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Predator-Prey Interactions in Ciliated Protists

Federico Buonanno and Claudio Ortenzi

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Abstract

Protists appeared relatively early in evolution, about 1.8 billion years ago, soon after the first prokaryotic organisms. During this time period, most species developed a variety of behavioral, morphological, and physiological strategies intended to improve the ability to capture prey or to avoid predation. In this scenario, a key role was played by specialized ejectable membrane-bound organelles called extrusomes, which are capable of discharging their content to the outside of the cell in response to various stimuli. The aim of this chapter is to describe the two main strategies adopted in ciliate predator-prey interactions: (a) the first is mediated by mechanical mechanisms and involves, for example, extrusomes called trichocysts and (b) the second is mediated by toxic secondary metabolites and involves different kinds of chemical extrusomes.

Keywords: protists, ciliates, extrusomes, secondary metabolites, chemical offense

1. Introduction

A common definition for predatory behavior describes it as the process through which one animal, the predator, captures and kills another animal, the prey, before eating it in part or entirely [1]; however, according to the opinion of a number of microbiologists and protistologists, this definition should be also extended to different organisms included in other life Kingdoms, with particular regard to microorganisms. Indeed, especially in the last 30 years, a lot of studies have been devoted to describing the predator-prey interactions among unicellular eukaryotic organisms like protists. Whittaker [2] originally defined protists as those “organisms which are unicellular or unicellular-colonial and which form no tissues,” and for this reason they must carry out at the cellular level all the basic functions which can be observed in multicellular eukaryotes. Among these functions, self-nonsel

mechanisms are represented by a large repertoire in protists and can trigger either autocrine or paracrine processes in some ciliates (see [3] for a review), together with the capability to detect prey (food) or predators in others. In this regard, it is known that protists have developed a variety of strategies of feeding behaviors especially in response to different environmental factors, together with a diverse kind of food available in micro-habitats. **Figure 1** shows a general scheme of predator-prey interactions, where the predator recognizes the presence of the prey (step 1) and can attack it (step 2). On the other hand the prey recognizes the presence of the predator (step 1') and it can organize its defense mechanisms (step 2') [4]. This scheme should be considered functional for both animals and protists, and indeed several studies have shown that the food recognition and the offense-defense mechanisms adopted by some groups of protists can be compared, in terms of complexity and variability, with those observed in animals.

In this context, a common feeding mechanism found in heterotrophic protists is phagocytosis, a process which requires specific organelles for food assimilation and which occurs in three steps: food capture, phagosome formation, and food digestion [5]. Different techniques of phagocytosis have been described in various protists, where they have especially been investigated in ciliates [5–7]. Verni and Gualtieri [5] describe three main phagocytotic processes in ciliates: filter feeding, suctorial feeding, and raptorial feeding. The authors compare them to the strategies used in fishing, like netting, trapping, and harpooning. In filter-feeding ciliates, the food, represented by small organisms or edible debris of various types, was pushed into the ciliate buccal cavity by the rhythmical beats of the cilia located in its adoral apparatus. Suctorial-feeding ciliates are represented by sessile or sedentary species that for most of their lives remain attached to other organisms or various substrates, intercepting the food particles with their specialized tentacles. Finally, raptorial ciliates are able to directly catch other organisms using peculiar organelles to paralyze and/or kill their prey, generally called extrusomes.

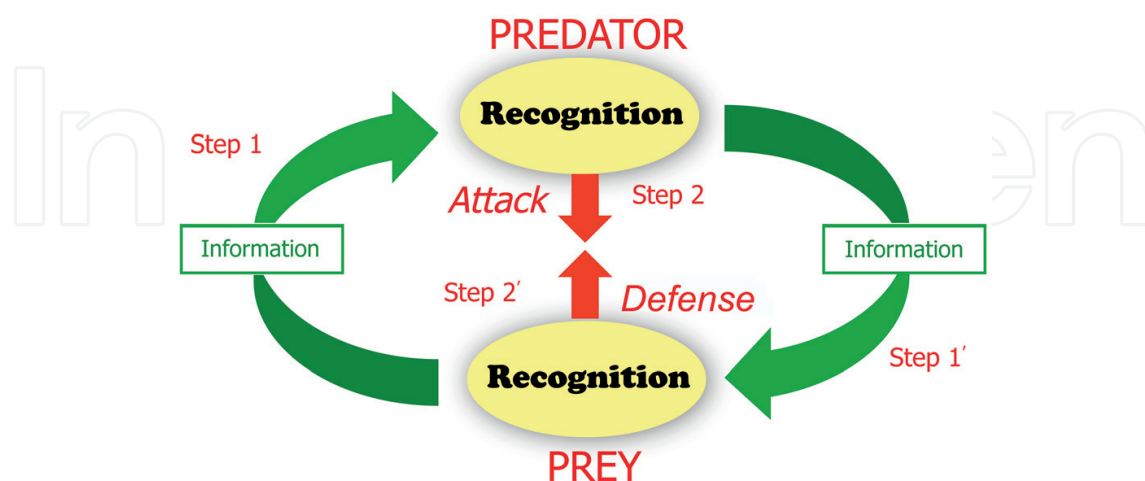


Figure 1. General scheme of predator-prey interactions. Redrafted from [4].

2. Extrusomes, the specialized organelles for predator-prey interaction

The term “extrusome” was proposed, for the first time, by Grell in 1973 for extrusive (ejectable) bodies, which occur widely in protists [8]. They are membrane-bound organelles usually located in the cell cortex, attached to the cell membrane. They can display differences in structure and morphology, but they share the common characteristic of discharging their contents to the outside of the cell in response to mechanical or chemical stimuli. Remarkably, when the extrusomes are discharged, the cell remains intact and functional. Studies on extrusomes and related organelles have been reviewed by Hausmann [9], Dragesco [10], Kugrens et al. [11], Hausmann and Hülsmann [12], and Rosati and Modeo [13]. Typical examples of these organelles include toxicysts, trichocysts, mucocysts, cortical, or pigment granules in ciliates and flagellates, haptocysts in suctorians, and kinetocysts in heliozoan actinopods. Some extrusomes are known to be related in predator-prey interactions, for example, to catch and kill the prey (such as toxicysts, haptocysts, kinetocysts, and some cortical granules), or used as defensive organelles (such as the trichocysts and various cortical or pigment granules), but the role of other kinds of extrusomes such as the mucocysts in *Tetrahymena* or the trichites in Strombidiidae [13] still remains obscure.

3. Offensive extrusomes

Offensive extrusomes generally possessed by raptorial protists and located usually at or near the feeding apparatus are discharged after contact with a possible prey, which is immobilized, damaged, or firmly bound to the predator. Among these, organelles, certainly the most widely studied, belong to the category of toxicysts (toxic extrusomes) and they play an essential role in capturing and killing prey [7, 13]. Toxicysts are synthesized in Golgi or ER vesicles and are usually localized in the cell cortex attached to the cell membrane. Most of them are observed in species belonging to the class Litostomatea and subclass Haptoria, but they are also present in other predatory ciliates. They are usually positioned in a specific region of the cell, near the oral apparatus, and generally in the first portion which contacts the prey during the raptorial feeding [13]. Independently of the specific differences in the morphology of the cytostome, the toxicysts are always present in an appreciable number, for example, in the genera *Didinium*, *Dileptus*, *Prorodon*, *Litonotus*, *Colpes*, *Homalozoon*, and many others. In resting position, the toxicysts appear generally as rod-like elements (**Figure 2**), and could be discharged in milliseconds, if exposed to an appropriate stimulus such as contact with a prey (**Figure 3**) [7]. In this case, the tubules of the toxicysts are suddenly introduced into the cytoplasm of the prey's body, like hypodermic needles, to inject the toxic material. Hausmann [7] reports essentially two ways by which the toxicysts may be discharged: in the first case, there is a fusion of the toxicyst's membrane with the plasma membrane, followed by the tubule discharge via evagination; in the second, observed in certain ciliate species, a telescopic

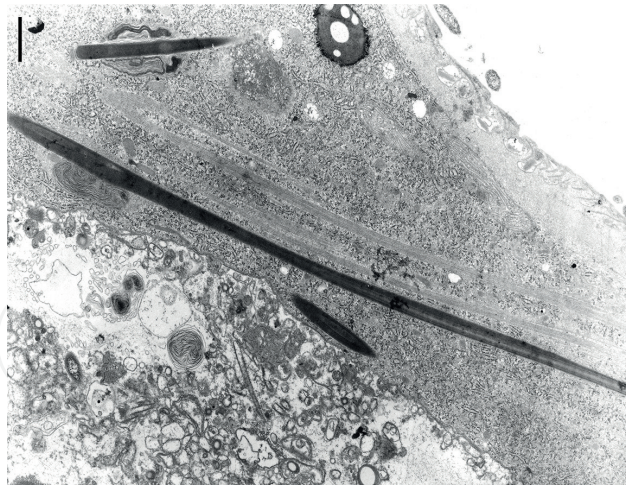


Figure 2. Transmission electron microscope (TEM) picture of the toxicysts in a dividing cell of a ciliate *Didinium nasutum*. Scale bar = 1 μm . Original picture by R. Allen from <http://www.cellimagelibrary.org/images/10010>.



