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PREY ATTRACTION IN CARNIVOROUS *GENLISEA* (LENTIBULARIACEAE)

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In this study we test three hypotheses. (1) Secretory hairs in the arms and the distal part of the neck of the carnivorous plant *Genlisea* (Lentibulariaceae) have a different principal function than the digestive hairs in the digestive chamber, that is, prey attraction. (2) Only bacteria and other organisms inside the trap and on the external trap surface lure prey. (3) Substances produced by the plant have a minor influence on prey attraction; more important is trap shape and morphology, because protozoa and microfauna may move to the small interspaces (traps or capillaries) by accidental, nonspecific wandering. We studied the structure of secretory hairs (glands) in the arms and the distal and proximal parts of the trap neck using light, fluorescence and electron microscopy. We tested the hypotheses with several experiments using sterile *Genlisea* traps as well as glass tubes acting as a *Genlisea* trap model, and various organisms as prey (*Blepharisma* sp., *Paramecium bursaria*, *Euglena* sp.). Hairs in the arms and the distal part of the *Genlisea* trap neck represent polysaccharide-protein-secreting hairs. Prey still moved to cleaned traps without chemical attractants. In the proximal part of the neck the secretory hairs have the same ultrastructure as digestive hairs in the digestive chamber of *Genlisea*. Sterile traps do not need commensals for catching prey. The results of the behavioral experiments reported here support the hypothesis that prey can move to the traps or capillaries by accidental, nonspecific wandering to small objects filled with water. Thus, the complex structure of the *Genlisea* trap with long arms may help catch prey simply by providing a large surface with many small openings which mimic the interspaces between soil particles, and the plant does not need special mediators for prey attraction.

Key words: *Genlisea*, Lentibulariaceae, prey attraction, carnivorous syndrome, carnivorous plants, secretory hairs, ultrastructure, mucilage-secreting hairs, *Paramecium*, *Euglena*.

INTRODUCTION

For survival in nutrient-poor environments, carnivorous plants need the benefits gained from trapped and digested prey (Givnish et al., 1984). For this reason, prey attraction is one of the major features of the carnivorous syndrome, although digestion of prey and absorption of their nutrients are the most commonly observed and confirmed features of carnivorous plants. Various strategies for prey attraction have evolved in carnivorous plants. Trap shape, morphology, colors, areoles and patterns of UV absorption by the trap surface are very important in luring potential prey (Joel et al., 1985; Joel, 1988; Juniper et al., 1989). Carnivorous plants with "air" traps have a strategy different from that of plants forming traps immersed in water or wet soil. However, in both pitcher- and bladder-shaped traps the attractants are generally produced near or in the trap entrance: nectar by nectaries and volatile compounds in Sarraceniaceae, *Nepenthes* and *Cephalotus* (e.g., Parkes, 1980; Juniper et al., 1989; Płachno, 2007; Płachno et al., 2007b) or mucilage by secretory hairs in some *Utricularia* species (Cohn, 1875), though in this genus the mechanism of prey attraction is still debated (Guisande et al., 2007). Sanabria-Aranda et al. (2006) did not consider the possible role of mucilage as an attractant

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and suggested that the plant-derived periphyton may lure prey by controlling the presence and amount of periphyton on the trap surface. It has also been suggested that bladder appendages such as antennae and bristles may guide prey to the trap entrance (Meyers and Strickler, 1979).

Prey attraction is even less understood in the related genus *Genlisea*, in which the eel (lobster-pot) trap type has evolved. *Genlisea* species are small, rootless wetland plants which produce underground corkscrew-shaped traps of foliar origin. A single *Genlisea* trap consists of a stalk, a vesicle (digestive chamber) and a tubular channel (neck), which divides into two helically twisted arms with openings (e.g., Juniper et al., 1989; Reut, 1993; Płachno et al., 2007a).

