Mutualism between the Carnivorous Purple Pitcher Plant and Its Inhabitants

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ABSTRACT: Larvae of the Diptera, *Wyeomyia smithii* and *Metriocnemus knabi*, accelerate the breakdown of prey and the rate of ammonia production in leaves of *Sarracenia purpurea* L. The leaves take up ammonia and carbon dioxide rapidly and infuse oxygen into the water contained in them. Brighter light and higher temperature promote more rapid uptake of ammonia. Thus, under the brightest, warmest times of day, inquiline metabolism and respiration can produce increased substrates for photosynthetic carbon reduction and nitrogen incorporation. These same conditions promote an aerobic metabolite-free environment within the leaves. When the system goes anaerobic as a result of superabundant prey decomposition, photosynthetic bacteria predominate and fulfill the roles of the previous aerobic inhabitants.

INTRODUCTION

The leaves of the insectivorous purple pitcher plant *Sarracenia purpurea* L. act both as a trap for insect victims and as a habitat for a variety of aquatic flora and fauna (inquilines). In the fluid of the leaves is found a variety of bacteria (Hepburn and St. John, 1927), protozoa (Hegner, 1926; Addicott, 1974), aquatic mites (Hunter and Hunter, 1964; Nesbitt, 1954), and three Diptera, a sarcophagid maggot, *Blaesoxipha fletcheri* (Aldrich), which feeds mainly in the surface from carcasses of freshly killed prey (Forsyth and Robertson, 1975; Fish and Hall, 1978), a chironomid midge, *Metriocnemus knabi* Coq., which feeds on dead victims at the base of the leaf (Knab, 1905; Judd, 1959; Cameron *et al.*, 1977), and a culicid, *Wyeomyia smithii* (Coq.), which grazes on suspended particulate material including bacteria and protozoa (Addicott, 1974; Smith, 1904; Fish and Hall, 1978). It has generally been assumed that the primary benefit of carnivory is derived nitrogen (Higley, 1885; Hepburn *et al.*, 1920; Wherry, 1929; Lloyd, 1942), but more recent investigators have suggested that in *Sarracenia flava* L., metallic ions (Plummer, 1963) and sulfur or phosphorus (Plummer and Kethley, 1964; Christensen, 1977) may also be important benefits. Among open leaves of *S. purpurea* in nature, nitrogen in the form of ammonia is omnipresent (Higley, 1885) and foliar ammonia is taken up by the host leaf of this species (Higley, 1885; Hepburn *et al.*, 1920).

Sarracenia flava may secrete digestive enzymes (Plummer and Jackson, 1963; Plummer and Kethley, 1964), but plant-originated enzymes in *S. purpurea* have not been conclusively demonstrated, despite claims to the contrary (Hepburn *et al.*, 1920). Even in *S. flava,* Plummer and Jackson (1963) believe that bacteria act rapidly to promote decomposition of prey. Degradation of prey in *S. purpurea* is then most likely carried out by its victims and inhabitants. Attention has focused mainly on autolytic enzymes of the prey themselves and exoenzymes of bacteria, but as early as 1882 Schimper (as quoted by Hepburn *et al.*, 1927) speculated that "innumerable worms" in the leaves "possibly participate in the transformation of the animal bodies into soluble compounds." Schimper thus implicated the macrofauna of pitcher plants in the conversion

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of prey to plant-available nitrogen. In the present paper, we pursue this implication, examining the roles of *Wyeomyia smithii* and *Metriocnemus knabi* in the acceleration of ammonia's appearance in the prey decomposition process. We further consider nitrogen and carbon dioxide uptake and oxygen production by pitcher plant leaves and the consequences of organic overload and resulting anoxia.

MATERIALS AND METHODS

Source and maintenance of organisms. — We obtained overwintering third instars of *Wyeomyia smithii* and third and fourth instars of *Metriocnemus knabi* from Ocean Co., New Jersey (40°N, 74°W) and maintained them in an ordinary refrigerator at 4 ± 1 C until used in experiments. We obtained *Sarracenia purpurea* from various localities along the Gulf Coast in western Florida, southern Alabama and southeastern Mississippi and maintained them on water tables beneath fluorescent light banks. Prey for plants consisted of meal worms (*Tenebrio* sp.) cultured on a mixture of dry bran, rolled oats and chunks of potato. For artificial leaves, we used glass jars, 4.5 cm high and 5.0 cm in diam.

