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Hydrogen cyanide release during feeding of generalist and specialist lepidopteran larvae on a cyanogenic plant, *Passiflora capsularis*

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Abstract. The hydrogen cyanide-based interaction of a strongly cyanogenic plant, *Passiflora capsularis*, and larvae of two insect herbivores, a generalist (*Spodoptera frugiperda*) and a specialist (*Heliconius erato*), is examined in terms of the combined kinetics of the feeding process and the simultaneous hydrogen cyanide (HCN) liberation, as compared with the natural kinetics of hydrogen cyanide evolution by plant-leaf tissue. There are marked differences in acceptance of *P. capsularis* by third-instar larvae of specialist and generalist species. The former, *H. erato*, display a parsimonious ingestion rate of 0.74 ± 0.15 mg (fresh weight) min^{-1} comprising 18% active feeding time, whereas *S. frugiperda* larvae show a more erratic and restrained feeding involving 4% of the time at 0.45 ± 0.14 mg min^{-1}. These *S. frugiperda* larvae ingest 124.4 ± 8.3 mg (fw) of the non-cyanogeneic *Spinacia oleracea* leaves in 24 h compared with only 74.7 ± 20.1 mg of *P. capsularis* in the same period. The total hydrogen cyanide released naturally from wild specimens of *P. capsularis* plants is in the range 326–3901 μg g^{-1}. Hydrogen cyanide evolution from macerated *P. capsularis* leaves takes place along a hyperbolic function with time and initial velocities of cyanide evolution are a linear function of total hydrogen cyanide. When feeding on *P. capsularis* leaves, *H. erato* releases only a minor fraction relative to total hydrogen cyanide (0.09%) and to the anticipated cyanide from the initial velocity (7%). By contrast, *S. frugiperda* evokes 5.8-fold more than the anticipated hydrogen cyanide release from the plant. The findings are interpreted as diverging strategies by generalist and specialist insects in the utilization of hydrogen cyanide in cyanogenic plants.

Key words. Cyanogenesis, generalist, herbivory, hydrogen cyanide evolution, kinetics, *Passiflora capsularis,* specialist, tropics.

Introduction

Cyanogenic glycosides are nitrogen-based phytoanticipins (Zagrobelny *et al*., 2004) useful in the defence of many plants (Thomsen & Brimer, 1997; Jones, 1998; Francisco & Pinotti, 2000) and invertebrates (Jones *et al*., 1962; Davis & Nahrstedt, 1984; Witthohn & Naumann, 1987) that regulate plant-insect and invertebrate–predator interactions (Zagrobelny *et al*., 2004). Cyanogenic glycosides are key elements in the development of modern ecological theory (Jones *et al*., 1978; Conn, 1979; Nahrstedt, 1985; Vetter, 2000; Puustinen & Mutikanen, 2001; Gleadow & Woodrow, 2002). In addition to their bitter taste, cyanogenic glycosides stochiometrically yield hydrogen cyanide (HCN) when in contact with the compartmentalized enzymes, β-glucosidases and α-cyanohydrin lyases by a two-step process, within the plant. Upon mechanical injury by herbivory, the various chemicals become mixed and HCN formation ensues. Cyanide is an almost universal toxin with a fast effect,

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although tolerance to cyanide is species dependent (Christensen, 1976; Vetter, 2000).

In view of HCN toxicity, strongly cyanogenic plants should remain relatively free of damage by generalist herbivores (Schappert & Shore, 1999; Viette *et al*., 2000), but are nevertheless attacked by specialists (Glander *et al*., 1989; Ferreira *et al*., 1997). For example, powerfully cyanogenic members of the *Passiflora* family of plants are selected by oligophagous *Heliconius* butterflies (Benson *et al*., 1976). On the other hand, when cyanogenic foliage is available to some non-adapted herbivores, by particular constraints of their habitat such as dominance of a single cyanogenic species or over-exploitation of more palatable plants, these insects may still utilize the resource (Scriber, 1978; Dirzo & Harper, 1982; Brattsten *et al*., 1983; Gould, 1984; Calatayud & Le Rü, 1996).

Cyanogenesis may be viewed either as a static protection offered by constitutive cyanogenic glycosides of bitter taste, or a dynamic formation of HCN during feeding episodes by chewing insects. Although the former depends strictly on the total amount of cyanogenic glycosides accumulated in plant tissue, the latter is the result of a time-dependent reaction of these cyanogenic glycosides, catalized by endogenous enzymes within the plant to furnish HCN.

Although tolerance or usage of cyanogenic glycosides by adapted insects has been explained by their transformation by enzymes of the insects' gut into innocuous materials (Fitzgerald *et al*., 2002), it is also conceivable that a substantial part of the plant's HCN may never enter the attacker and be lost to the atmosphere surrounding the herbivore by diffusion or wind. However, before the onset of diffusion, liberated HCN may have a deterrent effect. If plant tissue is ingested when HCN liberation is still occurring, it will be generated inside the herbivore's gut causing greater harm because of the confinement in a small volume and hence in high HCN concentrations.