Figure 3. Predatory behavior of *Coleps hirtus* on *Pseudokeronopsis erythrina*. The predator attacks the prey with its toxicysts (arrow). Micrograph extracted from a film clips. Scale bar = 200 μm .

discharge of the tubules was observed. During or near the end of the toxicysts' discharge, the toxic secondary metabolites were secreted by the tubules. It is worth noting that this mechanism of discharging toxic substances shows the structural and functional similarities that can be found between the toxicysts in ciliated protists and nematocysts in Cnidaria, despite the substantial differences in size [7].

In contrast with recent and less recent studies about the nature of the toxic secondary metabolites used by ciliates in chemical defense, no exhaustive data are yet available about the composition of the toxins stored in the toxicysts of predatory ciliates. This is essentially due to the difficulty in separating the content of extrusomes from other molecules produced by the ciliate, in order to purify them at homogeneity for subsequent chemical and structural analyses.

To date the presence of acid phosphatase has been demonstrated in the toxicysts of *Didinium nasutum* [14] and four other raptorial ciliates such as *Enchelys mutans*, *Lacrymaria olor*, *Homalozoon vermiculare*, and *Pseudoprorodon niveus* [15]. It has been supposed that this enzyme, generally present in lysosomes of animal cells, may probably be used by these ciliates to start the digestion of the prey.

3.1. The predatory behavior of *Coleps hirtus*

The complete analysis of the content of the toxicysts, together with observations of the predatory behavior, was also performed on another species, *Coleps hirtus*, a freshwater protostomatid ciliate. *C. hirtus* (40–65 × 20–35 μm) has an oral apparatus placed at the anterior end of the cell and its barrel-shaped body is covered by calcified armor arranged in plates. This ciliate is able to feed off bacteria, algae, flagellates, and ciliates, but it is also histophagous, that is, it feeds on living plant and animal tissue such as rotifers, crustaceans, and fish [16, 17]. *Coleps* is also reported to show a scavenger feeding on tissues of dead metazoans, such as *Daphnia*, *Diaphanosoma*, and chironomid larvae [18], as well as toward dead ciliates and dead specimens of its own species. *Coleps* is usually equipped with toxicysts used by the ciliate to assist its carnivorous feeding, and its predatory behavior has recently been analyzed against another ciliate species used as prey, *Euplotes aediculatus*. Observations conducted on a mixture of predators and prey showed several contacts between the specimens of *Colpes* and *Euplotes*, but only after 5–10 min did interactions between the anterior section of a predator with a specimen of *Euplotes* become effective. This time was probably essential for prey detection and recognition, followed by prolonged contact between predator and prey, generally ending with the rapid backward swimming of the latter which separated the two organisms. When the attacks became numerous some individuals of *Coleps* remained attached to their prey (**Figure 4**), which decreased their swimming speed and gradually stopped swimming. After 20–30 min, the prey was fragmented and eaten by several specimens of *Coleps*, and a similar predatory behavior was also observed using different ciliate species as prey [19]. On the contrary the toxicysts-deficient specimens of *Colpes* (**Figure 5**) obtained by means of the application of the cold-shock method capable of inducing an exclusive massive discharge of extrusomes in ciliates [20] appear unable to catch and kill their prey [19].

Unexpectedly, the analysis of the bioactive fraction of the toxicyst discharge of *Coleps hirtus* (performed by liquid chromatography-electro-spray-mass spectrometry and gas chromatography-mass spectrometry) showed the presence of a mixture of 19 saturated, monounsaturated and polyunsaturated free fatty acids (FFAs) with the addition of a minor amount of a diterpenoid (phytanic acid) but did not reveal the presence of enzymes, as reported for other



Figure 4. Multiple attacks by different cells *Coleps hirtus* on a cell of *Euplotes aediculatus*. Micrograph extracted from a film clips. Scale bar = 200 μm .

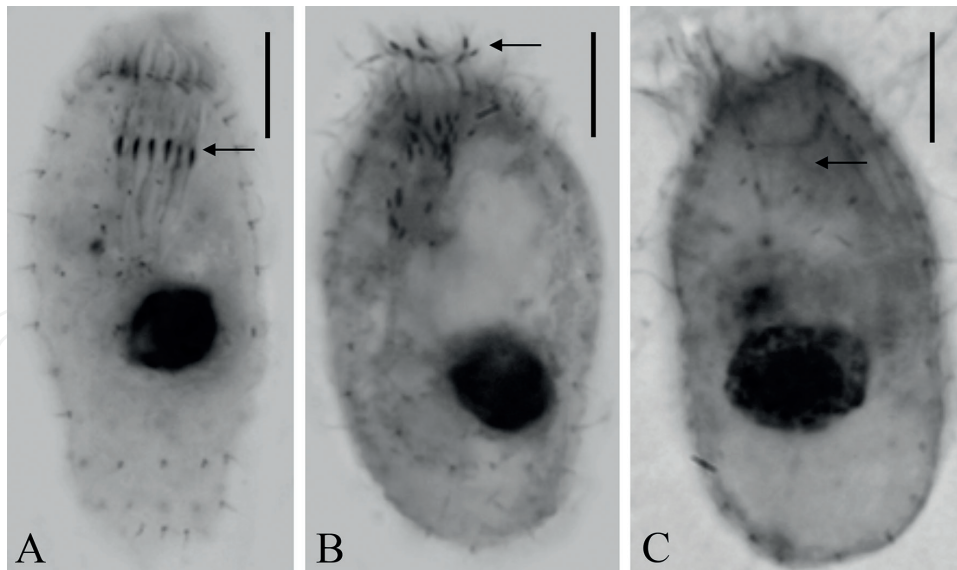


Figure 5. (A) The toxicysts in *Coleps hirtus* appear as rod-shaped organelles (arrow) in the oral basket of a cell. (B) The photomicrograph shows the toxicysts discharged (arrow) into the medium, immediately after a cold-shock treatment. (C) No toxicysts are detected in a toxicyst-deprived cell. Photomicrographs of fixed specimens by protargol stain. Scale bar = 10 μm . Pictures from [19].

predatory ciliates [19]. To date this is the only report on the presence of FFAs as toxic substances in the extrusomes of ciliated protists, but the use of this class of compounds as toxins by *Coleps* is shared with at least 15 freshwater, 13 marine, and 6 brackish water potentially

harmful microalgae, as well with some multicellular organisms. For example, a chemical defense by a mixture of FFAs was studied and demonstrated for the harmful microalga *Fibrocapsa japonica* (Raphidophyceae) [21–23], and also in animals, a defensive strategy mediated by FFAs was recently described for the fish *Barbus barbuis* which adopted it to protect its eggs from predators [24].

Very little is known about the role and source of phytanic acid in ciliates, this being the additional component detected in the toxicyst discharge of *Coleps*. Phytanic acid can be produced from the biodegradation of the side chain of chlorophyll [25], so one possible source arises from *Coleps*' carnivorous feeding on photosynthetic microorganisms [19]. Some insects, such as the sumac flea beetle, accumulate chlorophyll-derived metabolites as a chemical deterrent in excrements [26]. Komen et al. [27] demonstrated the toxic effect of phytanic acid on human skin fibroblasts, where it impaired mitochondrial respiration through protonophoric action. Regarding the role of phytanic acid in *Coleps*, it is possible to hypothesize that it can be used as a weapon, deterrent, or, at least, it could be stored in toxicysts given its potential toxic activity. In addition, it is known that ciliates themselves are also able to synthesize a huge number of terpenoids [28, 29]. This is the case of *Euplotes focardi* [30] and *Euplotes rariseta* [31] where the production of new diterpenoids was demonstrated. Terpene compounds and FFAs may also act together to exert cytotoxic effects [19]. FFAs may serve as a matrix to deliver toxic compounds to prey or predators and also to create a perfect environment where toxic metabolites can exert their functions.

It has been demonstrated that the substances discharged from the toxicysts by *Coleps* are highly toxic for a number of ciliate species such as *Euplotes aediculatus*, *Paramecium tetraurelia*, *Spirostomum teres*, and *S. ambiguum* or *Oxytricha* sp. [19], and their action mechanism appears to be related to a necrotic process. The term necrosis refers to a rapid (unprogrammed) cell death, with plasmatic membrane rupture, often caused by external factors such as toxins. On the contrary, the apoptosis is programmed cell death characterized by nuclear condensation, cytoplasmic shrinkage, and disintegration of the cell into small, membrane-bounded fragments. As shown in **Figures 6** and **7**, the purified toxin from *Coleps* is able to induce rapid cell death in *E. aediculatus* and in *S. ambiguum* preceded by cell membrane fracture without any changes in the morphology of the macronucleus. An action mechanism of this type seems to be a “good choice” for *Coleps* as it induces paralysis and a very rapid death in the prey.