Nitrogen measurement. — We measured ammonia nitrogen by pipetting 0.3 ml of a standard solution of ammonium sulfate or pitcher plant fluid into a 15 ml centrifuge tube, diluting with 7.0 ml of distilled water, and adding 3.0 ml of Nessler's solution (Folin-Wu, Fisher Supply Co.) and one drop of iodine solution (30 g KI + 20 g crystalline I per 1). After vigorous mixing and an incubation period of 5 min at room temperature (22 ± 1 C), we read the absorbance at 460 nm (A_{460}) in a Markson field colorimeter zeroed against 7 ml distilled water plus 3.0 ml Nessler's solution. We calculated experimental values of mg nitrogen per 100 ml (N mg %) by solving the regression equations for Nmg% as a function of A_{460} determined from known standards.

Absorption of nitrogen by leaves without inquilines. — To estimate the uptake of ammonium nitrogen by leaves of *Sarracenia purpurea,* we exposed intact plants in 0.3 m³ polystyrene humidity chambers to bright or dim light at warm or cool temperature. For "dim" light conditions we placed the plants 20-30 cm from a single air-cooled 4-w cool-white fluorescent lamp at 12 ± 1 or 22 ± 1 C; for "bright" light conditions we placed plants 40 cm from a single air-cooled Sylvania Circline 32-w cool-white fluorescent lamp at 12 ± 2 or 22 ± 1 C. The above dim and bright light conditions provided approximately $7 \mu W/cm^2$ and $13 \mu W/cm^2$, respectively. We provided photoperiods 14:10 = L:D under both conditions. After selecting leaves of similar size, we rinsed them three times with distilled water to minimize the number of microorganisms and rinse out any detritus. We measured 20 ml of 9.5 Nmg% of ammonium sulfate into each of three leaves and three control jars under the high intensity light and 20 ml of 12.0 Nmg% of ammonium sulfate into each of three leaves and one control jar under the low intensity light. We marked the 20 ml waterline on each leaf or jar and added sufficient distilled water daily to maintain the level at the 20 ml mark. We sampled the leaves or jars three times at intervals of 1-3 days during 8 days and calculated net foliar absorption after a given time as [(gross mg N absorbed by a leaf)-(gross mean mg N absorbed by the glass jar)].

Production of nitrogen by inquilines. — To estimate production of nitrogen by various inquilines, we filled 12 carefully matched leaves on intact plants and 12 jars with 20 ml of distilled water each and added 0.175 ± 0.025 g of fresh meal worms and 0.05 ml of a micro-flora/fauna inoculum obtained as undiluted, unfiltered, field-collected pitcher plant water from New Jersey. We placed the plants or jars into 0.3 m³ polystyrene humidity chambers at a distance of 40 cm from six 96-w cool white and two General Electric PL40 40-w plant lights at 22 ± 1 C and L:D = 16.8. To each of three leaves and three jars, we then added (1) nothing; (2) 10 size-matched third and fourth instar *Metriocnemus knabi*; (3) 10 third instar *Wyeomyia smithii*; or (4) 10 *M. knabi* plus 10 *W. smithii*. We maintained water level daily as above and sampled the jars and leaves every

2-3 days until death or metamorphosis of inquilines made further comparison pointless.

Identification of bacteria. — We carried out bacterial culture and isolation on a yeast extract and casein hydrolysate medium (Sistrom, 1977). To examine growth on various carbon sources, we started with a minimal medium (M22 in Sistrom, 1977) to create test media containing 0.2% casamino acids, malate, acetate, proprionate, citrate, glucose, mannitol, thiosulfate or tartrate or 0.5% caprylate, benzoate or para-hydroxy benzoate. To the latter two media we also added 0.01% acetate, and, as a blank, we made a minimal medium with 0.01% acetate added. We added sterile carbon sources to inoculated minimal media in 16 ml screw-top tubes and placed them 7 cm from a 60-w incandescent Lumiline lamp. We then determined growth by measuring turbidity in a Bausch & Lomb Spectronic 20 spectrophotometer over a 5-day period.

To determine absorption spectra of the bacteria, we spun down the contents of the caprylate medium, resuspended in 0.01 M Tris buffer without magnesium at pH 7.2. We disrupted the cells in a Bransom Instruments sonicator and centrifuged them to remove large cell fragments. We then recorded the absorption spectrum on a Cary 14 spectrophotometer.

