporator and re-suspended in 500 μ L of methanol.

Ten-microliter aliquots were injected and analyzed in negative ionization mode on a Hewlett-Packard

Model 1100 Series liquid chromatograph (Hewlett-Packard Development Company, L.P., Palo Alto CA, USA) coupled both to a Bruker Esquire-LC quadrupole Ion Trap Mass Spectrometer (IT-MS) equipped with an Electrospray Ionization (ESI) source (Bruker Optik GmbH, Ettlingen, Germany) and to a photo diode-array detector (DAD) (Agilent Technologies, Milan, Italy, Agilent 1100). Chromatographic separation was carried out on a Zorbax Eclipse XDB-C18 column (150 × 4.6 mm inner diameter, pore size 200 Å, particle size 3.5 µm; Hewlett Packard, Palo Alto, CA, USA), with a linear gradient of solvent A (methanol: water 7:3, containing 12 mM ammonium acetate) and solvent B (methanol containing 12 mM ammonium acetate) from 0%B to 100%B in 30 min, at a constant flow rate of 1.0 mL/min. Final conditions were kept for at least 30 min. Mass range was 50–1200 *m/z*, and high-voltage capillary was set at –4000 V. DAD was operated at 205, 215, 254, 574 and 590 nm. High-resolution MS was conducted using same chromatographic setup on an Orbitrap Fusion™ Tribrid™ Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA).

Results and discussion

Predator–prey interactions

It is known that one of the main functions of BPs in *B. japonicum* is chemical defense against unicellular predators (Harumoto et al. 1998; Terazima & Harumoto 2004). To investigate whether this function was also present in *B. stoltei* and *B. undulans* (Figure 1), we observed the predator–prey interaction of untreated or PGD cells of the *Blepharisma* species against one multicellular (*S. sphagnetorum*) predator and two unicellular predators (*C. hirtus* and *S. roeseli*). A significant difference in prey survival between untreated and PGD cells of *B. japonicum*, *B. stoltei* or *B. undulans* was observed in mixtures involving *C. hirtus* as predator. After 2 h, all untreated *B. japonicum* (5, *n* = 6), *B. stoltei* (5,

n = 6), and *B. undulans* cells (5, *n* = 6) lived, while the surviving PGD cells of *B. japonicum* (3.33 ± 0.2 , *n* = 6), *B. stoltei* (3.67 ± 0.2 , *n* = 6) and *B. undulans* (1.00 ± 0.4 , *n* = 6) were significantly fewer, strongly suggesting the presence of a chemical defense system in untreated *Blepharisma* cells, against *C. hirtus* (Figure 2).

Also in the mixtures involving groups of 10 prey, after 2 h, significant differences were observed in survival values between untreated cells of *B. japonicum* (9.83 ± 0.17 , *n* = 6), *B. stoltei* (10, *n* = 6), *B. undulans* (10, *n* = 6) and PGD cells of *B. japonicum* (7.17 ± 0.40 , *n* = 6), *B. stoltei* (8.17 ± 0.48 , *n* = 6), *B. undulans* (3.83 ± 0.31 , *n* = 6). The apparent reduction in the predator's efficiency, if compared to the data obtained with groups of five prey, appears to be linked to the different ratios between predators and prey as already observed in interactions involving other ciliates (Buonanno et al. 2012, 2014). However, the predatory action of *C. hirtus* was appreciably more effective on PGD of *Blepharisma* cells than on control cells, with statistically significant results, in any observed ratios.

A comparable result is also observed after 24 h for *B. japonicum* and *B. stoltei* cells, while both untreated and PGD groups of cells of *B. undulans* disappeared, probably because in long-period interactions, the predator is able to overcome the defense of *B. undulans* (Figure 2).

Parallel predator–prey experiments were also performed using the microturbellarian *S. sphagnetorum* and the ciliate *S. roeseli* as predators. In the mixtures involving *S. sphagnetorum*, all untreated or PGD cells of the three *Blepharisma* species disappeared after 2 h, suggesting that the chemical defense is ineffective against this predator (data not shown).