Interestingly, the cells of *Coleps* can also be damaged if exposed, *in vitro*, to their own toxin discharge [19]. Nevertheless, this cannot occur in nature, because on the one hand, the toxins are stored in the toxicysts of the ciliate, thus avoiding autotoxicity and on the other hand, the accidental exposure of *Coleps* to the toxicyst discharge dissolved in the medium is also unlikely, due to the choice of the predator to directly inject the toxins into the prey [19]. In this context, it is worth remembering the peculiar predatory behavior of *Coleps*, which usually leads to the observation that the same prey undergoes multiple attacks by several raptorial specimens, a behavior also adopted against young larvae of zebrafish [17]. It is likely that this behavior has evolved to ensure a fast immobilization of the prey, that after simultaneous multiple attacks, it can easily accumulate lethal concentrations of toxins injected by numerous predators. Therefore, essentially for the “wolf-like” group hunting behavior of *Coleps*, the species that appeared relatively resistant to its toxicyst discharge may also be easily caught and killed.

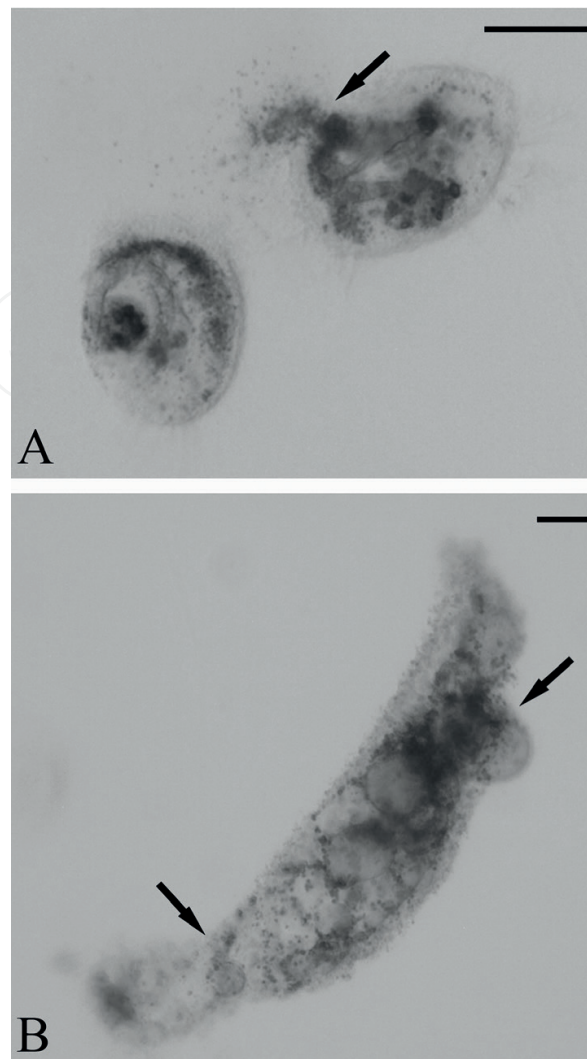


Figure 6. (A) Necrotic effects of the toxicyst discharge of *Coleps hirtus* on *Euplotes aediculatus* and (B) *Spirostomum ambiguum*. Arrows indicate the cell-membrane fractures. Scale bar = 100 μm . Pictures from [19].

3.2. *Didinium nasutum*, a specialized hunter

Differently to *Coleps*, other ciliate species have specialized in hunting and catching a few preferential prey. This is, for example, the case of *Didinium nasutum* that is capable of capturing and killing several species of *Paramecium* and few other ciliates. Generally, *Paramecium* species are able to defend themselves by means of mechanical extrusomes like trichocysts (that will be discussed later on this chapter) but *Didinium* seems to overcome the defense of *Paramecium* by means of a highly specialized combination of extrusomes. Present on the proboscis of *Didinium* are several units of two different kinds of extrusomes: toxicysts, as in other Litostomatea, and pexicysts, another specialized offensive extrusome observed only in this species [32]. These authors describe the discharge of pexicysts as the first response after the prey recognition [14], which is typically followed by the discharge of toxicysts. At the same time, the prey (generally a *Paramecium*) discharges its trichocysts which separate the two organisms, but the proboscis of *Didinium* remains attached to the prey by a tiny connection probably composed of a bundle of discharged pexicysts and toxicysts (**Figure 8**).

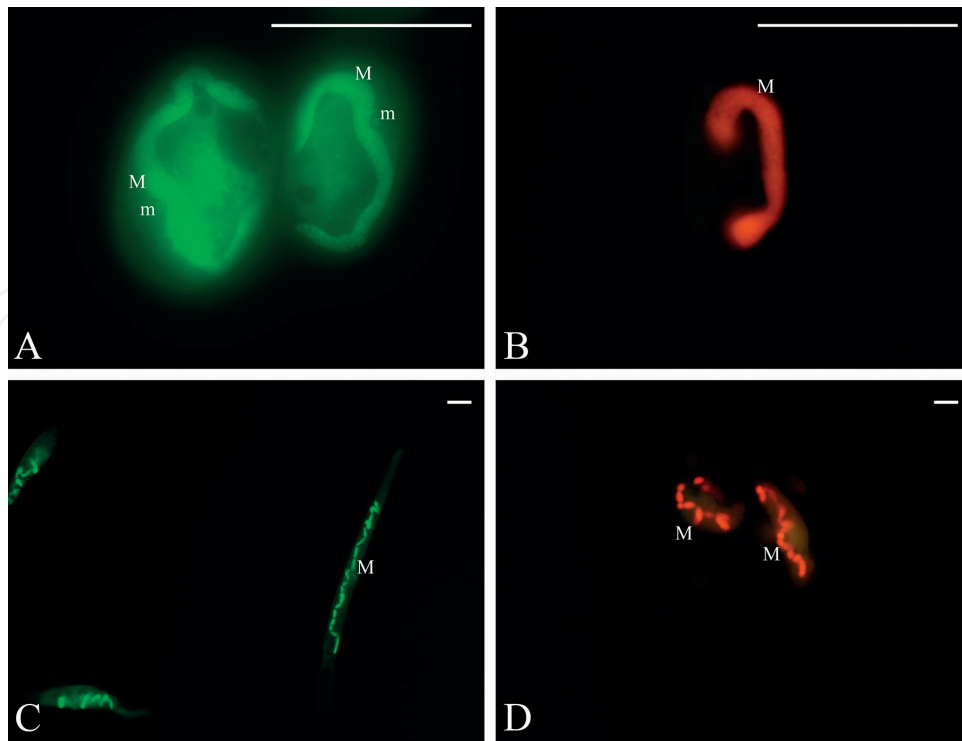


Figure 7. (A, B) Effects of the toxicyst discharge of *Coleps hirtus* on the macronuclear morphology in specimens of *Euplotes aediculatus* and (C, D) *Spirostomum ambiguum*. Cells were stained with acridine orange and ethidium bromide and observed by fluorescent microscopy. Viable cells show intact, bright green nuclei, nonviable cells show red/orange nuclei. M = macronucleus, m = micronucleus. Scale bar = 100 μ m. Pictures from [19].

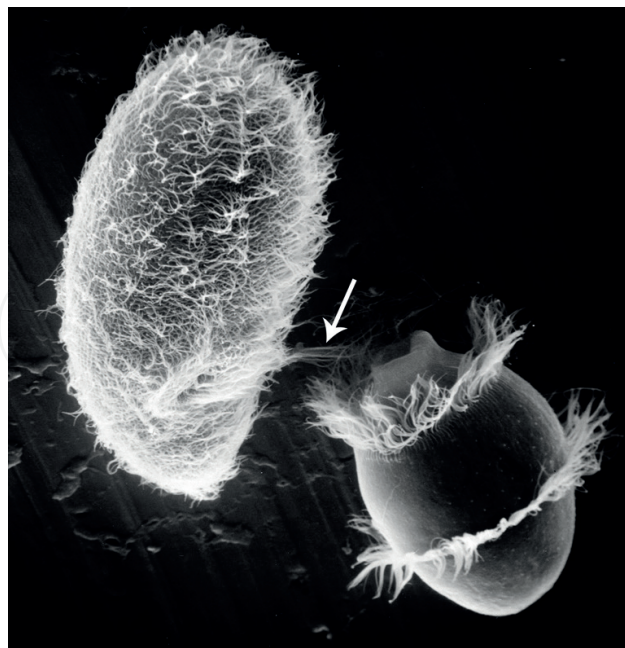


Figure 8. Scanning electron microscope (SEM) picture on the predator-prey interaction between a cell of *Didinium nasutum* and a cell of *Paramecium multimicronucleatum*. The bundle of toxicysts and pexicysts can be seen between the two organisms (arrow). Magnification $\times 50$. Original picture by G. Antipa from <http://www.cellimagelibrary.org/images/21991>.

Subsequently, the *Paramecium* will be reached again and captured by the predator. In the light of this observation, the pexicysts seem to act most by a mechanical function (as harpoon-like organelles) rather than with a chemical offense. This assumption is supported by the fact that another species of predatory ciliate, *Monodinium balbiani*, which is morphologically similar and phylogenetically close to *Didinium*, but without the presence of the pexicysts on its proboscis, unlike the *Didinium*, is sensitive to the defense mechanism possessed by *Paramecium*, which is often able to avoid capture [33].