To test for growth under anaerobic conditions, we inoculated minimal medium (M22) with either 0.1% acetate, 0.1% malate, or 0.1% glycerol solidified with agar in Gas Paks (Becton Dickson Co.) at a distance of 7 cm from four 40-w incandescent lamps with intervening water bottles to absorb heat.

Gas in leaves. — For the examination of carbon dioxide, we used leaves clipped from an intact pitcher plant. We stripped water of CO_2 and O_2 by continuously bubbling N_2 through it and used this stripped water directly or after charging with CO_2 by the addition of $NaHCO_3$. We filled each leaf to about ¾ capacity and then, while holding the leaf in ice water, sealed the surface with heated petroleum jelly (Vaseline). We made hooks of aluminum wire and suspended individual leaves from the edge of a 500-ml beaker so that the cut petioles were continuously submerged in distilled water. We placed two of these beakers in 3 cm of distilled water in a 40 liter aquarium, covered one with a 3 liter metal can (dark), left the other exposed (light), and covered the aquarium with a 2.5 mm polycarbonate plate. Illumination was then provided by a 20-w cool-white fluourescent lamp at a distance of 8 cm.

For gas determination, we removed water samples by piercing the base of individual leaves (one leaf per sample) with a needle attached to a syringe and withdrawing 2 ml of water which was injected directly into a Fisher Model 25V gas partitioner. Analysis of CO_2 and O_2 in these samples followed the procedure used by Morris (1967).

Statistical treatment of data. — Regressions, analysis of variance (ANOVA) and, where indicated by ANOVA, Duncan's New Multiple Range Test (DMRT) were carried out according to methods provided in Steel and Torrie (1980).

RESULTS

Nitrogen uptake by leaves. — Nitrogen uptake was not correlated with leaf size but was proportional to the concentration of nitrogen in the leaf (Fig. 1). We therefore calculated rate of uptake as $(mg_t - mg_{t+1})/(mg_t)(\triangle t)$ where mg_t is the mg of nitrogen present as ammonia nitrogen at time t and $\triangle t$ is the interval from t to t + 1. After log-transformation to achieve homogeneity of variance, rate of uptake was affected by both light ($F_{1,32} = 31.36$; $P < 0.001$) and temperature ($F_{1,32} = 11.18$; $P < 0.01$) with no interaction ($F_{1,32} = 0.23$; $P > 0.05$). Rate of nitrogen uptake is then faster in brighter light or at higher temperature (Fig. 1).

Nitrogen production from prey. — Nitrogen levels in leaves (Fig. 2) did not vary from day 2 onwards ($F_{5,48} = 2.34$; $P > 0.05$), regardless of the presence, absence or combination of *Wyeomyia smithii* and *Metriocnemus knabi* ($F_{3,48} = 1.70$; $P > 0.05$). After the 2nd

day, nitrogen levels in jars were always higher than in leaves. Nitrogen levels in jars were greater when inquilines were present but did not vary among jars with *W. smithii, M. knabi* or both ($F_{3,48} = 17.24$; $P < 0.001$; followed by DMRT). Among all jars, nitrogen levels rose from days 2-9 but did not differ significantly among days 9-13 ($F_{5,48} = 27.54$; $P < 0.001$; followed by DMRT).

Concomitant with the rise and plateau of nitrogen levels in jars, inquilines decreased gradually due to mortality and pupation. To take this attrition into account, we first calculated the net increase in nitrogen due to the presence of inquilines as:

net increase = $(N_{t+1,E} - N_{t+1,c}) - (N_{t,E} - N_{t,c})$

where $N_{t,E}$ and $N_{t,c}$ are the nitrogen levels in the jars at time t with (E) or without (C) inquilines. Finally, we calculated the net rate of nitrogen production per individual as:

net rate = (net increase)/(number of inquilines) (Δt).

Net rate of nitrogen production (Fig. 3) was then positive in jars containing *Metriocnemus knabi* for days 2-6 and negative thereafter. The net rate in jars containing *Wyeomyia smithii* was positive for all days but day 13. Peak rate of nitrogen production occurred on day 4 in jars with *M. knabi* and on day 9 in jars with *W. smithii*. Jars containing both *M. knabi* and *W. smithii* showed a pattern intermediate to that of jars containing only one of them.

These results show that the presence of inquilines enhances the production of nitrogen in water with decaying prey but that the effect of *Wyeomyia smithii* was delayed with respect to that of *Metriocnemus knabi*.