S. roeseli is a sedentary ciliate which beats its adoral cilia rhythmically to drive food, such as bacteria or other protists, into its cytostome. It has been systematically observed (20 independent experiments) that this ciliate is not able to ingest specimens of *B. japonicum* or *B. stoltei*, due to the large cell body of

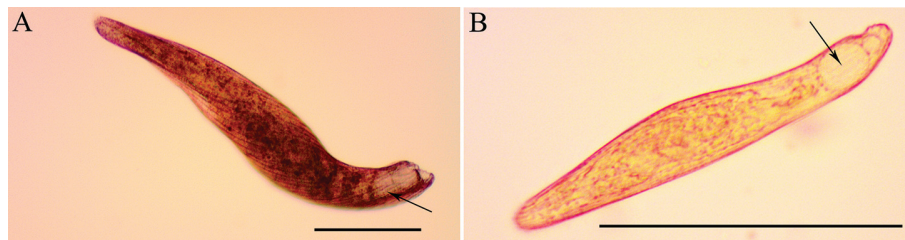


Figure 1. Micrographs of living cells of **A**, *Blepharisma stoltei* and **B**, *Blepharisma undulans*. The pigment granules are slightly visible as red/pink dots (arrows) located in the region between ciliary lines, in correspondence to large and transparent contractile vacuoles at the posterior end of the ciliates. Scale bars = 50 µm.

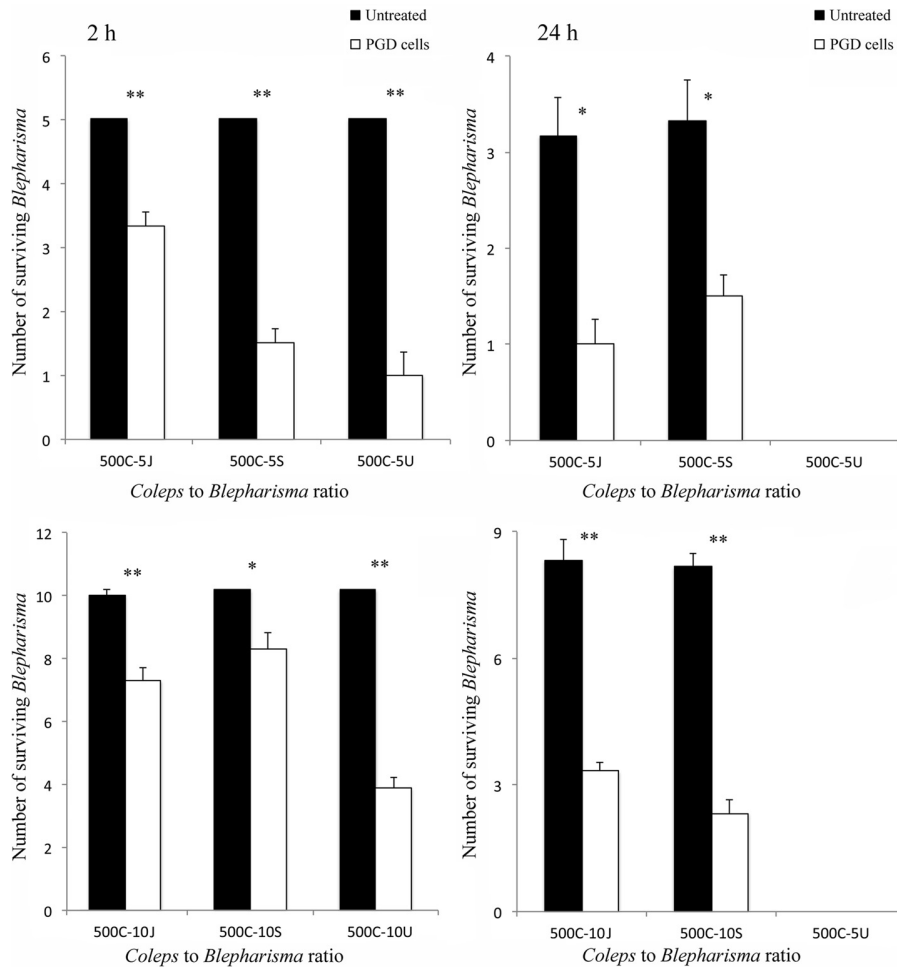


Figure 2. Effect of pigment granule removal in cells of *Blepharisma japonicum*, *Blepharisma stoltei* and *Blepharisma undulans*, obtained by cold-shock treatment, on the predator–prey interaction against *Coleps hirtus*. Groups of five or 10 cells of either pigment granule-deprived (PGD) or untreated cells of *B. japonicum*, *B. stoltei* or *B. undulans* were mixed with groups of 500 cells of *C. hirtus* (the 500C-5J, 500C-5S, 500C-5U and 500C-10J, 500C-10S, 500C-10U, respectively). Each bar represents the mean (\pm standard error, SE) of six independent experiments. * $P < 0.005$ and ** $P < 0.0001$.