3.3. The peculiar tentacles of suctorians

In this context it is also relevant to mention the subclass Suctoria, represented by ciliates which become sessile during development and consequently lose the ciliary structure. Suctorians are able to feed on other protists and frequently on other ciliates by means of specialized tentacles. The distal ends of these tentacles are often equipped by peculiar extrusomes called haptocysts that are involved in prey capture. When a tentacle touches a possible prey, the discharge of haptocysts is able to penetrate the prey's membrane, forming a connection between the predator and the prey and injecting the extrusome content into the latter, which also concurs to the fusion of the membranes belonging to the two organisms [13, 34]. However, the fusion of the two membranes is not always immediate, for example, in *Heliophrya erhardi*, Spoon et al. [35] observed that many specimens of *Paramecium* contacting the tentacles of the suctorian escaped discharging trichocysts at the point of contact, suggesting that *Paramecium* is able to defend itself from the puncture of the haptocysts.

4. Defensive extrusomes

In addition to predatory behavior, ciliated protists have also evolved different defense strategies, many based on the discharge of extrusomes. Two different mechanisms involved in their defense behavior are essentially observed: the first is mediated by the mechanical actions of trichocysts as in *Paramecium* or *Frontonia* and the second is mediated by the toxic secondary metabolites of different kinds of chemical extrusomes.

4.1. The mechanical defense

Spindle trichocysts (or simply, trichocysts) are spindle-shaped organelles which discharge their content in the form of a thread. They are found in some ciliates and flagellates and are sometimes furnished with a specially constructed tip [9]. The best known and studied trichocysts are those in the genus *Paramecium*. Trichocysts in *Paramecium* are 3–4 μm long, carrot-shaped membrane-bounded organelles armed with a sharply pointed tip, and are present in thousands all over the cell surface, except at the oral apparatus (**Figures 9** and **10**). When paramecia are subjected to various stimuli, the membranes of the trichocysts and the cell membrane blend together, and the content of the extrusomes is immediately discharged to the outside of the cell, forming a spear-like structure in milliseconds (**Figure 11**) (see [13] for a review). Trichocyst discharge has therefore been extensively studied as a model system

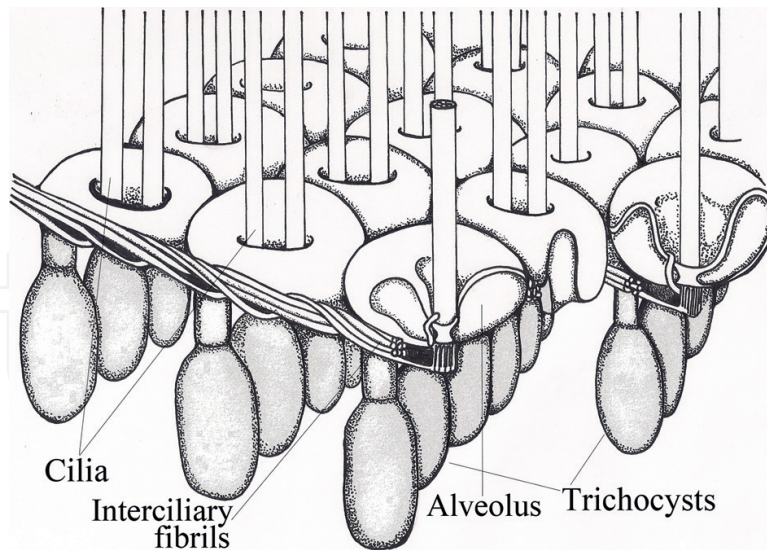


Figure 9. Scheme of the ciliary structure and the trichocysts of *Paramecium*. Picture from <http://biodidac.bio.uottawa.ca>, redrafted by R. D'Arcangelo.

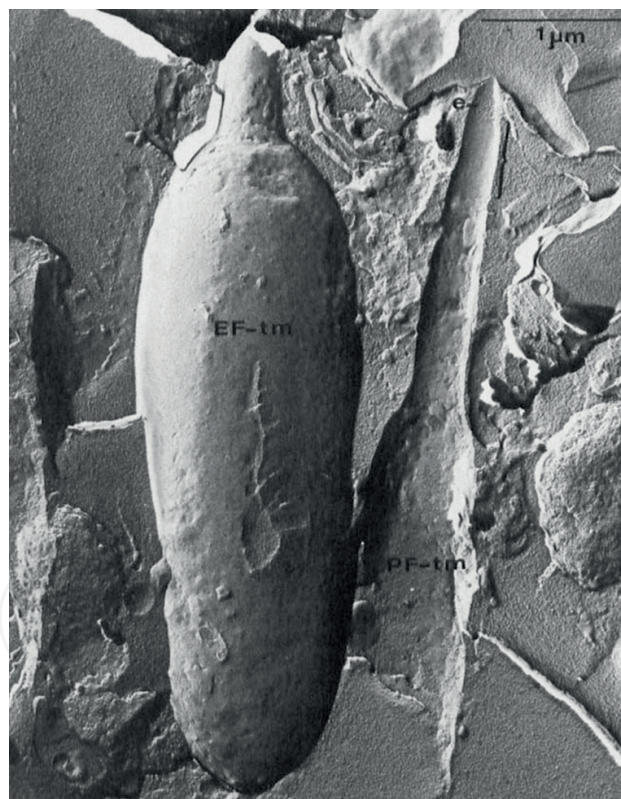


Figure 10. Membrane details of resting trichocysts under the freeze fracture. The trichocyst tip (tt) and body (tb) are covered by the same membrane. The A-face of this membrane (A-tin) possesses randomly distributed particles whereas the B-face (B-tin) shows corresponding depressions. Scale bar = 1 μ m. Picture from [9].

of exocytosis [36] (see [37] for a review). Synthesis, processing, and sorting of component proteins in trichocysts are also studied as model systems of protein biosynthesis [36] for a review.



Figure 11. The trichocysts discharged by a cell of *Paramecium tetraurelia* exposed to picric acid solution. Scale bar = 100 μm .

Maupas, one of the pioneers of protozoology, first proposed the defensive function of trichocysts in *Paramecium* in 1883, observing its morphological features and judging it as self-evident [38]; however, this point was questioned for years. The main controversy was due to the fact that *Paramecium* species are easily preyed upon by *Didinium* in spite of massive trichocyst discharge by paramecia. Pollack reported that *Didinium* preys on wild-type cells as easily as trichocyst-defective mutants in *P. tetraurelia* [39]. However, further studies have unequivocally indicated that trichocysts in *Paramecium* exert an effective defensive function against unicellular predators, including the raptorial protists *Dileptus margaritifer*, *Monodinium balbiani*, *Climacostomum virens*, *Echinosphaerium akamae*, and *E. nucofilum* [33, 40–43]. In addition, a more recent paper also analyzed the defensive function of trichocysts in *P. tetraurelia* against some microinvertebrate predators, such as a rotifer (*Cephalodella* sp.), an ostracod (*Eucypris* sp.), and a turbellarian flatworm (*Stenostomum sphagnetorum*) [44]. The results of this study show the success in the defensive function of trichocysts against the rotifer and the ostracod while the mechanism seems ineffective against the flatworm. The authors speculate that the efficiency of the defense by means of trichocysts depends essentially on the kind of prey-capture behavior displayed by the predators. In particular, the success of the defense mediated by trichocysts appears positively related to the time that the predator requires to capture and manipulate the prey before ingestion. Consequently, and different from the turbellarian flatworm that directly swallows paramecia, predators such as the rotifer and the ostracod that, prior to ingesting paramecia, contact it with a ciliated corona or articulated appendices, give the prey sufficient time to activate the trichocysts discharge that allows it to escape [44]. Essentially this looks like the same phenomenon observed during the interaction between *Paramecium* and the predatory ciliate *Dileptus margaritifer*, that attempts to paralyze its prey with the toxicysts on its proboscis before ingestion, thereby inducing an explosive extrusion of trichocysts by *Paramecium*, which then swims away [44]. In this regard, another interesting observation was made when *Paramecium* was placed in a cell-free fluid containing the toxic material derived from the toxicysts from *Dileptus* [45] (Miyake A. personal communication); indeed after contact with this toxic solution,

Paramecium cells violently reacted by immediately discharging most of their trichocysts before being killed. In this reaction, sometimes a single specimen (cell) of *Paramecium* was completely surrounded by its discharged trichocysts. When this occurred, the *Paramecium* survived long after other cells were killed, moving slowly in the narrow space in the capsule of discharged trichocysts. But when it happened that one of these encapsulated cells managed to squeeze out of the capsule, it was quickly killed. This observation suggests that discharged trichocysts of *Paramecium* function as a barrier against the *Dileptus* toxins and hence the locally discharged trichocysts in the *Paramecium-Dileptus* interaction function as an instant shield against *Dileptus*.

To summarize, the mechanical defense by trichocysts and related extrusomes appear to be multiple, including quick physical displacement, the thrust into a predator, and protection against the predator's toxins, increasing the chance for the prey to survive and escape. However, especially in ciliates and flagellates, other kinds of extrusomes used for defense were found, ones that, unlike trichocysts, are capable of discharging toxic materials in response to predatory behavior.