Gas in leaves. — To mimic a situation in which inhabitants of pitcher plants, through their metabolism, generate CO_2, we started with $NaHCO_3$-charged leaves producing an initial CO_2 concentration of 18.93 ml/liter. Figure 4A shows that between the starting date and the 3rd day, CO_2 levels declined at the same rate in both the light- and dark-exposed leaves. Thereafter, CO_2 levels remained between about 8 and 14 ml/liter in the dark-exposed leaves but declined to about 2-8 ml/liter in the light-exposed leaves. ANOVA of CO_2 concentration from days 3-9 revealed significant differences between the light- and dark-exposed leaves ($F_{1,10} = 5.02$; $P < 0.05$). In a separate experiment, we stripped gases from the leaves as above and placed only the stripped water into the leaves. After 2 days, CO_2 levels in the dark-exposed leaf had risen to between 10 and 13 ml/liter (Fig. 4A) but to less than 3 ml/liter in the light-exposed leaves.

To examine the influx of oxygen into leaves, we added water stripped of other gases by bubbling nitrogen through it to a series of leaves and recorded the appearance of O_2 into these leaves (Fig. 4B). ANOVA revealed significant differences between light and dark-exposed leaves ($F_{1,10} = 6.33$; $P < 0.05$).

These results indicate that leaves of the pitcher plant, especially in the light, take up CO_2 from and infuse O_2 into the water contained therein.

Anaerobic bacteria. — Both in the field and in the laboratory, leaves or jars with an ex-
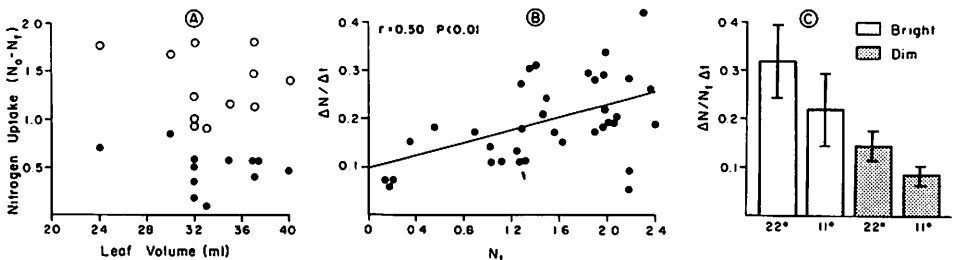

Fig. 1. — Nitrogen (ammonia) uptake by leaves of *S. purpurea*. (A) Effects of leaf size on nitrogen uptake after 2 (●) and 6 (○) days. N_t = ammonia nitrogen (N mg%) remaining at time t. (B) Dependence of rate of uptake (mg per day) on total nitrogen content of a leaf at time t (N_t). (C) Net uptake of nitrogen ($\Delta N/N_t \Delta t$ day) at warm (22 C) or cool (11 C) and bright (13 $\mu W/cm^2$) or dim (7 $\mu W/cm^2$) light. Vertical bars show 2 SE