It is evident that the proportion of both fractions of HCN, diffused and ingested, will depend on the natural kinetics of HCN evolution by the plant's tissue, the rate at which this tissue is devoured by the attacker and its particular feeding strategy. Conceivably, adapted insects using cyanogenic glycosides as nutrient should inhibit HCN production during feeding to preserve valuable nitrogen for digestion and metabolism, whereas nonadapted insects should enhance the formation of HCN when chewing to reduce its potential harm in the gut.

The rate of HCN formation in cyanogenic plants has been studied quantitatively only recently in *Pteridium arachnoideum* (Alonso-Amelot & Oliveros, 2000, 2005; *Eucalyptus polyanthemos* (Goodger *et al*., 2002) and *Phaseolus lunatus* (Ballhorn *et al*., 2005). This rate depends on the plant species and the total content of cyanogenic glycoside for the genotypes also possessing the corresponding β-glucosidase. In kinetic studies using macerated leaves of *P. arachnoideum* and *P. lunatus*, the time-dependent HCN formation shows an initial pulse that wanes within a relatively short time period. However, this pulse implies the decomposition of only a minor fraction of the total cyanogenic glycoside load in the plant, meaning that, in principle, most cyanogenic glycosides should be left intact to decompose inside the herbivores lumen. Thus, it is necessary to examine experimentally the release of HCN under a more realistic situation in which a cyanogenic plant is devoured by chewing insect larvae. Although Ballhorn *et al*. (2005) investigated this question in *Schistocerca gregaria,* a polyphagous insect, the present study examines HCN formation during feeding of a monophagous and a polyphagous herbivore on a cyanogenic plant in a tropical habitat. This approach provides information about the time-dependent defence response of the plant upon attack and its expression as HCN release, so far unknown outside the species mentioned above, as well as the adaptive response of the insect to this plant defence depending on its specialist vs. generalist status.

Although earlier studies on *P. arachnoideum* show it to be a strongly cyanogenic and widely distributed plant (Alonso-Amelot & Oliveros, 2000, 2005), the few arthropod herbivores that successfully exploit this fern are generally small, and hence unsuitable for quantification experiments. A more accessible model is provided by cyanogenic leaves of tropical *Passiflora capsularis* (L.) (Passifloraceae). Two relatively large (> 100 mg) lepidopteran larvae were selected, which are naturally found in wild populations of this plant, *Heliconius erato* (L.) (Lepidoptera: Heliconinae) and *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae). The first species is an obligate *Passiflora* feeder and is part of a guild of adapted butterflies that severely defoliate members of the *Passiflora* and *Turnera* plant families (Benson *et al*., 1976). *Spodoptera frugiperda* is a generalist moth found only on *P. capsularis* during events such as population explosions and a lack of sufficient alternative food (personal observation), as occurs irregularly in the study area between May and August

The present study aims to quantitatively study the cyanogenic glucoside–HCN dynamics during larval feeding. First, the kinetics of HCN formation by *P. capsularis* leaves at the average temperature found in the field (30 °C) are determined. Next, the rate of leaf intake by *H. erato* and *S. frugiperda* larvae, as well as the amount of HCN simultaneously liberated to the atmosphere, are studied. Finally, the cyanide balance in food and faeces of *P. capsularis* fed larvae is analysed. Comparison of the first two sets of data allows determination of the extent to which insects induce or inhibit the formation of HCN during feeding, whereas the third series of experiments help to understand the insects' strategy in tolerating the plant's chemical defence, according to their specialist vs. generalist status. The physio-ecological consequences that this might entail are discussed.

Materials and methods

Study sites

Four secondary forests composed of medium to large trees (canopy levels of 10–25 m, respectively) with

interspersed lower vegetation were selected between 1330 and 1600 m above sea level around the city of Mérida, in Mérida State, Venezuela (8°34′N 71°11′W), an intramontane valley with a bimodal annual rain regime of approximately 1800 mm. Specific location names were: Las Tapias, Paseo Los Pinos and La Hechicera Baja. These plant communities are of the seasonal sub-Andean semideciduous forest type. Sampling of *P. capsularis* plants and *H. erato* eggs was conducted during the rainy months (May–November) of 2001 and 2004 in forest clearings.

Insects

The finding of *H. erato* larvae in the field on *P. capsularis* was restricted by the scarcity of wild plant material. Further restrictions were due to the oviposition strategy of females of *H. erato*, which consists of laying only one egg, and occasionally two per plant, at the growing tip of the pod. The occurrence of parasitoids in a large proportion of the field-recovered larvae (Ehrlich & Gilbert, 1973) was also a limiting factor. Collected eggs were transferred onto *P. capsularis* leaves until the emergence of adults. Rearing was performed at 23–25 °C in 1-L plastic pots with netting for ventilation. Caged fertile females were allowed to oviposit on potted plants and the F_1 generation was used for the experiments. Voucher specimens were submitted to the insect collection of the Laboratory of Insect Ecology, Universidad de Los Andes, and classified by the curator Dr Luis Daniel Otero. For feeding and cyanogenesis measurements, only third-instar larvae of similar size and weight were used from the laboratory colony.