4.2. The chemical defense

Pigment granules (also called pigmentocysts) and **cortical granules** are extrusive organelles containing pigmented or colorless toxic material, respectively, and they were originally classified as a special type of mucocysts [9]. Pigment and cortical granules are mainly present in heterotrich and karyorelictean ciliates, such as *Blepharisma*, *Stentor*, *Loxodes*, and *Trachelonema*, but they may also exist in other groups of ciliates. They are usually present in great numbers throughout the cell cortex, sometimes providing bright colors to their bearers. Examples are *Stentor coeruleus*, whose coloration is due to the pigment called stentorin, and several red species of *Blepharisma*, whose coloration is due to blepharismins, formerly overall called zoopurpurin by Giese [46]. The coloration of these common heterotrichs has long attracted attention and most studies on pigment granules have been carried out using *S. coeruleus*, and a few red species of *Blepharisma*. *B. japonicum* (**Figure 12**) is the best studied species of the genus *Blepharisma* and it presents pigment granules usually in a size of 0.3–0.6 μm diameter, arranged in stripes between the rows of cilia that confer a red-pink coloration to the ciliate (**Figure 13**). These granules have been shown to contain a mixture of five compounds called blepharismins that are multifunctional quinone derivatives structurally related to hypericin, a photodynamic toxin of *Hypericum perforatum* (St. John's Wort), and stentorin, produced by the ciliate *S. coeruleus* [47, 48] (**Figure 14**). To date, two primary functions of blepharismins have been demonstrated: light perception and defense function against predators [47–52]. 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 blepharismins [53]. In addition to light perception, blepharismins were found to act as chemical weapons via their light-independent cytotoxic effect against predatory protozoans and methicillin-resistant Gram-positive bacteria [49, 50, 54]. A possible explanation for this cytotoxicity can be found in the capability of blepharismins to form cation-selective channels in planar phospholipid bilayers [51], a phenomenon also expected to occur in the cell membranes of microorganisms exposed to toxic concentrations of ciliate pigments.



Figure 12. External morphology of a living cell of *Blepharisma japonicum*. Scale bar = 100 μm .



Figure 13. Extrusive pigment granules in *Blepharisma japonicum* (arrow) visible as red/pink dots under a vacuole. Scale bar = 100 μm .

The defensive function of blepharismins was initially proposed by Giese in 1949 who found that crude extracts of *Blepharisma* were toxic to various ciliates but not to *Blepharisma* itself [55]. Unfortunately, however, his preliminary tests did not support this assumption, that is, *Blepharisma* was easily eaten by predators such as the heliozoan *Actinospherium eichhorni* and small crustaceans [46, 55]. Some predators, *Didinium nasutum*, *Woodruffia metabolica*, and

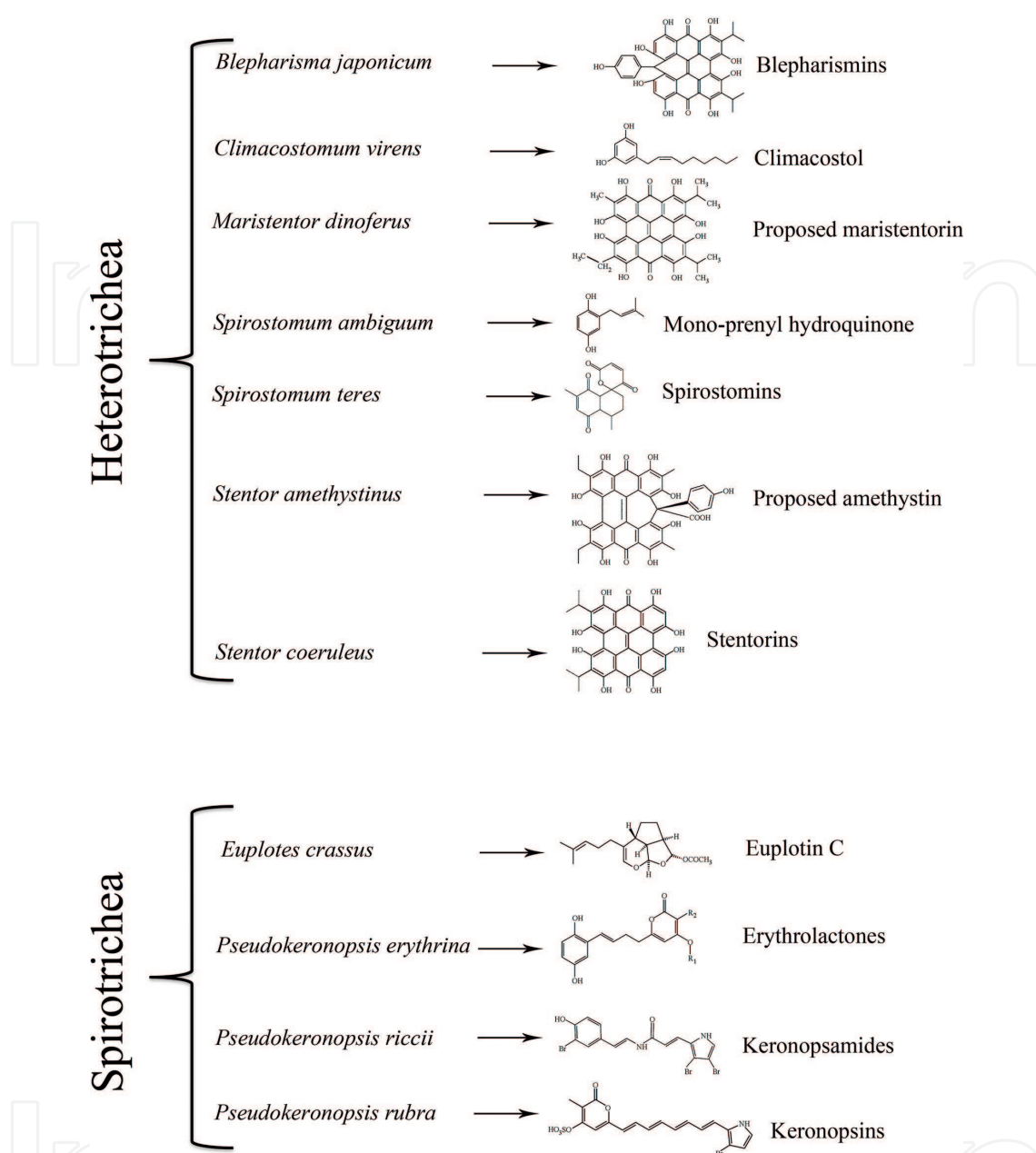


Figure 14. Main secondary metabolites produced by ciliated protists. Erythrolactones: A1 ($R_1 = \text{SO}_3^-$; $R_2 = \text{C}_6\text{H}_{13}$ (n-hexyl)); B1 ($R_1 = \text{SO}_3^-$; $R_2 = \text{C}_7\text{H}_{15}$ (n-heptyl)); C1 ($R_1 = \text{SO}_3^-$; $R_2 = \text{C}_8\text{H}_{17}$ (n-octyl)); A2 ($R_1 = \text{H}$; $R_2 = \text{C}_6\text{H}_{13}$ (n-hexyl)); B2 ($R_1 = \text{H}$; $R_2 = \text{C}_7\text{H}_{15}$ (n-heptyl)); C2 ($R_1 = \text{H}$; $R_2 = \text{C}_8\text{H}_{17}$ (n-octyl)).

Podophrya fixa, did not eat *Blepharisma*, but they also ignored some other ciliates including uncolored ones. In the absence of further evidence, Giese was skeptical about the assumption [46]. This hypothesis was further unequivocally demonstrated by Miyake, Harumoto, and collaborators, comparing normally pigmented red cells of *B. japonicum*, albino mutant cells, and light-bleached cells (a phenocopy of the albino mutant) as prey for the raptorial ciliate *Dileptus margaritifer* and evaluating the toxicity of purified blepharismins on various ciliate species [49, 50]. As a response to the attack by *D. margaritifer* versus one cell of *B. japonicum*,

the latter releases the toxic blepharismins, visible as spherical bodies of 0.2–0.6 μm in diameter under scanning electron microscopy (**Figure 15**). The discharge takes place within a second and it is able to repel the predator, while the albino and light-bleached cells are much more sensitive to the attacks of *D. margaritifera* [49, 50]. Recently the defensive function of blepharismins was also investigated in two additional species of *Blepharisma*, *B. stoltei*, and *B. undulans* against two predatory protists (*C. hirtus* and *Stentor roeseli*) and one metazoan, the turbellarian *S. sphagnetorum* [56]. The results indicate that the chemical defense mechanism present in *B. stoltei* and *B. undulans* is mediated by the same five blepharismins present in *B. japonicum*, although produced in different proportions [56]. Authors speculate that the conservation of this panel of toxic secondary metabolites suggests that distinct roles for these molecules are likely required at least for the fine control of photophobic reactions, as initially proposed by