these two species, whereas it is able to ingest the substantially smaller specimens of *B. undulans* (both untreated and PGD cells). Although *S. sphagnetorum* and *S. roeseli* seem to overcome *Blepharisma*'s chemical defense, it was observed that after the ingestion of intact cells of the toxic ciliates these predators are not able to reproduce, suggesting the presence of post-ingestion toxicity phenomena. To investigate whether *S. sphagnetorum* could be affected by the toxic pigments of *B. undulans*, one specimen of the predator was fed with 150 cells of *B. undulans* or with 150 cells of *Paramecium multimicronucleatum* (a non-toxic prey was used as control) in 500 μ L of SMB, and the number of specimens of *S. sphagnetorum* was counted after 120 h. As shown in Figure 3, *S. sphagnetorum* fed with *B. undulans* can reproduce only

when fed with *P. multimicronucleatum*, whereas this ability is lost when it is fed with *B. undulans* and some predator specimens start to die after 48, strongly suggesting the presence of post-ingestion toxicity phenomena in the turbellarian. Similar results were also obtained using *S. roeseli* as predator (data not shown).

These results are in apparent contrast with the data collected in a previous study, where it was observed that *S. sphagnetorum* is able to recognize and avoid toxic prey such as the ciliates *Spirostomum teres* Claparede et Lachmann, 1858 or *Spirostomum ambiguum* Ehrenberg, 1835 (Buonanno 2011). Nevertheless, in the interaction with *Blepharisma*, the toxic pigments do not immediately affect the microturbellarian as in the case reported above involving *S. teres* or *S. ambiguum*,

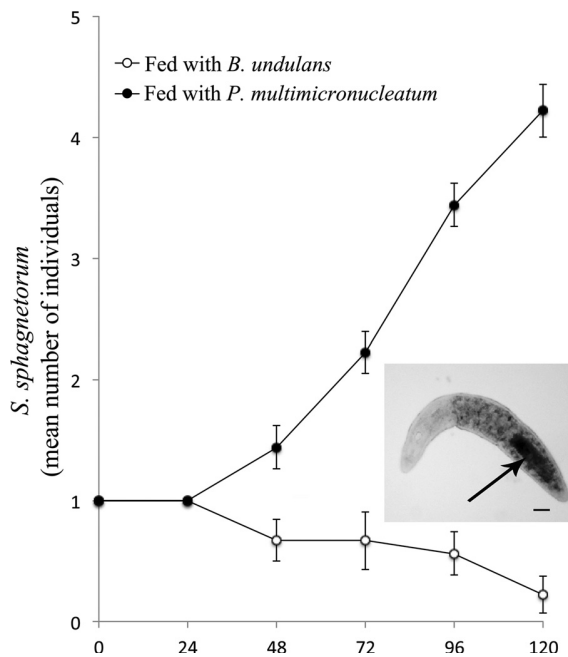


Figure 3. Comparison between growth curves of *Stenostomum sphagnetorum* fed either with *Blepharisma undulans* or with *Paramecium multimicronucleatum*. One specimen of *S. sphagnetorum* was mixed with 150 cells of *B. undulans*, or with 150 cells of *P. multimicronucleatum* as control. The specimens of *S. sphagnetorum* were counted for 120 h. Each point represents the mean (\pm standard error, SE) of nine independent experiments. Inset, a specimen of *S. sphagnetorum* with engulfed cells of *B. undulans* (arrow) visible as a dark area in the intestine. Scale bar = 50 μ m.

and a long time of “exposure” to the ingested prey is needed to damage *S. sphagnetorum*.

Overall, these results show that like *B. japonicum*, *B. stoltei* and *B. undulans* also present a chemical defense mechanism against predators.

BPs of *B. stoltei* and *B. undulans*

Toxin-enriched supernatants (TES) containing the discharge from the pigment granules of *B. japonicum*, *B. stoltei* and *B. undulans* were fractionated by reverse-phase chromatography (RP-HPLC) to obtain the elution profiles reported in Figure 4. We can reasonably state that pigments isolated from *B. stoltei* and *B. undulans* are equivalent to BPs found in *B. japonicum*, as they showed (a) the same retention times under RP-HPLC, (b) the same m/z under low- and high-resolution MS, and (c) the same UV-visible spectra recorded for BPs of *B. japonicum*.