Larvae of *S. frugiperda* obtained from a certified collection at Instituto de Zoología Tropical of Universidad Central de Venezuela, Caracas, were reared individually on an artificial diet composed of a homogenate of white bean meal (400 g), calcium caseinate (30 g), agar (30 g), ascorbic acid (500 mg), sodium benzoate (5 g) and distilled water (1250 mL). Approximately 5 g of this material was placed in each one of 50-mL plastic cups, with replacement every 48 h. Third-instar larvae were used for feeding experiments.

Plant samples

Mature leaves (29.9 ± 1.6 cm^2) were excised from wild plants in the field between nodes 3 and 6, packed in plastic clip bags and transported in a cold box (10–15 °C) within 1–3 h to the laboratory for analysis. Several collections were made in as many field trips to avoid storing the excised leaves for extended periods. For feeding experiments, young plants were removed from the study site with the soil surrounding the roots, transferred to 10-L clay pots, watered three times a week and placed in a nursery under conditions resembling their natural habitat.

Commercial spinach plants were grown in loamy soil using two applications of 20-15-20 NPK (Pequiven, Morón, Venezuela) standard fertilizer (5%, 100 mL of distilled water per plant) over a growth period of 3 months. Leaves were excised, washed with distilled water, allowed to dry on adsorbent paper towels and presented whole to each *S. frugiperda* larva.

Feeding behaviour

At time zero, one third-instar larva of each species was exposed to a previously weighed *P. capsularis* leaf of measured area, in a 9-cm Petri dish. The activity of the larva was continuously recorded in terms of actual feeding activity and resting times for 6 h. After each pause in feeding, leaf weight and area was determined with minimal disturbance of the larva. Five replicates were observed for each species.

The spinach–*Passiflora* choice experiment was carried out by exposing individual larvae of both species to four 1-cm circles of spinach and *Passiflora* leaves arranged radially around the centre of a 9-cm Petri dish using filter paper (Whatman No. 1, Maidstone, Kent, U.K.) for support. Larvae were allowed to feed for 2 h and five replicates were completed per species.

Quantification of cyanogenesis: general method

The sodium picrate-based spectrophotometric gas flow method of Alonso-Amelot & Oliveros (2000) was used. Briefly, undisturbed test tissue (0.1–0.2 g) was macerated with acid-washed fine sand in a mortar at –15 °C for 5 min. This allows the plant tissue to be crushed mostly to subcellular size particles, hence furnishing adequate mixing of cell components because examination of a water suspension of the sand homogenate under a light microscope (× 400) only showed a few residual cellular clumps in dispersed masses. The homogenate was then placed inside a flow reactor made of a 15-mL syringe connected to moist air inlet and outlet, and submerged in a water circulation bath at 30.0 ± 0.1 °C. After 1 min, air was passed at two bubbles per second (maximum rate 6 mL min^{-1}) and collected in a 18-mL test tube used as gas trap, containing 10 mL of aqueous alkaline 8% sodium picrate. After 18 h of bubbling, the picrate solution was transferred to a 1-cm optical path quartz cuvette and absorbance at λ_m 515 nm was measured. The HCN concentration was determined against calibration standards.

Small samples for HCN analysis were processed in a microdiffusion apparatus. A 50-mL stoppered bottle containing the sample was fitted with a 2-mL beaker containing 1.2 mL 8% alkaline sodium picrateg attached to the cap with the aid of a fine steel wire. At time zero, the bottle thus prepared was immersed in a water bath at 37 °C for no less than 15 h, after which the concentration of HCN trapped in the picrate solution was determined spectrophotometrically, as described above.

Kinetics of HCN liberation

The method of Alonso-Amelot & Oliveros (2005), using gas flow apparatus (Alonso-Amelot & Oliveros, 2000), was

employed at 30.0 ± 0.1 °C, a temperature commonly found in the study sites during the hours of maximum insect activity. Fresh *P. capsularis* leaf samples weighed 200–250 mg. Aliquots were extracted every 5 min during the first 30 min and every 10 min subsequently, until 340 min with a final reading after 24 h. A total of 42 independent kinetic runs were performed.