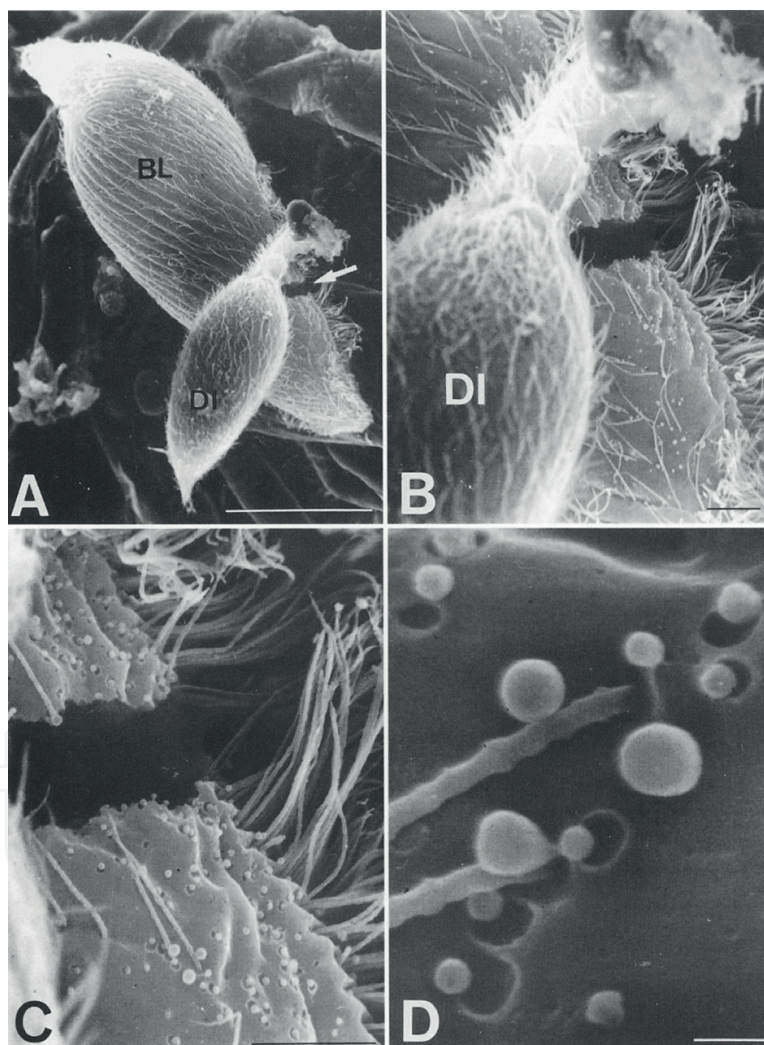


Figure 15. SEM micrographs of the predator-prey interaction between a cell of *Dileptus margaritifera* (DI) and a cell of *Blepharisma japonicum* (BL). (A) *Blepharisma* being attacked by *Dileptus*. Arrow indicates the site of the damage inflicted by the proboscis of the *Dileptus*. The rupture runs across the adoral zone of membranelles of the *Blepharisma*. Scale bar = 50 μm . (B) Enlargement of the region near the rupture in A. Scale bar = 5 μm . (C) The rupture magnification in B, showing the surface of *Blepharisma* peppered with spherules discharged from pigment granules. The surface is also pitted with small depressions presumably formed at the spots where the spherules have passed through the cell membrane. Scale bar = 5 μm . (D) Enlargement of a part of C. Scale bar = 0.5 μm . Pictures from [50].

Matsuoka et al. [48]. Summarizing, the *Blepharisma* species studied are able to defend themselves against *C. hirtus*, although *S. sphagnetorum* and *S. roeseli* seem to overcome *Blepharisma*'s chemical defense, but 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 the post-ingestion toxicity phenomena [56]. Additional toxic pigments, structurally related to hypericin, were found in other heterotrich ciliate species, such as stentorin in *S. coeruleus* (see [57] for a review), amethystin in *S. amethystinus* [58], and maristentorin in the marine ciliate *Maristentor dinoferus* [59], but the defensive function was experimentally proved only for *S. coeruleus* [60].

Karyorelictean ciliates also possess pigment granules which are similar in size, structure, and distribution to those in the heterotrichs, but principally due to the difficulties to the growing species of karyorelictid in the laboratory, the chemical nature of their pigments is still unknown. The most studied species is freshwater *Loxodes striatus*, which presents yellow-brown pigment granules previously examined as photoreceptors [61]. More recently it has been proved that the pigment granules in *L. striatus* are extrusive organelles which contain a toxic photodynamic pigment used for chemical defense against predators [62]. *Loxodes* are able to discharge the toxic pigment as response to attacks of the ciliate *D. margaritifer* (**Figure 16**) or of the turbellarian *S. sphagnetorum* repelling predators. Intriguingly Finlay and Fenchel already proposed a defensive function for the pigment granules in *Loxodes* (*L. striatus* and *L. magnus*) based on different evidences; specifically, they found that light induces in *Loxodes* a characteristic behavior to escape from toxic water and that the pigment granules are the photoreceptors for this reaction [61]. They assumed that this reaction may serve to localize *Loxodes* in regions of low oxygen tension where predators, such as planktonic metazoan, are rare and therefore the pigment may function as a predator-avoidance strategy. If this is the case, pigment granules of *Loxodes* participate in two very different kinds of defense, chemical defense and the behavior-based predator-avoidance, conferring to the ciliate an ability to defend itself against a wider range of predators [62].

Pigmented granules are found also in other groups of ciliates as the Spirotrichea, and mainly in the genus *Pseudokeronopsis*, which shows species equipped with reddish-brown pigment granules morphologically similar to those in heterotrichs [63]. Particularly in *P. carnea* [64] and in *P. erythrina* [65], these granules are reported as extrusive organelles. New secondary metabolites, keronopsins and keronopsamides, respectively, produced by *P. rubra* and *P. riccii*, were recently isolated together with their sulfate esters (**Figure 14**) [66, 67]. In the case of *P. rubra*, it was demonstrated that a crude extract of this organism containing keronopsins, A1 and A2, and their sulfate esters B1 and B2, is capable of paralyzing or even killing ciliates and flagellates [66]. For these reasons a defensive function for these secondary metabolites has been proposed; however, no data relative to their cellular localization and mechanism of action are available to date. On the other hand, in the case of *P. riccii*, the function of the alkaloid secondary metabolite keronopsamide A and its sulfate esters B and C has not been investigated, and the possible localization of the pigments in the cortical granules is only presumed [67]. The most extensively studied species is *P. erythrina*; previously described as an estuarine one, it was successively found also in the freshwater environment and hence reported as a euryhaline organism [68]. This ciliate shows an elongated body (**Figure 17**) equipped with spherical, dark-reddish, brown, or brick red colored pigment granules of about 1 μm in diameter that are mainly arranged around ciliary organelles [69]. As the content of pigment granules, three new secondary metabolites have

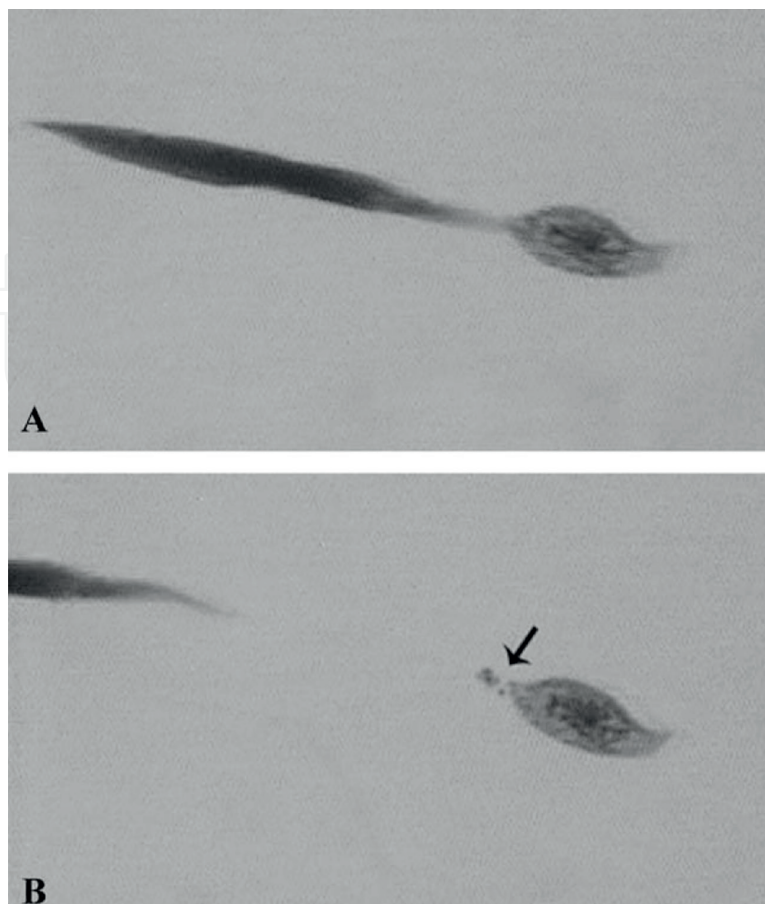


Figure 16. Predator-prey interaction between *Dileptus margaritifer* and *Loxodes striatus*. (A) *Dileptus* (the slender cell at the left) starts swimming backward after hitting a *Loxodes* with its proboscis. (B) The same cells as in A, about a second later, showing the retreated *Dileptus* and a mass of brownish material (arrow) near the *Loxodes*. Micrograph extracted from a film clips. Magnification $\times 70$. Pictures from [62].