cess of organic material turn a reddish pink and become viscous and fetid. We initially streaked samples from red-colored water in a jar with *Wyeomyia smithii, Metriocnemus knabi,* a pitcher plant water inoculum, and rotten mealworms on a yeast extract-casein hydrolysate plate and from this plate isolated red bacterial colonies. When grown on minimal medium with various carbon sources, no turbidity occurred after 5 days on the blank, casamino acids, citrate, glucose, mannitol, thiosulfate, or tartrate-enriched
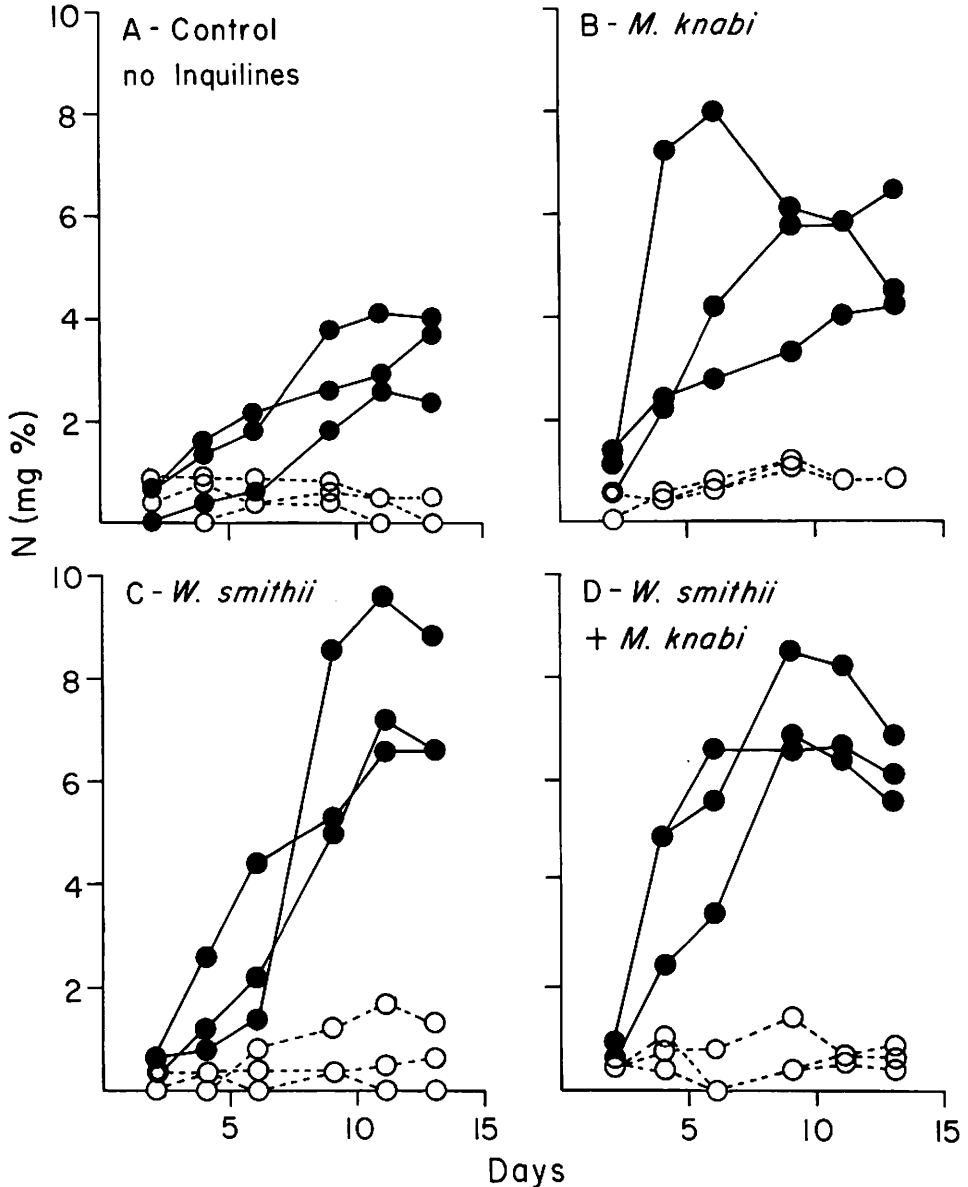

Fig. 2. — Nitrogen levels in leaves (O) and jars (●) after the addition of 0.175 g of mealworms and an inoculum of pitcher plant water and midges (*M. knabi*), mosquitoes (*W. smithii*), both or neither (control)

media. There was growth on propionate (greatest turbidity), para-hydroxy benzoate and, to a lesser extent, malate, acetate and benzoate. These results are closest to those obtained from *Rhodopseudomonas palustris* by Trüper and Pfennig (1978). The absorption spectra from the supernatant of disrupted cells exhibited bacterial chlorophyll peaks at 804 and 861 nm with carotenoid peaks at 465, 492, and 525 nm. While the chlorophyll peaks are compatible with several bacteria, the carotenoid peaks are closest to those for *R. palustris* (Biebl and Drews, 1969).

When we plated pitcher plant water from northern Maine on an acetate-malate-glycerol medium, we obtained two types of colonies, one red, the other orange. Under the microscope, both resembled *Rhodopseudomonas palustris* in shape and size (2-2.5 μm). The red colony had a color indistinguishable from *R. palustris* while the orange colony was clearly different. Under anaerobic conditions, both formed black-red colonies but the orange colony appeared to grow more slowly than the red one. From these observations, we conclude that the red colonies were *R. palustris* whereas the orange one remains unknown, but both colonies are clearly capable of rapid anaerobic growth in bright light.