For each species, the mean relative abundances of BPs obtained from a suspension of 100,000 cells were as follows:

B. japonicum: BP-A, 76 μ g; BP-B, 287 μ g; BP-C, 331 μ g; BP-D, 58 μ g; BP-E, 133 μ g. *B. stoltei*: BP-A,

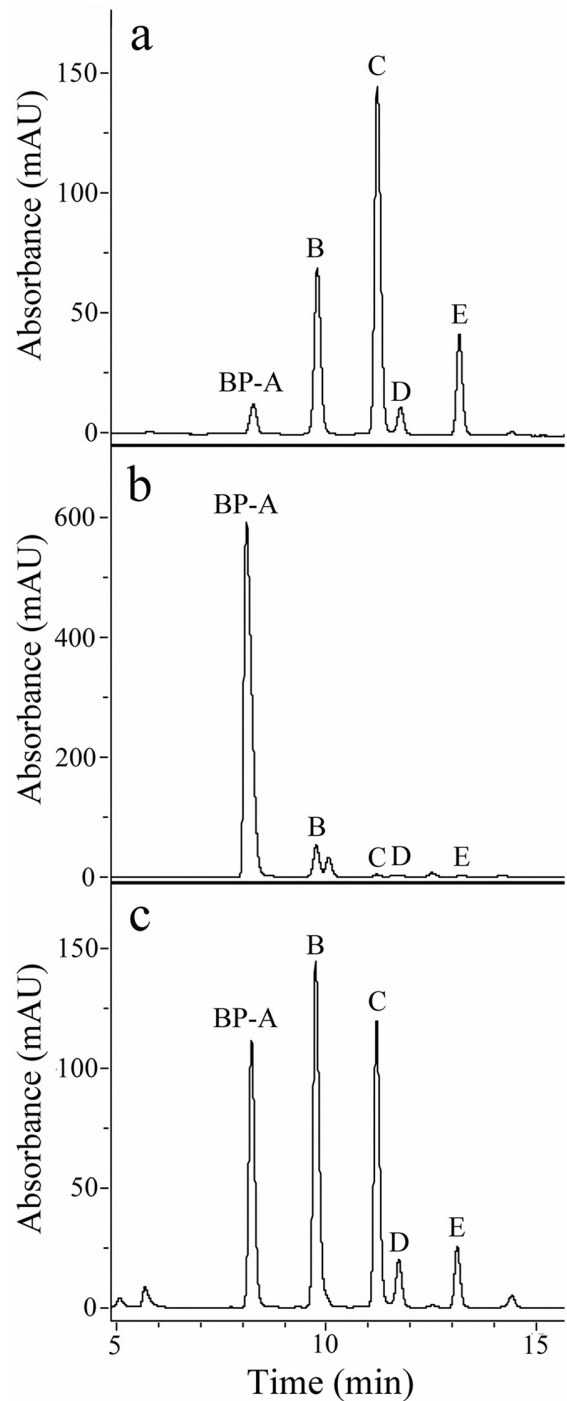


Figure 4. HPLC fractionation of the pigmented toxin enriched supernatant (TES) of (a) *Blepharisma japonicum*, (b) *Blepharisma stoltei*, and (c) *Blepharisma undulans*.

1.3 mg; BP-B, 179 μ g; trace amounts ($\geq 1\%$ of total BPs) of BP-C, -D, and -E. *B. undulans*: BP-A, 285 μ g; BP-B, 322 μ g; BP-C, 297 μ g; BP-D, 69 μ g; BP-E, 87 μ g.

It is worth noting that, differently from the situation described for other ciliate genera, such as *Euplotes* Ehrenberg, 1831, *Pseudokeronopsis* Borror & Wicklow, 1983, *Spirostomum* Ehrenberg, 1834 or *Stentor* Oken, 1815, in which different species can produce diverse or even biogenetically distant specific secondary metabolites (Guella et al. 2010; Buonanno 2011; Buonanno et al. 2012, 2017; Höfle et al. 2014; Sera et al. 2015; Anesi et al. 2016), in the genus *Blepharisma* different species appear to conserve equivalent structures, although produced in different proportions.

Toxicity of BPs

To evaluate the contribution of each of the five BPs present in the pigment granules of *Blepharisma* to the toxic effects of their discharge, we analyzed the effects of purified aliquots of these molecules on four species of grazing and raptorial ciliates, and on one microturbellarian predator. For this purpose, a set of dose–response experiments was performed and the results indicated that all BPs exerted an appreciable toxic effect on all species examined (Table I).