Figure 17. External morphology of a living cell of *Pseudokeronopsis erythrina*. Scale bar = 100 μm .

recently been characterized and named erythrolactones A2, B2, and C2. These are characterized by a central 4-hydroxy-unsaturated δ lactone ring bearing an alkyl saturated chain at carbon-2 and a butyl-benzenoid group at carbon-5 [65, 68]. These molecules were detected in the crude extract of whole cells together with their respective sulfate esters, erythrolactones A1, B1, and C1 (**Figure 14**). After the application of the cold-shock method on massive cell cultures of *P. erythrina* to induce the exclusive discharge of pigment granules, it was demonstrated that only

non-sulfonated molecules A2, B2, and C2 were contained in the extrusomes of the ciliate [65]. The mixture of these three molecules has been proven to repel some predators, such as the ciliate *C. hirtus*, and to be toxic for a panel of ciliates and microinvertebrates [65]. Erythrolactones A2, B2, and C2 are the only toxins present in the extrusome discharge of *P. erythrina*, whereas their respective sulfate esters A1, B1, and C1 remain confined inside the cell environment [68]. It is known that the process of sulfonation of endogenous molecules is a major metabolic reaction in eukaryotes that can increase water solubility and influence conformational changes but can also lead to the activation or inactivation of a biological effect (see [70] for a review). Buonanno and collaborators [64] speculate that the exclusive maintenance of the sulfate esters of the erythrolactones inside the *P. erythrina* cell may be associated with their temporary inactivation, in order to prevent the phenomenon of self-toxicity that could occur before their definitive storing, as non-sulfonated and active compounds, in the cortical pigment granules.

Other organelles strictly related to pigment granules are the colorless **cortical granules** in the heterotrich, sometimes reported as granulocysts to underline their extrusive nature. These organelles show a greatest morphological similarity to pigment granules, as in the case of the cortical granules of *Climacostomum virens* [71] and *Blepharisma hyalinum* [72]. The function and biological activity of the secondary metabolites contained in the cortical granules seem to be primarily related to chemical defense or offense, and the cortical granules in *C. virens* are to date the most studied. This freshwater heterotrich ciliate, if properly stimulated, is able to repel predators by discharging the colorless toxin climacostol (**Figure 14**) and some related analogues.

This toxin may be chemically classified within a large group of natural compounds known as resorcinolic lipids (also called alkylresorcinols or 5-alkylresorcinols), widely detected in prokaryotes and eukaryotes [73] and with reported antimicrobial, antiparasitic, antitumoral, and genotoxic activities (see [74] for a review).

A typical defensive behavior of *C. virens* occurs when a predator, such as the ciliate *D. margaritifer*, contacts a *C. virens* cell with its toxicysts bearing proboscis (**Figure 18A**). *D. margaritifer* swims backward while dense material is visible under dark field microscopy, emerging from the site where the proboscis touched the *C. virens* (**Figure 18B**) which swims away [75]. Sometimes, together with the discharged material from *C. virens*, it is also possible to detect some hazy material consisting of needle-like structures which appear to be discharged toxicysts of *D. margaritifer* (**Figure 19**), suggesting a possible further protection against the toxic extrusomes of predators [75]. Interestingly, the chemical defense adopted by *C. virens* against *D. margaritifer* is also effective against some other protists and metazoans [44, 76].

If the defensive function of cortical granules in *C. virens* is widely demonstrated, some evidences indicate that these extrusomes could be also successfully used for chemical offense. Differently from the *Paramecium* species which do not have trichocysts (exclusively for defense) localized in the oral apparatus, *C. virens* presents a wide number of cortical granules in the buccal cortex suggesting an additional offensive function for these extrusomes [71]. *C. virens* is able to catch and ingest prey of different sizes, from small flagellates such as *Chlorogonium elongatum* to large ciliates, such as *B. japonicum* or *Spirostomum ambiguum* [43, 77]. These prey are sucked up into the buccal cavity of *C. virens*, which is formed of a peristomial field and a buccal tube, and then ingested in a food vacuole, which arises at the end of the tube [43]. A cell of *P. tetraurelia* which is entirely taken into the buccal tube of *C. virens* is able to discharge the trichocysts and escape from

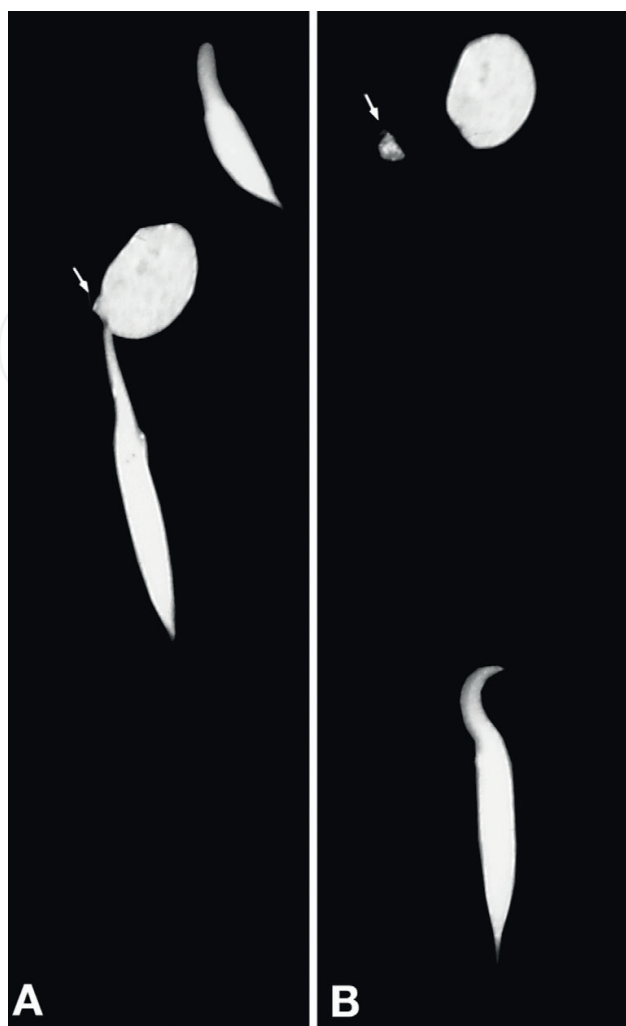


Figure 18. Predator-prey interaction between *Dileptus margaritifera* and *Climacostomum virens*. (A) *Dileptus* (the slender cell at the center) starts swimming backward after hitting with the proboscis *Climacostomum*. A small bulge (arrow) is developing on the surface of the *Climacostomum* at the site where the proboscis has just hit. (B) The same cells as in A, about a second later, show the retreated *Dileptus* and a small cloud (arrow) near the *Climacostomum*. Dark field micrographs of living cells. Magnification $\times 70$. Pictures from [75].

the predator [43], different to what happens when an individual of the same species is totally caught in the pharynx of the microturbellarian *S. sphagnetorum* [44]. Perhaps, as in the case of contact with the toxicysts of the raptorial ciliate *D. margaritifera*, the trichocysts were discharged after contact with climacostol released from *C. virens* to kill the prey. A similar phenomenon also occurs with different preys which possess chemical extrusomes for defense such as the ciliate *S. ambiguum*. In this case, after a cell-cell contact, the *S. ambiguum* displays rapid cell contraction, and according to the authors, it is likely that this contraction is induced by the discharge of extrusomes by *C. virens* [77]. If this is the case, it is likely that the cortical granules of *C. virens* could be equally used as multifunctional extrusomes, both for chemical defense and offense.

Besides the natural role of climacostol and thanks to the availability of a straightforward method for its chemical synthesis [78], other bioactivities of the toxin and its potential application to human health are, to date, investigated in various biological systems. The toxicity of climacostol proves very effective against pathogenic Gram-positive bacteria such as *Staphylococcus aureus* or

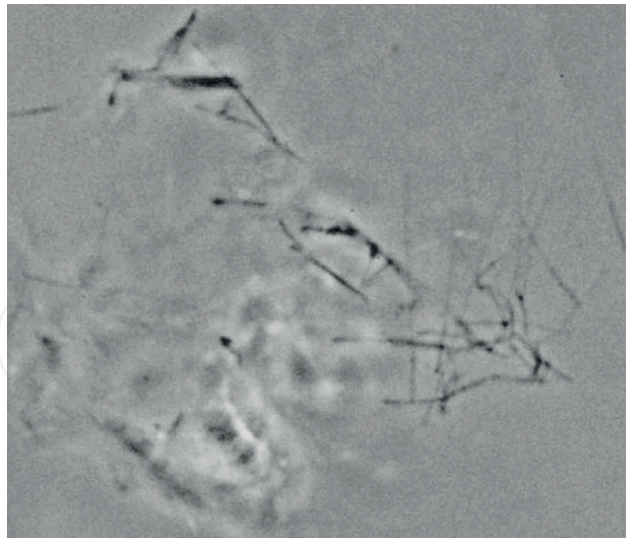


Figure 19. Hazy cloud consisting of needle-like structures discharged from the toxicysts of *Dileptus margaritifer*. Magnification $\times 720$. Pictures from [75].