DISCUSSION

Our results confirm those of Cameron *et al.* (1977), that pitcher plant leaves infuse oxygen into the water they contain (Fig. 4B). In addition, we have found that the leaves, especially in the light, take up carbon dioxide from this water (Fig. 4A). Our results (Fig. 1) also confirmed earlier reports (Higley, 1885; Hepburn *et al.*, 1920;
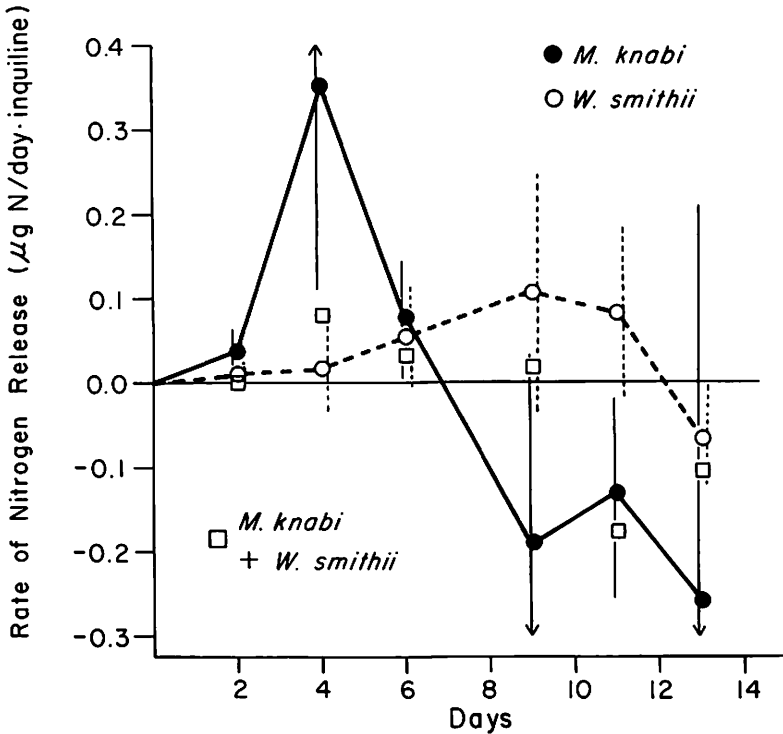

Fig. 3.—Net rate of nitrogen release (net increase over control per day per individual inquiline). A positive rate indicates that insect inquilines are producing nitrogen at a rate greater than that attributable to bacteria, protozoa, autolytic and leaf-originated enzymes. Vertical bars show the standard deviation

Plummer and Kethley, 1964; Christensen, 1977) that ammonia is taken up by pitcher plant leaves and show further that this uptake is enhanced at either high temperature or light intensity (Fig. 1). The rate of ammonia uptake does not appear to taper off at higher concentrations (Fig. 1B) and exhibits a Q_{10} of about 1.5 (Fig. 1C). Both these observations suggest that uptake is the result of passive diffusion along an electrochemical gradient.

It is clear (Fig. 2) that leaves are capable of absorbing the combined end products of bacterial, protozoan and insect metabolism. With or without midges or mosquitoes, jar ammonia rose to higher levels than plant ammonia, which remained at a relatively low, steady level. It is therefore from rates of nitrogen production in jars that we draw our conclusions regarding the roles of inquilines in the breakdown of prey. In the absence of insects (Fig. 2A), the action of bacteria, protozoa and autolytic enzymes produces a steady supply of soluble nitrogen. The addition of midges initially greatly enhances the rate of nitrogen production while mosquitoes have a less dramatic, delayed effect (Fig. 3).

Temporal patterns of nitrogen production can be interpreted from the feeding biology of the inquilines. The midge larvae feed on both large and small detritus. They are routinely observed boring through dead victims at the base of the leaf. In so doing, they disrupt the physical integrity of the victim's cuticle and organ systems, accelerating the release of endogenous bacteria and of autolytic enzymes. Mosquito larvae are filter-feeders and browsers, eating bacteria, protozoa and/or particulate material. These food sources would have to become available before mosquitoes could consume them and hence the delayed appearance of the mosquito-produced nitrogen. When both are present, midges might create more opportunity for bacteria and protozoa, but the effect of the latter would be diminished from grazing by mosquitoes, resulting in intermediate rates of nitrogen production.