BP-A proved to be the most toxic compound against ciliates, with an LC₅₀ ranging from 0.8 to 5.15 µg/mL. Similar to BP-A, BP-B also showed high toxicity against target ciliates (LC₅₀ from 1.01 to 10.94 µg/mL), whereas BP-C, -D and -E showed a weaker toxicity (LC₅₀ from 1.03 to 24.28 µg/mL), particularly against *E. aediculatus* and *S. roeseli* (LC₅₀ from 11.38 to 24.28 µg/mL). Comparable values of toxicity were observed for all BPs against *S. sphagnetorum* (LC₅₀ from 20.45 to 23.89 µg/mL). In addition, it was observed that *B. japonicum*, *B. stoltei* and

B. undulans are substantially immune to all BPs, showing an LC₅₀ > 100 µg/mL (data not shown).

Interestingly, BP-A is particularly abundant in *B. stoltei* that also produces an appreciable amount of BP-B, and trace amounts of the other BPs. It is likely that in this ciliate the defensive role is essentially played by BP-A, especially in consideration of its relative abundance, with the less-represented BP-B primarily mediating the step-up photophobic response of the ciliate, according to the function proposed for this BP in *B. japonicum* by Matsuoka et al. (1997).

Even if the two most hydrophilic BPs, BP-A and BP-B, showed the highest activity, it appears that exposure to toxic concentrations of each purified BP can result in lethal damage to target organisms, as shown in Table I. This observation is in full accord with the cytotoxic mechanism previously proposed for all BPs, which proves capable of aggregating in clusters of different sizes and forming channels of different conductance in planar phospholipid bilayers (Muto et al. 2001). Assuming that the same action mechanism may also occur *in vivo*, resulting in the formation of transmembrane pores and subsequent cell lysis, channel formation might be indicated as the main physiological effect of BPs on both unicellular and multicellular organisms.

Conclusion

The data collected in this work show that three species of *Blepharisma* share a common panel of multifunctional secondary metabolites (BPs), whereas intra- and interspecific molecular diversity appears to be a constant for the secondary metabolites characterized in the other genera of ciliates

Table I. Comparison of the toxic effects of the five purified blepharismins (BP-A; BP-B; BP-C; BP-D and BP-E) on the ciliates *Coleps hirtus*, *Euplotes aediculatus*, *Paramecium multimicronucleatum* and *Stentor roeseli* and on the microturbellarian *Stenostomum sphagnetorum*. Viability was assessed after 24 h of incubation and the LC₅₀ values were obtained by nonlinear regression analysis of three independent experiments, with the 95% confidence limits calculated using GraphPad Prism 6 software.

	Cytotoxicity at 24 h (LC ₅₀ µg/mL; 95% CI)				
	BP-A	BP-B	BP-C	BP-D	BP-E
Ciliated protists					
<i>Coleps hirtus</i>	0.80 (0.78–0.81)	1.01 (0.91–1.11)	1.21 (0.87–1.68)	1.03 (0.72–1.47)	1.39 (1.03–1.87)
<i>Euplotes aediculatus</i>	4.52 (2.41–8.47)	8.18 (7.03–9.52)	11.38 (8.85–14.67)	11.61 (8.11–16.62)	11.65 (3.32–40.82)
<i>Paramecium multimicronucleatum</i>	1.87 (1.69–2.06)	2.16 (1.79–2.61)	2.57 (1.82–3.63)	2.70 (0.71–10.21)	4.63 (3.34–6.43)
<i>Stentor roeseli</i>	5.15 (2.94–9.02)	10.94 (9.61–12.46)	24.28 (4.22–139.7)	18.21 (0.02–172.0)	17.95 (3.01–107.4)
Metazoan					
<i>Stenostomum sphagnetorum</i>	20.45 (15.88–26.32)	21.56 (19.50–23.83)	23.28 (20.12–26.94)	22.64 (19.83–25.84)	23.89 (23.89–23.89)

LC: lethal concentration; CI: confidence index.

investigated previously. The conservation of this panel of secondary metabolites can be observed even in the limited case of *B. stoltei*, where two BPs are predominant, but the presence of all five BPs is confirmed, suggesting that distinct roles for these molecules are likely required at least for the fine control of photophobic reactions, as proposed by Matsuoka et al. (1997). Otherwise, with regard to the defensive role ascribed to the title compounds, our data confirm that each of the five BPs can exert an effective toxic/lethal effect on raptorial ciliates or multicellular predators, in full accord with the action mechanism proposed by Muto et al. (2001). Here we focused our attention on three pigmented species of *Blepharisma*, but an extension of the analyses among this genus to secondary metabolites produced by colorless species, such as *B. hyalinum* Perty, 1852, may pave the way to an increased body of data and to obtain a more representative picture of the metabolomics of single-celled ciliated eukaryota.

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