S. pneumoniae and against a fungal pathogen, *Candida albicans* [79]. In addition, on the basis of the anticancer properties of other resorcinolic lipids, the toxic potential of climacostol is also studied against cancerous and non-cancerous mammalian cells, including human cell lines. The results show that climacostol effectively inhibits the growth of some tumor cell lines in a dose-dependent manner by inducing programmed cell death, with non-tumor cells proving significantly to be more resistant to the toxin [73, 80]. More recently the anti-tumor therapeutic activity of this toxin was also proved *in vivo*, using a melanoma allograft model in mice [81]. These results are quite interesting also in light of the fact that different molecules produced by other ciliate species show some particular pharmacological properties such as the sesquiterpenoid euplotin C or the cell type-specific signaling protein pheromone *Er-1* from *Euplotes* species (see [82] for a review).

Returning to the topic of this chapter, different secondary metabolites have been also isolated and characterized from other heterotrics, such as *Spirostomum ambiguum*, and *S. teres*. *S. ambiguum* (**Figure 20**) is a colorless freshwater species and one of the largest and elongated existing ciliates ($800\text{--}2000 \times 48\text{--}60 \mu\text{m}$). The species is very common in the sludge-water contact zone of wells, ponds, sewage ponds, lakes, oxbows, ditches, and in the sediments of alpha- to beta-mesosaprobien rivers [77]. The defensive function of its cortical granules was recently demonstrated against different predators and the toxicity of its content was tested on a panel of freshwater ciliates [77, 83]. *S. ambiguum* has numerous cortical granules which, under a phase contrast microscope, appear as dots placed in the region between ciliary lines that could be observed in a large transparent contractile vacuole placed at the posterior end of the cell (**Figure 21A**) [77]. The cold-shock method was applied to *S. ambiguum* to obtain the cortical granule-deficient cells, which showed a markedly reduced number of extrusomes (**Figure 21B**). Both untreated and cortical granule-deficient cells were exposed to the attack of *C. virens*, and when the buccal apparatus of the predator makes contact with an untreated cell of *S. ambiguum*, it showed a rapid contraction while the predator swam backwards (**Figure 22A**). Similarly to untreated cells, cortical granule-deficient cells of *S. ambiguum* also showed rapid contraction after attack by *C. virens*, but they were successfully captured and sucked up by the predator



Figure 20. External morphology of living cells of *Spirostomum ambiguum*. Scale bar = 200 μm .

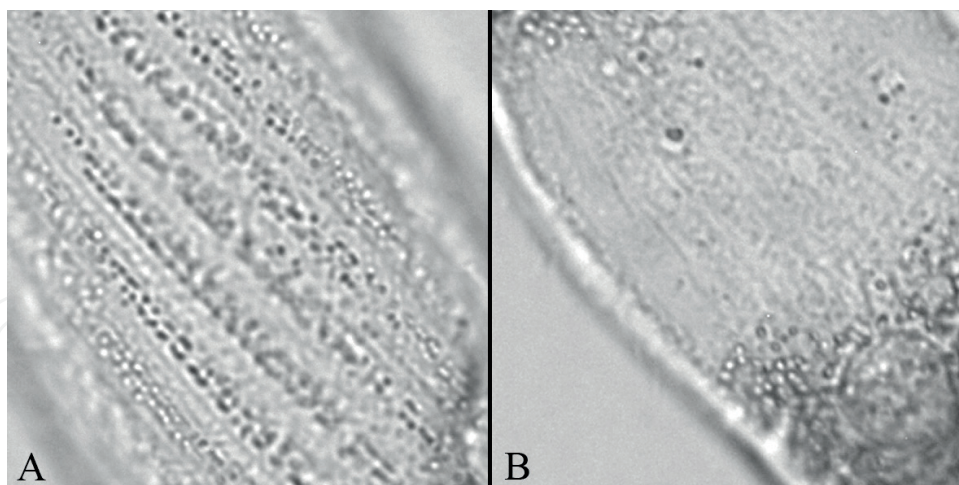


Figure 21. Reduction in the number of extrusomes (cortical granules) in *Spirostomum ambiguum* obtained by cold-shock treatment. (A) Extrusomes in an untreated cell. (B) Extrusome-deprived cell after cold shock. Magnification $\times 900$. Pictures from [77].

into its buccal cavity (**Figure 22B**) [77]. The toxin involved in this interaction was purified by reversed phase high-performance liquid chromatography (RP-HPLC), and its structural characterization was carried out through nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) measurements and revealed as 2-(3-methylbut-2-enyl)benzene-1,4-diol(mono-prenyl hydroquinone) (**Figure 14**). Prenylated-hydroquinone derivatives are

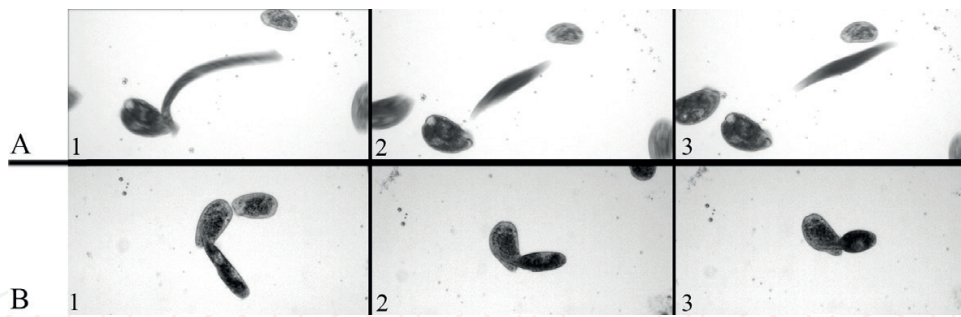


Figure 22. Predator-prey interaction between *Climacostomum virens* and *Spirostomum ambiguum*. (A) 1: Cell of *C. virens* contacts a cell of *S. ambiguum* with its buccal apparatus. 2: *S. ambiguum* shows rapid contraction while the predator swims backwards. 3: The same cells as in 2, a second later, showing a retreated *C. virens*, while *S. ambiguum* swims away. (B) Predator-prey interaction between *C. virens* and extrusome-deficient cells of *S. ambiguum* obtained by cold-shock treatment. 1: *C. virens* cell contacts a *S. ambiguum* cell which instantly shows contraction. 2: *C. virens* engulfs the contracted *S. ambiguum* cell and continues to eat the *S. ambiguum* cell (3). Micrographs extracted from a film clip. Magnification $\times 50$. Pictures from [77].

metabolites of abundant occurrence and have been isolated from fungi, algae, plants, animals, and bacteria [77]. In this case the involvement of this molecule in predator-prey interaction is clear. Interestingly, another freshwater species of the genus *Spirostomum*, *S. teres*, possesses a different colorless toxin used for defense, characterized as spiro[(2,5-dimethyl-5,6,7,8-tetrahydronaphthalene-1,4-dione)-8,6'-(pyrane2',5'-dione)] and named spirostomin (**Figure 14**) [84]. It is no novelty that closely related organisms can produce different or even biogenetically distant specific secondary metabolites [77], and it is very common for ciliates [56]. To date, the only reported exception to this phenomenon is related to the genus *Blepharisma* in which the three species *B. japonicum*, *B. stoltei* and *B. undulans* share the same mixture of blepharismins even if produced in different proportions [56].

4.3. The inducible defense

Another peculiar defensive mechanism, reported as inducible defense, has been described for some *Euplotes* species as the response to the presence of some predators, such as microturbellarians, ciliates, or amoebas. These predators can release active substances, called kairomones, which induce some behavioral and morphological changes (such as the formation of spines in *Euplotes*) as a defensive mechanism in response to the presence of the predator [85–88] for a review.

It could be interesting to study the efficiency of the inducible defenses, if compared to mechanical and chemical defense by means of extrusomes. In this regard, a first study was performed to compare the efficiency of the defense mediated by trichocysts in *P. aurelia* with that mediated by cortical granules in *C. virens* and *S. ambiguum* [44]. The authors reported that the mechanical defense in *Paramecium* against metazoan predators appears to be equally effective as the chemical one, but can be successfully activated only during the very early interactions with the predator, whereas it is ineffective after the ingestion of the ciliate. In contrast, the chemical defense adopted by a toxic ciliate against metazoan predators can also be activated after the ingestion of the prey by the predator, but its effectiveness appears to be strictly linked to the cytotoxic potency of the compound stored in the protozoan cortical granules. It would also be interesting to compare these two mechanisms against unicellular predators.

5. Conclusions

In a general perspective, it is clear that the researches on predatory behavior and on the related defensive mechanisms in protists not only represent progress in knowledge about the ecological role played in nature by predator-prey interactions in aquatic microhabitats but will also provide new research opportunities for evolutionary biology and may also represent a relevant source of new natural products.

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Conflict of interest

The authors have declared no conflict of interest.

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