Prey may initially be broken down on the surface by *Blaesoxipha fletcheri* (Forsyth and Robertson, 1975; Fish and Hall, 1978), in the detrital zone by *Metriocnemus knabi*, or by the immediate or combined effects of bacterial and autolytic enzymes. *Wyeomyia smithii* can graze these bacteria or particulate materials directly or via protozoa. Each step in this pathway releases nitrogen available to plants. Bacteria, protozoa and autolytic degradation of prey would eventually result in the latter's complete breakdown (Fig. 2A). The role of the insect inhabitants is then one of accelerating this
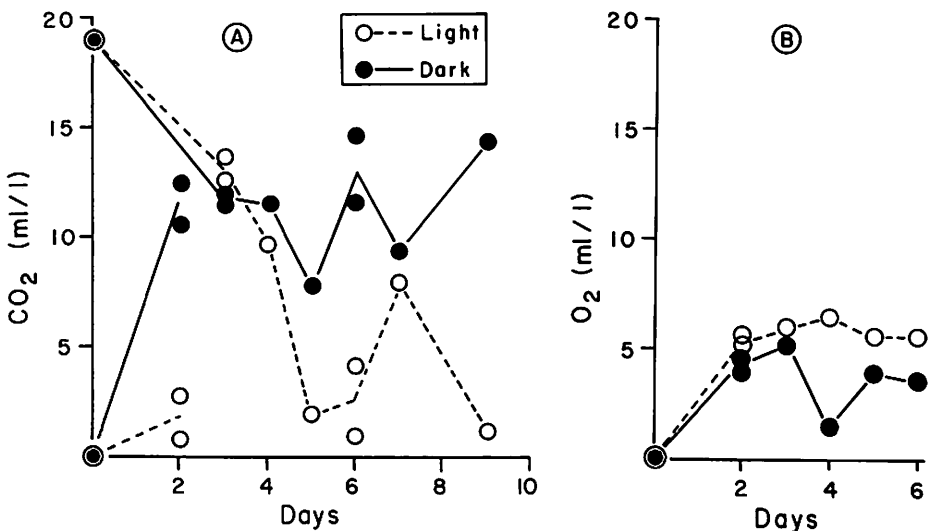

Fig. 4.—Carbon dioxide and oxygen levels in sealed leaves under light and dark conditions

process, either by direct action (*M. knabi* and *B. fletcheri*) or by preventing the buildup of nitrogen in bacterial and protozoan cells (*W. smithii*).

Our results and biochemical pathways surrounding photosynthesis in other higher plants suggest that pitcher plant photosynthesis and inquiline respiration and metabolism are complementary systems (Fig. 5).

Inquiline respiration and metabolism will be greatest at higher temperatures. Midday and afternoon summer temperatures in pitcher plant leaves range from 25-40 C (Evans and Brust, 1972; Kingsolver, 1979; Bradshaw, 1980). Temperatures within leaves on the open bog mat exposed to the sun are higher than either air or surface temperature (Kingsolver, 1979), suggesting further that convection around the leaves is low within the boundary layer of bogs. Under these conditions of high light and temperature and low convection, the availability of carbon dioxide may become
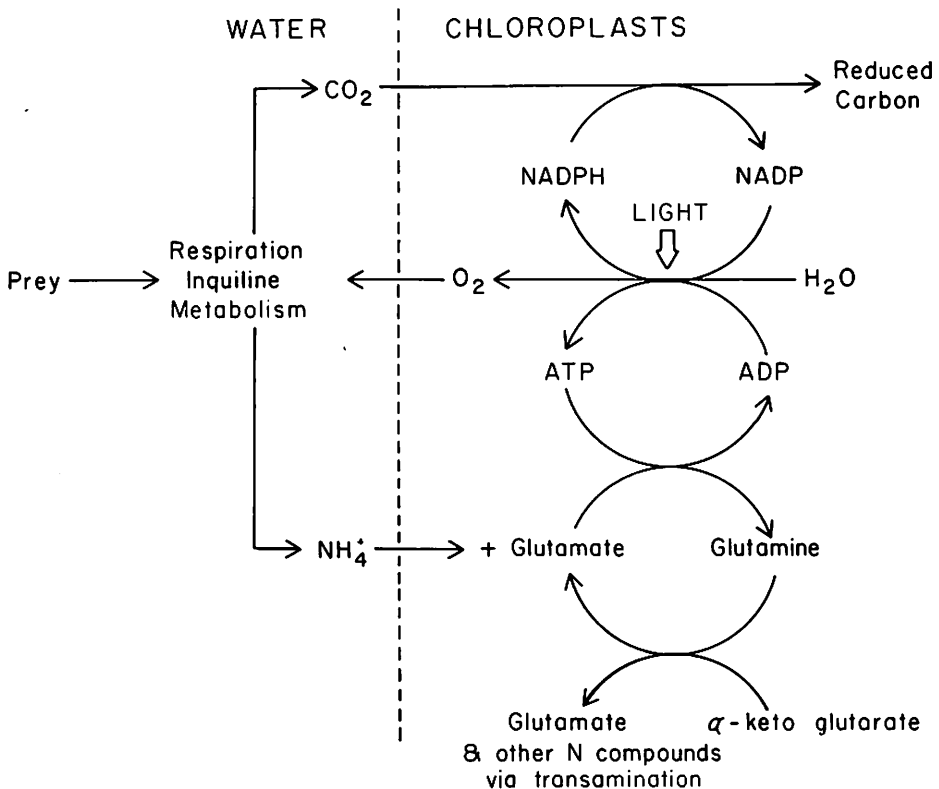