Quantification of HCN released and ingested by larvae during feeding

The cyanogenic capacity of many plants varies with leaf age and environmental conditions (Kaplan *et al*., 1983; Gleadow & Woodrow, 2000; Woodrow *et al*., 2002). To avoid this natural source of variance, freshly-excised *P. capsularis* leaves of appropriate size (29.9 ± 1.6 cm^2) and position (nodes 3–6) were cut into half along the central vein. One half included the petiole and central vein, and the petiole's end was wrapped with a moist cotton swab to maintain turgor for the feeding experiment. The other half was used for the analysis of the total HCN content $[HCN]_t$. The surface fresh weight and surface area of each half were obtained, the latter with the aid of a scanner and imaging analysis software. $[HCN]_t$ of the second half was determined by induction with toluene in the gas flow apparatus because toluene effectively disrupts cell walls and membranes, causing contact between cyanogenic glucosides and β-glucosidases (Alonso-Amelot & Oliveros, 2000). The first leaf half was placed inside a 125-mL Erlenmeyer flask inserted in a specially-designed bubbling apparatus (Alonso-Amelot & Oliveros, 2000). At time zero, one weighed third-instar larvae that had been starved overnight (12 h) was introduced inside the flask, which was then tightly stoppered and the air flow started. Feeding was allowed to continue undisturbed for 24 h at 24 °C under an LD 12 : 12 h photoperiod, with five replicates per insect species. The HCN liberated during feeding was measured as described above. A parallel control was performed by placing an undisturbed leaf fragment in the gas tight apparatus for the same time period. Free HCN was absent in the samples. After removing and weighing insects and faeces, the weight and area of the remaining plant material was determined. Hydrogen cyanide released during consumption of spinach leaves by *S. frugiperda* was determined under similar conditions by placing plant material inside the simple flask in the gas-flow apparatus for the same time period and temperature (24 °C). No cyanogenesis could be recorded from the spinach samples thus studied.

Insects recovered from the *Passiflora* experiment were starved for 24 h, additional faecal pellets were collected and weighed after drying at 35 °C for 6 h, during which time no HCN was detected. The content of HCN in faecal fractions was obtained by maceration of a known amount (several mg) under liquid nitrogen and after thawing to 0 °C, 50 μL of 0.1 M phosphate buffer (pH = 6.8) and 50 μL of β-glucosidase from almonds (Sigma, St Louis, Missouri) (0.2%, 12.4 U) was added. Earlier experiments had shown that this enzyme effectively decomposes *P. capsularis* cyanogenic glycosides after ingestion in the insect's lumen (personal obsvervation). The homogenate was then transferred to the microdiffusion apparatus described above and incubated at 37 °C for 15 h.

Statistical analysis

The nonparametric Mann–Whitney test ($\alpha = 0.05$) was employed for mean comparisons of the consumption parameters of *P. capsularis* using the STATISTIX Version 7.0 package (Analytical Software, Tallahassee, Florida). Image analysis was performed with MOCHA Analytical software V 2.0 (Jandel Scientific, San Rafael, California).

Results

Hydrogen cyanide release capacity of Passiflora capsularis leaves

Passiflora capsularis total hydrogen cyanide content potential $[HCN]_t$ varied from 325.5 to 3901.0 μg g^{-1} (fw) (Fig. 1) in the wild specimens. The majority of leaves (> 88%) contained > 1000 μg g^{-1} (fw) of cyanide [median = 1551.9 μg g^{-1} (fw)]. Leaves used for the feeding experiments were selected from those yielding 2090–3150 μg g^{-1} (fw) of HCN due to the small amounts of HCN evolved when the insects fed, and thus allowed for more accurate measurements. These leaves were accepted readily by larvae of the two insect species in spite of the higher content of cyanogenic glycosides.

Feeding behaviour

Heliconius erato consumed a large portion of the leaves presented to them whereas *S. frugiperda* accepted only a limited amount (Table 1). The amount eaten by *S. frugiperda* was compared with their consumption of spinach (*Spinacia oleraceae*) leaves [124.4 ± 8.3 mg (fw), area 14.6 ± 0.6 cm^2 in 24 h vs. only 74.7 ± 20.1 mg (fw), area 8.8 ± 1.6 cm^2 of *P. capsularis*]. In dual choice assays, *S. frugiperda* ate only spinach discs whereas *H. erato* ate only *Passiflora* discs.

Because insects generally pause when feeding, the feeding behaviour of 24 h-starved larvae was observed on *P. capsularis* leaves by measuring the time they spent actually feeding, or not, during the first 300 min of exposure. The results for *H. erato* (Fig. 2A) showed that the intense feeding activity at the beginning of the observation period for 12 ± 2 min was followed by an intermission of 79.3 ± 4 min comprising not feeding. Further feeding and nonfeeding periods were proportionally shorter. The feeding rate tended to stabilize at 0.74 ± 0.15 mg (fw) min^{-1} following a linear pattern ($r^2 = 0.9718$). The total feeding time was 57.3 ± 3.8 min out of 300.7 ± 17.0 min of exposure, or approximately 18.2% of the total time. Assuming that, in the 24-h experiment, the feeding period maintained the same proportion, the total