Barthlott et al. (1998) suggested that *Genlisea* can attract protozoa chemotactically, but did not describe the chemical attractants or their source. Later Płachno et al. (2005) showed experimentally that metazoans such as annelids can also be attracted and trapped. Studnička (2003a) suggested that the *Genlisea* trap acts as false soil interspaces partially filled with air, which may be an attractant for oxygen-dependent microfauna. Adamec (2005) showed that *Genlisea* traps radially release O_2 from traps to an anoxic medium, which could act as an attractant. Lloyd (1942, p. 94) thought that glands in *Genlisea* traps "may supply only mucilage to lubricate the interior and facilitate the movements of prey downward through the arms and neck, or they may secrete digestive enzymes or both." Two classes of secretory hairs (glands) occur in the trap cavity: those with two terminal cells (Fig. 1a,b) and those with four (most commonly four, up to eight in some cases) terminal cells (e.g., Lloyd, 1942; Reut, 1993). Recently the ultrastructure of secretory hairs in the digestive chamber (with four terminal cells) was described in detail by Płachno et al. (2007a). These hairs are responsible for prey digestion and later for nutrient absorption. Studnička (2003a,b) suggested that the hairs in the arms and the distal part of the neck of *Genlisea* (with two terminal cells) are similar to bifids of *Utricularia* and may pump water as in *Utricularia*. An experimental study by Adamec (2003) contradicts this idea. Active transport of water in *Genlisea* traps has not been documented; that experiment used isolated traps, which might have altered trap physiology. Płachno et al. (2005) did not observe active water transport with prey transport in *Genlisea* traps.

In this study we test three hypotheses. (1) Hairs in the arms and the distal part of the neck have a different principal function than the digestive hairs in the digestive chamber (bulb); their primary function is prey attraction. We also study the architecture of these hairs to determine whether there are similarities to *Utricularia* bifids, and compare their ultrastructure with glands of other carnivorous genera. (2) Only bacteria and other organisms in the trap and on the external trap surface lure prey. (3) Substances produced by the plant have a minor influence on prey attraction; protozoa and microfauna are generally attracted to small hollow objects.

MATERIALS AND METHODS

Plants of the subgenus *Genlisea* (*Genlisea hispidula, G. repens, G. margaretae*) and subgenus Tayloria (*Genlisea violacea* f. Giant, hybrid of *Genlisea lobata* × *G. violacea* f. Giant) were cultivated in the Department of Plant Cytology and Embryology, Jagiellonian University in Cracow. They were grown under a 16 h photoperiod in pots containing a mixture of wet peat and sand (Płachno et al., 2007a).

LIGHT, FLUORESCENCE AND ELECTRON MICROSCOPY

The procedures for preparing samples for SEM, cryo-SEM and TEM were as described earlier (Płachno et al., 2007a). Cytochemical tests included periodic acid-Schiff (PAS) reaction for insoluble polysaccharides, Coomassie brilliant blue R-250 and aniline blue black for proteins, and Sudan Black B for lipids (for details see Kozieradzka-Kiszkurno, 2003). For phosphatase activity, traps were hand-sectioned with a razor blade and assayed with ELF®97 phosphatase substrate (ELFP, Molecular Probes) following the protocol of Płachno et al. (2006).

EXPERIMENTS

Genlisea violacea plants from sterile in vitro tissue culture were used. This was done in order to avoid contamination by bacteria and other organisms inside or on the external surface of the traps, which could have potentially influenced prey attraction, and to investigate whether the plants themselves can attract prey. The sterile culture protocol followed the one Darnowski (2004) employed for *Drosera*. Traps were taken from sterile tissue culture and washed several times in distilled water.

EXPERIMENT 1. To determine whether hairs from the arms and the distal part of the neck produce attractants, in contrast to hairs from the digestive chamber (vesicle), we cut traps into separate arms and digestive chambers and placed them in Petri dishes. Three pairs of arms were put into each of three Petri dishes with 20 ml of a 1:1 mixture of filtered pond water and distilled water, and all of the following organisms (2 ml culture each) were added to each dish: *Blepharisma* sp., *Paramecium bur-*