Fig. 5. —Gas and nitrogen flow in the pitcher plant microcosm under aerobic conditions. Reactions taking place in the water are inferred from the present study; reactions taking place in chloroplasts are inferred from photosynthetic processes in other higher plants. Prey are broken down by bacteria, protozoa, midges and mosquitoes. Through respiration and metabolism, these inquilines provide a ready supply of carbon dioxide and ammonia to their host while consuming oxygen produced by the latter. Light drives the cleavage of water to reduce NADP and to phosphorylate ADP. The generated NADPH is used to reduce carbon dioxide and the generated ATP powers the amination of glutamate to glutamine via glutamine synthetase. Glutamine may then combine with α-keto glutarate to form two molecules of glutamate via glutamate synthetase (Mifflin and Lea, 1976). One of the glutamate molecules thus generated reenters glutamine synthesis; the other glutamate serves as an amide donor to form other nitrogenous compounds in plants by a variety of transamination reactions (Bryan, 1976). Each reaction in this diagram is accelerated by temperature in the direction of the arrows

limiting for photosynthesis. Yet, it is under these same conditions that inquiline respiration would be generating the most carbon dioxide. The net result is that peak oxygen production and carbon dioxide demand by *Sarracenia purpurea* should coincide with peak requirements and production, respectively, of leaf inhabitants. While it is generally assumed that nitrogen enrichment is the primary advantage gained by pitcher plant carnivory, we propose that increased photosynthetic efficiency due to enhanced carbon dioxide availability may make an equally important contribution to *S. purpurea.*

While both plant and inquilines clearly benefit from one another, there is no compelling reason to ascribe this mutualism to co-evolved responses. Leaves of higher plants in general take up carbon dioxide and evolve oxygen during photosynthesis; other higher plants that do not contain water or harbor inquilines are capable of foliar nitrogen adsorption (Whittwer and Teubner, 1959). Similarly, bacteria, protozoa and insects in general produce carbon dioxide and nitrogenous end products as a result of respiration and metabolism. Whatever factors led to carnivory in ancestral pitcher plants at the same time provided a food supply for bacteria, protozoa and other inquilines, resulting incidentally in enhanced carbon dioxide as well as nitrogen production. The attributes which enable each member of this community to exist with the others were probably possessed by the constituent species at the time of their first association with *Sarracenia purpurea.*

The above scheme all depends upon the system remaining aerobic; but, our own observations, as well as those of others (Knab, 1905; Plummer and Kethley, 1964), show that superabundant prey can overload the system. At such times, oxygen levels decline, the normal inquilines die (Knab, 1905; Cameron *et al.,* 1977), and the water becomes fetid and takes on a red or wine color (Higley, 1885; Hepburn *et al.,* 1920). Our results indicate that this characteristic red color is due to at least *Rhodopseudomonas palustris* and perhaps our unidentified orange bacterium as well. Both are clearly capable of thriving in bright anaerobic conditions. *Rhodopseudomonas palustris* is a widespread photosynthetic nonsulfur bacterium, especially in acid bogs and waterlogged soils (Biebl and Drews, 1969; Biebl and Pfennig, 1982), and there is no reason to suppose that it has evolved in response to the specific environment of pitcher plants. Rather, it travels on or in the inquilines and victims of *Sarracenia purpurea* and, being omnipresent, is able to exploit, opportunistically, anaerobiosis in the light. As such an opportunist, it provides a backup or fail-safe system which incidentally assures that, even under conditions hostile to the normal inquilines, degradation of prey will continue in the leaf of its carnivorous host.

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