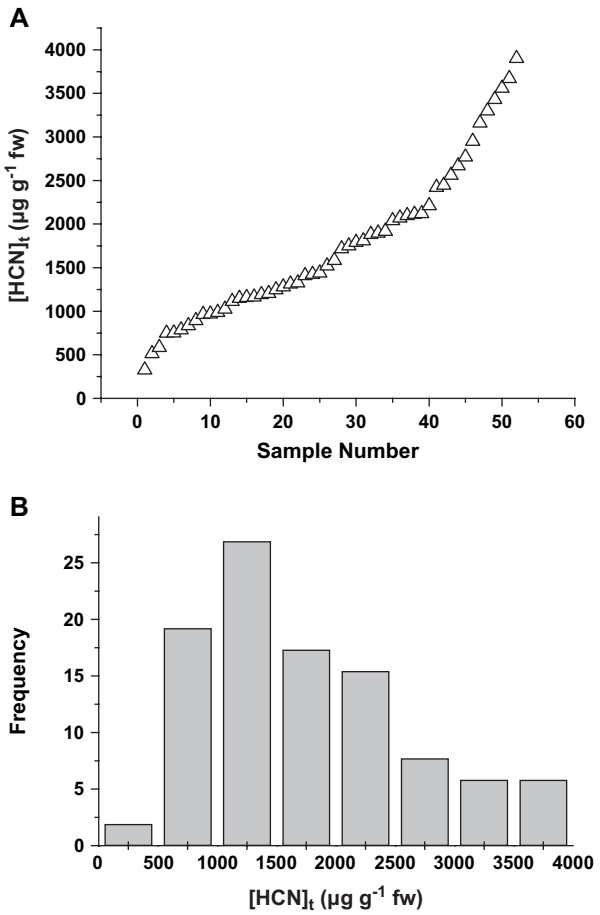

Fig. 1. (A) Total capacity of hydrogen cyanide formation $[HCN]_t$ [µg g^{-1} (fw)] in middle leaves of wild *Passiflora capsularis* collected at four sites with generally similar ecological characteristics in Mérida state, Venezuela. (B) Histogram of relative frequency distribution of $[HCN]_t$ in middle leaves of average size (see text) in four populations of this plant.

amount of 224 mg (fw) ingested by *H. erato* (Table 1) meant a rate of 0.85 ± 0.12 mg (fw) min^{-1} which is still within the values recorded for individual feeding intervals

Larvae of *S. frugiperda* showed a more erratic feeding behaviour (Fig. 2B). For a short period after time zero, they began eating the *P. capsularis* leaf, but stopped after ingesting only 2 ± 1.1 mg, which is less than 17% of that accepted by *H. erato* in the same time period, resulting in larval weight differences (Table 1). The height and width of the feeding bars in the lower portion of the plot and the ordinate scales in Fig. 2(A,B) illustrate these differences. The feeding curve of accumulated material ingested by *S. frugiperda* larvae showed a hyperbolic profile and the feeding rate tended to stabilize at 0.32 ± 0.13 mg (fw) min^{-1}. However, considering that *S. frugiperda* consumed 74.7 ± 20.1 mg (fw) of leaf in 24 h, and the fact that, according to the data shown in Fig. 2(B), the larvae spent approximately 4.6% of the total time actually feeding, extrapolation of these values to the 24-h data (Table 1) reveals an average feeding rate of 1.13 mg min^{-1}, which is approximately the same as at the beginning of the feeding behaviour experiment. This clearly indicates that *S. frugiperda* was capable of feeding more actively after an initial trial period and may mimic the situation in the field during population explosions.

Kinetics of HCN formation from Passiflora capsularis leaves

Having calculated the content and distribution of cyanide in *P. capsularis* foliage, it was necessary to determine the capacity for formation of HCN per unit time by the plant after tissue injury under the constraints of its own resources. *Passiflora capsularis* leaf tissue yields HCN following a hyperbolic time-dependent correlation (Fig. 3) ($\chi^2 = 0.001$, SS = 0.0109). From these data, it was possible to derive a mathematical model (Eq. 1) to calculate the expected amount of evolved hydrogen cyanide $[HCN]_E$ into the air when the herbivore eats an amount (W) of leaf during T min:

$$[HCN]_E(\mu g) = [HCN]_t[P_1T/P_2 + T]W \quad (1)$$

where: $[HCN]_E$ = expected amount of evolved HCN at time T; $[HCN]_t$ = total HCN (µg) released ($T = \infty$) per g (fw) of the leaf tissue devoured by herbivor; W = weight (g) eaten; $P_1 = 1.16 \pm 0.04$ and $P_2 = 224.4 \pm 19.7$ (P_1 and P_2 are unit-less curve-fitting coefficients).

Most important for the protection of *Passiflora* leaves is the rate at which HCN is released immediately after herbivore attack because chewing insects separate leaf tissue from the plant, grind it in their mouth parts and ingest it in only a few moments, giving only a short period of time for the release of HCN into the atmosphere or their mandibles. This was best assessed by the initial velocity $[HCN]_i$ of cyanide formation. This quantity was estimated by a set of 17 kinetic

Table 1. Consumption parameters of *Passiflora capsularis* leaves by *Heliconius erato* and *Spodoptera frugiperda* third-instar larvae during 24 h of exposure at 24 °C in the gas-flow apparatus.

	Consumption [mg (fw) per larva]	Larval body live weight (mg)	HCN in accepted plant per larva (µg)	Liberated HCN per larva (µg)	Ingested HCN per larva (µg)	Dry faecal pellets per larva (mg)	HCN in faecal pellets (µg)
Heliconius erato	224.2 ± 47.6[a]	306.8 ± 36.3[a]	469 ± 122[a]	0.4 ± 0.5[a]	468 ± 120[a]	38.8 ± 6.0[a]	19.8 ± 6.6[a]
Spodoptera frugiperda	74.7 ± 20.1[b]	167.7 ± 15.8[b]	194 ± 52[b]	13.3 ± 4.3[b]	201.6 ± 84.6[b]	37.9 ± 14.7[b]	39.8 ± 11.8[b]

The hydrogen cyanide (HCN) content of faecal pellets was determined with the aid of the microdiffusion bottle, as described in the text. Values are mean ± SE ($n = 5$). Different superscript letters denote a statistical difference ($\alpha = 0.05$, Mann–Whitney test).