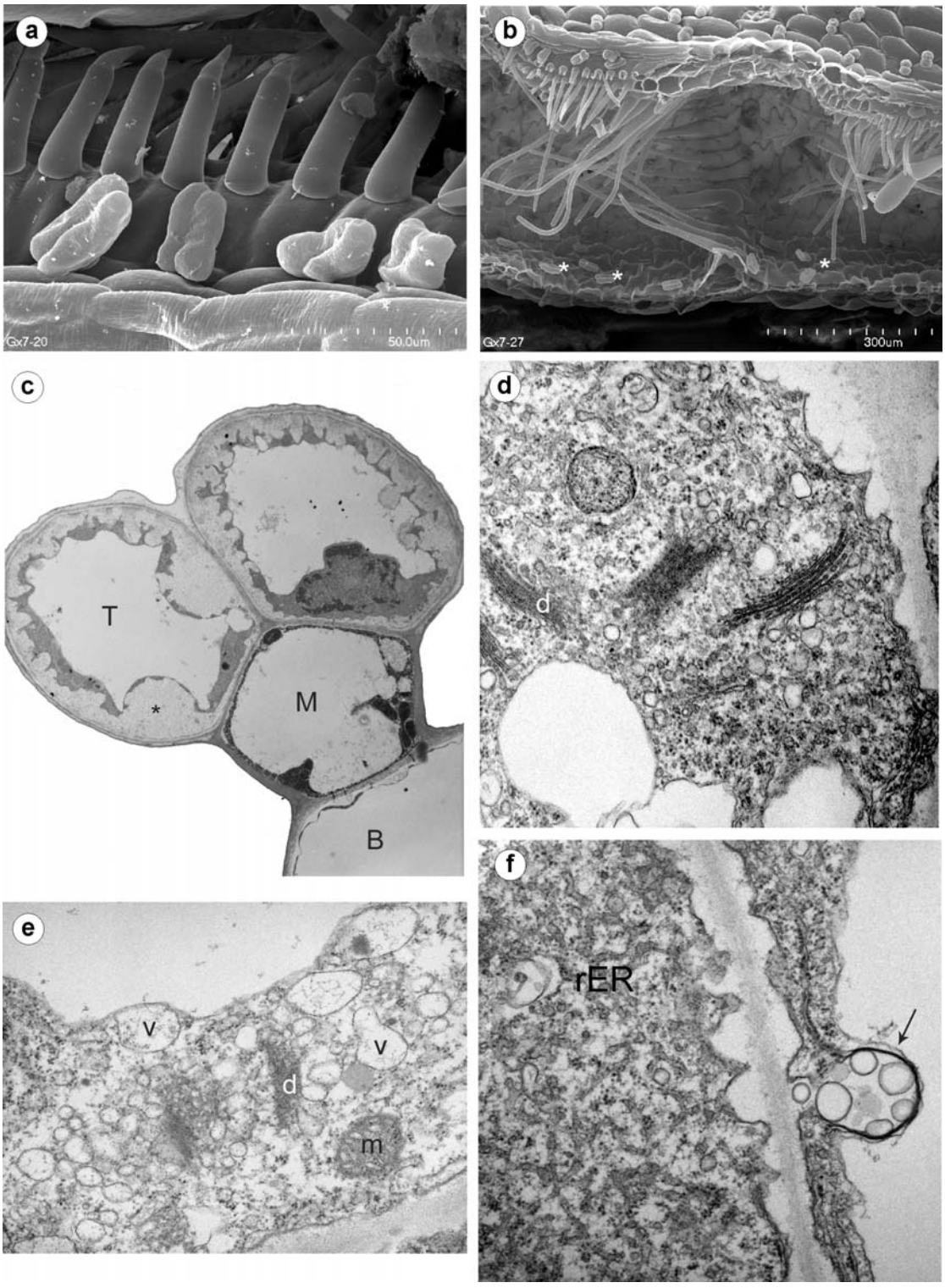

Fig. 1. (**a**) Trap opening of *Genlisea lobata* × *G. violacea* arm with secretory and nonsecretory retaining hairs, (**b**) Part of section through *G. lobata* × *G. violacea* arm: star – secretory hair, (**c**) Section through secretory hair of *Genlisea lobata* × *G. violacea*. B – basal cell; M – middle cell; T – terminal cell; star – mucilage. Bar = 1.6 μm, (**d**) and (**e**) Part of terminal cell cytoplasm of *Genlisea* secretory hair with active dictyosomes (d). m – mitochondrion and secretory vesicles (v). Bar = 283 nm for (d) and 357 nm for (e), (**f**) Part of section through terminal cells of *Genlisea* secretory hair, visible are well-developed rER (rough ER) and vesicle exocytosis (arrow). Bar = 306 nm.

saria and *Euglena* sp. The same procedure was followed for digestive chambers. All cultures of organisms were obtained from Carolina Biological Supply Company (Burlington, NC, U.S.A.). Barthlott et al. (1998) also used *Blepharisma* and *Paramecium* in experiments; both are large, easily observed ciliates. After 15 min, and later at 1 h, 2 h, and 4 h intervals, the contents of arm and digestive chambers were checked directly under a stereomicroscope at 20× and 40×. Later this plant material was fixed in 2.5% glutaraldehyde (GA) in 0.05 M cacodylate buffer (pH 7.0) at 4°C for 4 days. Samples for SEM were prepared as described earlier (Płachno et al., 2005).