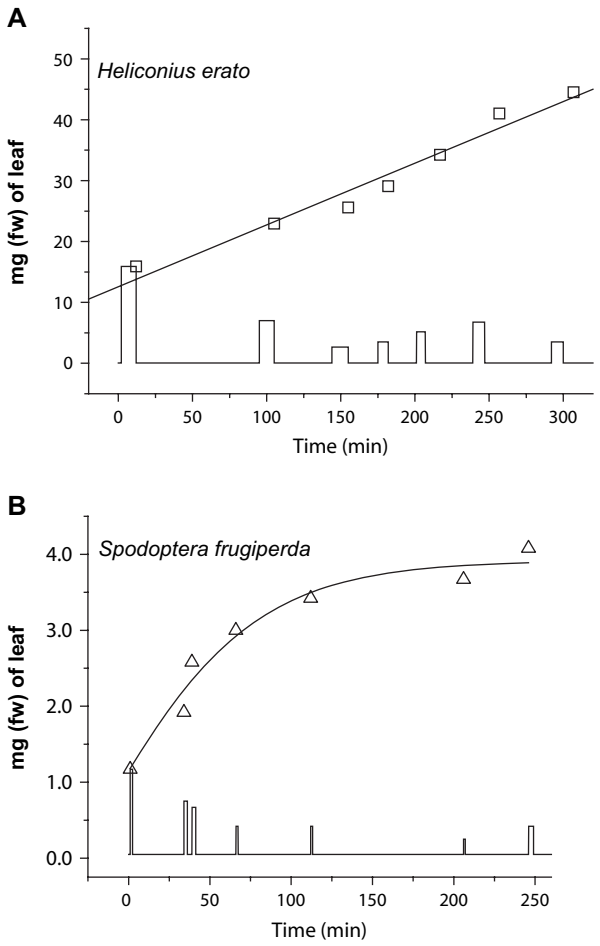

Fig. 2. Examples of time-dependent feeding cycles of (A) *Heliconius erato* and (B) *Spodoptera frugiperda* measured in individual third-instar larvae on *Passiflora capsularis* leaves containing 2060–2600 μg g^{-1} (fw) of hydrogen cyanide (HCN). Open squares (A) and open triangles (B) represent the accumulated plant material ingested by individual larvae, whereas line plots indicate the amount ingested per feeding period, its duration and frequency along the observations over 260–300 min. Although individual feeding events vary, the accumulated ingestion is comparable among larvae of the same species.

runs drawing aliquots every 5 min for the first 30 min and then after the rest of the measurements up to 24 h as above. Values varied widely between 1.76 and 30.27 μg g^{-1} (fw) min^{-1} of HCN but correlated linearly with $[HCN]_t$ (Fig. 4). This led to a Eq. 2 estimating the amount of HCN released by the plant being devoured at the beginning of the feeding process as a function of the total cyanide present and the rate of feeding *R*:

$$[HCN]_i = (A + B \times [HCN]_t) \times T \times R \qquad (2)$$

where: $[HCN]_i$ = initial velocity of HCN release (μg min^{-1}); $A = -4.8 \pm 2.0$ (μg g^{-1} min^{-1}); $B = 0.0118 \pm 0.0015$ (min^{-1}); T = time (min); R = feeding rate (fresh weigh of

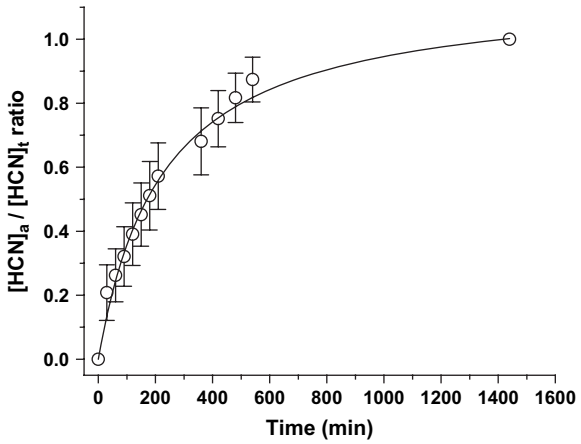

Fig. 3. Time-dependent accumulation of hydrogen cyanide $[HCN]_a$ relative to $[HCN]_t$ released from *Passiflora capsularis* leaves. Error bars indicate SD (n = 29).

ingested leaf (g) per minute); and $r^2 = 0.8054$ (regression coefficient).

Release of HCN during feeding

By knowing the amount eaten and rate of feeding of both larval species on *P. capsularis* leaves and the rate equation (Eq. 2), the anticipated HCN evolution into the air when insects fed on leaf lamina could be calculated.