EXPERIMENT 2. Another experiment to check the same hypothesis was performed. Traps were cut to obtain digestive chambers, and later the chambers were placed in a 1:1 mixture of filtered pond water and distilled water overnight to wash the digestive chambers and avoid contamination from the fluid from other trap parts. This experiment was done in 6 replicates. The next day, three digestive chambers were placed in each dish with 20 ml of a 1:1 mixture of filtered pond water and distilled water. Then the organisms were added to the dishes as in Experiment 1. After 2 h the digestive chamber contents were studied under a stereomicroscope.

EXPERIMENT 3. To check whether substances produced by the plant have a minor influence on prey attraction, and that protozoa or invertebrates are generally attracted to small objects (cf. Jobson and Morris, 2001), we used empty glass capillaries (~1.6 μl volume). This experiment was done in 3 replicates. Three glass capillaries were put into each Petri dish with 20 ml of a 1:1 mixture of filtered pond water and distilled water, and 2 ml culture of the organisms were added as above. After 40 min the contents were checked under a stereomicroscope. As *Euglena* formed resting stadia and were not active, we repeated this experiment as above, with *Euglena* but without the other organisms. After 24 h the tube contents were examined under a stereomicroscope.

EXPERIMENT 4. We also wanted to check whether the trap could attract prey after water-soluble substances were removed from the surface of *Genlisea*. For this experiment the traps were washed in 0.1% SDS (sodium dodecyl sulfate) solution for 10 min, and then twice in distilled water. The rest of the first part of the treatment followed Experiment 1, except using whole traps. After 15 min and later at 1 h the contents of traps were examined under a stereomicroscope. Control traps were not washed with SDS solution, but the other conditions were the same (6 treatments, 3 traps per dish). After 4 h the water with organisms was removed, and clean water was added. After 24, 48 and 72 h, trap contents were observed to see what happened to trapped prey.

STATISTICAL ANALYSIS

The frequencies within populations of protists were counted in each experiment. The variance of frequencies of occurrence between populations within each experiment was tested against the null hypothesis of equal occurrence frequencies in all experiments with the contingency chi-squared test.

RESULTS

STRUCTURE OF SECRETORY HAIRS

The basic structure of the secretory hairs in the arm and the distal part of the neck is uniform throughout the genus (Fig. 1c). The middle cell has a Casparian strip-like lateral wall and forms a symplastic connection between the basal cell and two terminal cells. Both basal and middle cells are highly vacuolated. Occasionally a large amount of lipids is present in the middle cell.

Two stages of terminal cells were found. At the first stage a large vacuole occupies most of the cell and is surrounded by a thin peripheral layer of cytoplasm rich in mitochondria, active dictyosomes with numerous vesicles (Fig. 1d,e) and tubular elements of ER forming a complicated network (Fig. 1f). Part of the vesicles contains content resembling polysaccharides (Fig. 1e). Some evidence of vesicle exocytosis was observed (Fig. 1f). Portions of ER elements are in close association with the plasmalemma. Unlike in immature hairs (Fig. 2a), in mature hairs the surfaces of the radial walls are irregular and are easily visible in TEM (Fig. 2b,c) and cryo-SEM. The plasma membrane has a very irregular outline. In section, "cytoplasm islands" are visible in the wall (Fig. 2b). Microtubules occur near the plasma membrane and form a cortical cytoskeleton (Fig. 2c). Plasmodesmata were not observed in the radial walls, indicating the absence of a symplastic connection between terminal cells.

At the second stage, terminal cells are also highly vacuolated; however, in the periplasmic space there is a layer of secreted material which is very irregular in outline, like an invaginated plasma membrane (Fig. 1c). This material shows polysaccharide character (PAS-positive reaction, Fig. 2d).

Hairs from the proximal part of the neck have ultrastructure similar to hairs from the digestive vesicle of *Genlisea* (see Płachno et al., 2007a). Phosphatase activity was detected in terminal cells in the outer cell wall, cytoplasm and vacuoles (data not shown).

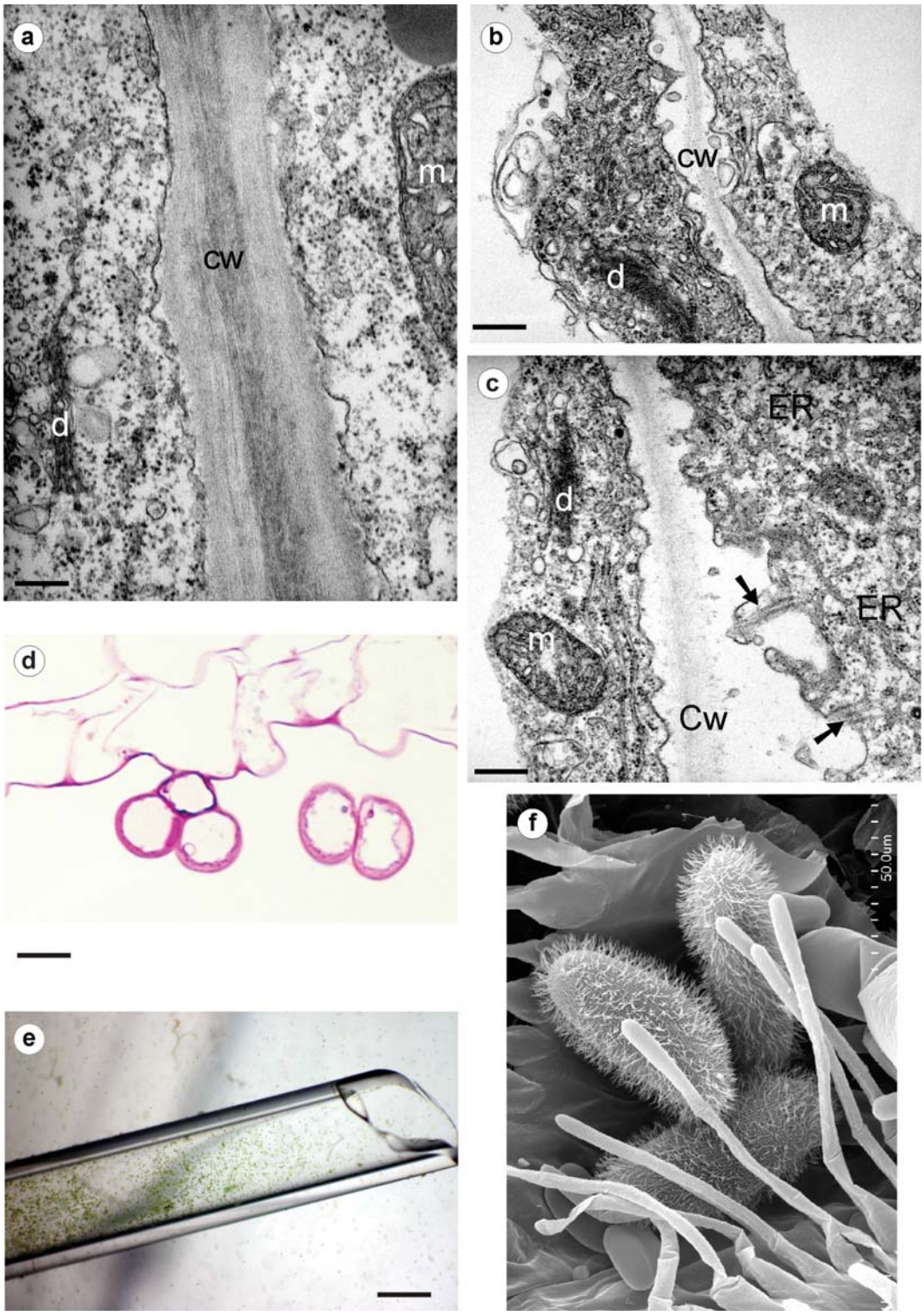

Fig. 2. (**a**) Part of section through terminal cells of immature *Genlisea hispidula* secretory hair. cw – radial cell wall; m – mitochondrion; d – dictyosome. Bar = 211 nm, (**b**) and (**c**) Part of section through terminal cells of mature *G. hispidula* secretory hair, visible are very irregular surfaces of radial walls (cw). m – mitochondrion; ER – endoplasmic reticulum; d – dictyosomes; arrow – microtubules. Bar = 246 nm for (b) and 417 nm for (c), (**d**) Section through secretory hair of *Genlisea lobata* × *G. violacea*, cytochemical test for insoluble polysaccharides, positive reaction in periplasmic space, layer of secreted material. Bar = 7 μm, (**e**) Glass tube (model of *Genlisea* trap) with numerous *Euglena* sp. Bar = 0.6 mm, (**f**) *Paramecium bursaria* in arm of *G. violacea*.