Heliconius erato larvae were offered leaves with $[HCN]_t = 2090 \pm 340$ μg g^{-1} (fw) of HCN as cyanogenic glycosides. They ingested 224 ± 48 mg ($n = 5$) of this material at a rate $R = 0.85 \pm 0.12$ mg (fw) min^{-1} assuming that they employed 18.4% of the observation time of 24 h (i. e. $T = 265$ min, as

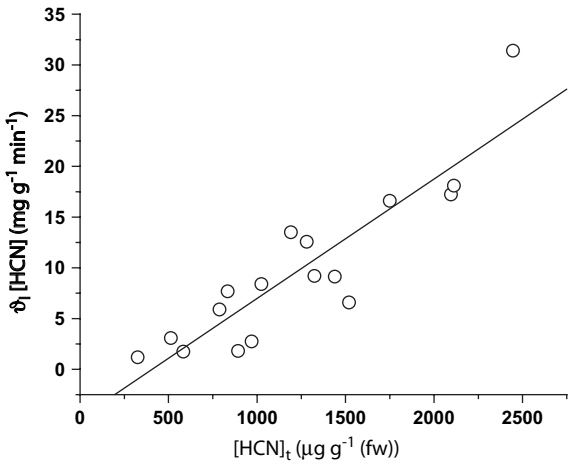

Fig. 4. Relationship between initial velocity of hydrogen cyanide (HCN) release and total contents of cyanide ($[HCN]_t$) in the plant's tissue. Equation 3 (see text) governs this regression (n = 17).

calculated above). By replacing these numbers in Eq. 2, it is possible to estimate the quantity of evolved HCN that would be anticipated in the air chamber when feeding was in progress. For *H, erato,* Eq. 2 gave $[HCN]_i = 5.56 \pm 0.79$ μg HCN. This represented only a small fraction of the cyanide contained in the ingested material (469 ± 122 μg) (Table 1). The actual evolution of HCN when *H. erato* larvae fed was measured simultaneously with the feeding experiment in the gas-flow apparatus. Surprisingly, even less HCN was detected in the gas traps (0.41 ± 0.46 μg HCN) corresponding to only 7% of the anticipated $[HCN]_i$ and 0.09% of $[HCN]_t$.

Spodoptera frugiperda larvae were given *Passiflora* leaves with $[HCN]_t = 2600 \pm 130$ μg g^{-1} (fw) of HCN as cyanogenic glycoside. They were able to eat only 74.7 ± 20.1 mg ($n = 5$) of leaf with $R = 1.13 \pm 0.3$ mg (fw) min^{-1} assuming that they employed 4.6% of the 24-h observation time in actual feeding activity (i.e. $T = 66$ min, as calculated before). Using these parameters, Eq. 2 gave $[HCN]_i = 2.29 \pm 0.38$ μg. Again, this was only a small fraction of the contained cyanide ($[HCN]_t = 194 \pm 52$ μg HCN) in the ingested sample but the actual cyanide detected in the traps was much larger (13.28 ± 4.33 μg HCN).

Discussion

Cyanogenic glycosides play a central role in the defence of plants against herbivore predators through their direct deterrence effect or capacity to yield HCN. The amount of cyanogenic glycosides in plant tissue varies considerably, depending on the organ, ontogeny and environmental conditions, and is distributed unevenly among plant populations (Schappert & Shore, 1999; Gleadow & Woodrow, 2000; Alonso-Amelot & Oliveros, 2005). *Passiflora capsularis* follows these precepts because of its exceptionally large range of cyanide content (Fig. 1), probably due to cyanogenic polymorphism. No morphological differences were noted between weakly and strongly cyanogenic plants and insect herbivores will find an ample range of cyanogenic responses in *P. capsularis* plants in one single area.

All known natural cyanogenic glycosides yield HCN. Because this compound is also toxic to the plant, its formation must be restricted to the moment plant tissue is disrupted by the attacker. The mixing of cyanogenic glycosides and β-glucosidases takes place during the trophic interaction with HCN evolution, albeit not instantaneously but subjected to kinetic restraints. Despite the central importance in defining the time-dependent availability of cyanide to deter or intoxicate the intruder, few studies focus on this subject: in *E. polyanthemos, P. arachnoideum* and *P. lunatus*. Values of initial velocity of release $[HCN]_i$ vary greatly: *E. polyanthemos*: 0.33×10^{-3}–2.3×10^{-3} μg min^{-1} g^{-1} HCN (Goodger *et al*., 2002); *P. arachnoideum* (croziers): 0.06–5.74 μg min^{-1} g^{-1} HCN at 30 °C (Alonso-Amelot *et al*., 2005); *P. lunatus* (young leaves): 0–6.4 μg min^{-1} g^{-1} HCN at 30 °C (Ballhorn *et al*., 2005). By comparison, *P. capsularis* middle leaves, with $[HCN]_i$ 1.76–30.27 μg g^{-1} (fw) min^{-1} HCN at this temperature, makes it the highest HCN yielding plant known so far. Notably, the profile of time-dependent HCN release relative to $[HCN]_t$ (Fig. 3) in both *P. capsularis* and *P. arachnoideum* follows a hyperbolic curve. Although, in ecological terms, this might be interpreted as convergence of HCN-based defence strategies, from a biochemical point of view, it could result from the natural Michaelis–Menten kinetics followed by β-glucosidases and hydroxynitrile lyases involved in the cyanogenic-glycoside–HCN transformation (Bauer *et al*., 1999).