EXPERIMENTS

EXPERIMENT 1. *Paramecium bursaria* specimens were observed in 88.9% (16) of the digestive vesicles. *Blepharisma* sp. were observed in 11.1% (2) of the digestive chambers. *Paramecium bursaria* were observed in 100% (18) of the arms and *Blepharisma* sp. in 38.9% (7) of the arms (Fig. 2f). The occurrence *Paramecium* and *Blepharisma* in chambers and arms did not significantly differ (2=1.804, P > 0.05). Most *Euglena* formed resting stadia and were not active, but we did find *Euglena* in 16.8% (3) of the arm pairs (in one Petri dish).

EXPERIMENT 2. *Paramecium bursaria* were observed in 77.4% (14) of the digestive chambers, and *Euglena* sp. in 44.4% (7) of them. Other organisms were not observed in the digestive chambers.

EXPERIMENT 3. *Paramecium bursaria* were observed in all glass capillaries. *Blepharisma* sp. were in 4 tubes (44.4%). *Euglena* formed resting stadia and were not active. After 24 h, *Euglena* were observed in all glass tubes in high numbers (Fig. 2g).

EXPERIMENT 4. Cleaned (with SDS) traps: *Euglena* sp. – 100% (18), *Paramecium* bursaria – 39.9% (7). Traps not cleaned: *Euglena* sp. – 88.9% (16), *Paramecium bursaria* – 55.6% (10), *Blepharisma* sp. – 27.8% (5). After 24 h all organisms were still alive inside the traps. After 48 h and after 72 h, both *Euglena* sp. and *Paramecium bursaria* were still observed inside the traps, and some of them were still moving. The occurrence of *Euglena* and *Paramecium* did not significantly differ between clean traps and the ones not cleaned (χ^2=0.628, P>0.05).

COMPARISON OF EXPERIMENTS 1, 2 AND 3

The χ^2 test indicated a significant difference in the frequency of occurrence of protozoa in digestive vesicles between Experiments 1 and 2 (χ^2=2.25 P=0.32). The frequency of occurrence of protozoa in digestive vesicles from Experiment 2 and trap arms from Experiment 1 was similar (χ^2=13.22 P=0.0013). It did not differ between digestive vesicles from Experiment 1 and glass capillaries from Experiment 3 (χ^2=18.25 P=0.0001). Nor did we find any difference in their frequencies between arms from Experiment 1 and glass capillaries from Experiment 3 (χ^2=75.1 P=0.0001).

DISCUSSION

LINK BETWEEN STRUCTURE AND FUNCTION OF SECRETORY HAIRS

Unlike *Utricularia* bifids, the middle cell of *Genlisea* arm and distal neck hairs lacks a wall labyrinth. Terminal cells of *Genlisea* are structurally simpler than the terminal cells of *Utricularia* bifids. Our observations do not support Studnička's (2003a,b) suggestion that these hairs are similar to *Utricularia* bifids and that they pump water. The similarity of structure of these hairs is only superficial.

The *Genlisea* hairs described here produce mucilage which is stored in a periplasmic space, resembling the situation in the digestive hairs of *Pinguicula vulgaris* (Vassilyev and Muravnik, 1988) and to some extent in the glandular hairs of *Urtica dioica* (Vassilyev, 1994; cf. our Fig. 1c with *Urtica dioica* in Fig. 21). Most mucilage-secreting cells have a hypersecretory Golgi apparatus (for carnivorous plant mucilage glands, see Schnepf, 1961, 1963; Vintéjoux and Shoar-Ghafari, 1997, 2005). Moreover, the protoplast commonly is pushed by mucilage into the cell center and finally disappears (e.g., Fahn, 1979). This process occurs in long-stalked mucilage hairs in the related genus *Utricularia*, but not in some mucilage-protein-secreting hairs (e.g., in *Urtica*, *Dendrocnide*; Vassilyev, 1994, 1994a), nor *Genlisea*. If we compare *Genlisea* hairs from trap arms with typical mucilage-secreting hairs as in *Mimulus* (Schnepf and Busch, 1976) or *Utricularia* (Vintéjoux and Shoar-Ghafari, 1997), they clearly differ. *Genlisea* does not have a hypersecretory Golgi apparatus, and the dominant organelle of *Genlisea* hairs is the vacuole (like secretory hairs from the digestive chamber; see Płachno et al., 2007a). Slime may be important for the commensals that occur in *Genlisea* traps, but the mucilage may also have another function. Studnička (2003b) suggested that the viscous substances in the *Genlisea* trap might serve as a semi-permeable plug and block nutrients leaking from the trap.

Phosphatase activity also occurs in arm and distal neck hairs (see Płachno et al., 2006); however, we suggest that these hairs are not responsible for prey digestion. Enzymes might be secreted into the trap cavity and to the external aqueous medium in trap openings, where they might release inorganic phosphate from organic substances, especially since the arm surface area is so large. However, killing prey and carcass digestion both occur in the digestive chamber and the upper part of the neck, as described by Lloyd (1942, p. 94): "I have observed that prey only half-way down the tubular neck shows signs of a far degree of disintegration." We found that the hairs from the proximal part of the neck have the same ultrastructure as hairs from the digestive chamber of *Genlisea*. This result accords with Studnička's (2003b) suggestion that the proximal neck may be an extension of the digestive chamber.

PREY ATTRACTION

Secretion from secretory hairs apparently is not necessary for prey attraction. It is clear from our data that live *Genlisea* traps from plants grown in vitro are no more attractive to prey than glass capillaries. Possibly both the prey seen inside traps or trap fragments and those inside the capillaries ended up there by accidental, nonspecific wandering.

Barthlott et al. (1998) observed accumulation of protozoa near non-sterile *Genlisea* traps, which might have contained commensals potentially luring ciliates. In traps of cultivated *Genlisea* plants there is a rich community of commensals including bacteria, desmids, *Chlamydomonas* div. spec., and euglenoids (Płachno et al., 2005; Płachno, 2006; Płachno and Wołowski, 2008). In this context we cannot rule out the potential existence of chemical mediators emitted by these commensals, especially since some of them produce mucilage and enzymes in the trap environment (Płachno and Wołowski, 2008). Similarly, bacteria and algae attached to the trap surface might be prey for the protozoa and small invertebrates that visit *Genlisea* traps. Seine et al. (2002) found that protozoa were attracted to places where traps of *Utricularia* traps were put and later removed (mediated exposure tests), but it is not clear whether attractants were generated by traps or by the bacteria adhering to trap surfaces.

Jobson and Morris (2001) shed some light on the behavior of prey creatures. They used boiled *Utricularia uliginosa* traps and sand grains to test the behavior of the blind copepod *Elaphoidella*. They found that passive objects might hold some attraction for these phytophilous copepods, which prefer to climb on different objects rather than move in open water. We suggest that *Genlisea* may also take advantage of this typical invertebrate behavior, because not only crustaceans but many ciliates prefer to "walk" on these surfaces. Moreover, traps in the natural habitat are surrounded by soil particles, and trap openings mimic the spaces between soil particles. In this scenario the prey would not be attracted specifically to the traps but would arrive there accidentally by "walking" on soil and trap surfaces.

We can improve our glass capillary model of the *Genlisea* trap in future work. In these experiments the protozoa entered a capillary but could escape, and the shape and entrance area of the glass tubes differed from those of the traps, where prey could accumulate. Our next experiments should use models more closely matching the studied trap.

Studnička (2003a) suggested that oxygen in the trap cavity might be an attractant, and Adamec (2007a) found anoxia in traps. Thus, radial oxygen loss from arms to the ambient anoxic medium (Adamec 2005) might be an attractant. According to Adamec (2007a), suffocation might be the main reason for prey death in *Genlisea* and *Utricularia* traps. We argue that Studnička's and Adamec's hypotheses are valid only when prey are dependent on a critical level of oxygen; however, *Genlisea* traps grow in an anoxic environment (Adamec, 2007a). Organisms that occur in this environment should tolerate these conditions well. In part we agree with Adamec (2007a) that *Utricularia* may use suffocation for killing prey. This mechanism may work especially well in aquatic floating species that catch mainly aquatic crustaceans, many of which are rather oxygen-dependent (Newrkla, 1985; Weider and Lampert, 1985). Still, this is problematic in terrestrial and affixed-aquatic species with dimorphic shoots (e.g., *U. stygia, U. intermedia*), which have traps in substrate with low oxygen levels (Adamec, 2007b) where fauna are well adapted to facultative anoxia.

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