As opposed to *P. arachonoideum* in which prunasin is the only cyanogenic glycoside, the kinetics recorded in the present study for *Passiflora capsularis* is the combined response of six cyanogenic glycosides, including lotaustralin, linamarin and three minor cyclopentanoid cyanogenic glycosides, all of which may accumulate in variable proportions (Fischer *et al*., 1982; Olafsdottir *et al*., 1989). Although their individual kinetic peculiarities, possible product-controlled reaction rate and reversibility to the intermediate cyanohydrin configure a complex chemical scaffold for the cyanogenic response, it is their combined production of HCN that constitutes an impact of ecological importance for *P. capsularis* protection. In addition, these cyanogenic glycosides may be produced by the plant for its own primary metabolism (e.g. nitrogen-storage and mobilization as observed in the seeds of *Hevea brasiliensis*) (Selmar *et al*., 1988). In these seeds, cyanogenic glycoside enzymatic splitting to yield HCN, immediately followed by β-cyanoalanine synthase-mediated trapping to assimilate cyanide as a noncyanogenic aminoacid precursor, takes place during germination and early seedling growth. Thus, the rate of HCN evolution measured in *P. capsularis* and other species may be an evolutionary gauged trade-off between the requirements imposed by defence demands and the metabolic needs for an essential element via a toxic material (cyanide) that cannot be accumulated in the plant cell before its transformation to noncyanogenic derivatives.

Wild *P. capsularis* and other members of the *Passiflora* family show few signs of active herbivory at the study sites used in the present study probably because of the high cyanide content and capacity for its release. When damage appears in their leaves, almost always larvae of the various *Passiflora* specialists, and only occasionally polyphagous insects, are responsible. *Heliconius erato* and *S. frugiperda* are common examples of these types in this region. Although *H. erato* larvae develop into normal imagoes by feeding on *P. capsularis* leaves only, *S. frugiperda* does not and accepts only a reduced amount of this plant, as shown by its preference for spinach leaves and the much greater consumption of this material. Being generalists and highly mobile, the larvae can forage on a variety of other plants to satisfy their nutritional needs. Leaf digestibility by both insect species, as shown by the ratio of ingested material and faecal pellet production (Table 1), is another indication of xenobiosis in *Passiflora* acting more effectively against *S. frugiperda.*

The contrast of feeding behaviour on *P. capsularis* leaves between these oligophagous and polyphagous insects becomes

evident in their feeding cycles (Fig. 2A,B). Although, in both species, there are short periods of active feeding followed by long non-feeding intervals, presumably when digestion is taking place, the difference in the amount eaten and the time of feeding is obvious. The regular trend of accumulated feeding observed in *H. erato,* which reflects the uneventful intake and digestion of plant material, is different in *S. frugiperda* and shows a rapid satiation after an initial moderate activity. If it is true that this species must become adapted to accept the available diet driven by the need to feed, as indicated by the average rate of 1.13 mg (fw) min^{-1} in 24-h, it spends only 4.6% of the exposure time actively feeding vs. 18.4% for *H. erato*. This reveals the need for longer digestion intervals, the effect of feeding deterrents or the lack of feeding stimulants. A similar shortening of feeding intervals and overall inhibition associated with cyanide contents has been reported recently for another polyphagous herbivore, *S. gregaria*, when offered cyanogenic accessions of *P. lunatus* (Ballhorn *et al*., 2005).

The feeding behaviour dissimilarities are additionally underlined by the amount of HCN released during feeding on *P. capsularis*. The evolution of only 7% of the amount of HCN that the plant would be capable of yielding during the feeding process of *H. erato* may be interpreted as an evolutionary adaptation to prevent the loss of valuable plant organic nitrogen to the atmosphere to be utilized metabolically (e.g. for sequestration of unaltered cyanogenic glycosides to support the chemical defence potential of the herbivor). Selective sequestration of *Passiflora* cyanogenic glycosides has been established in the body integument of *Heliconius charitonia* and *Heliconius sara* (Engler *et al*., 2000). Some *Heliconius* butterflies are also known to synthesize *de novo* linamarin and lotaustralin in a redundant defence pattern (Nahrstedt & Davis, 1983). It is also conceivable that the transformation of ingested cyanogenic glycosides into useful amino acids and other nitrogen-containing metabolites because enzymes involved in cyanide metabolism, such as rhodanase, are ubiquitous in insects. For third-instar larvae feeding on 224 mg of *P. capsularis* middle leaves in 24 h (Table 1), the average amount of cyanide is 468 μg. After discounting the cyanide remaining in the faecal pellets ejected in the feeding period (20 μg in 24 h), an average of 448 μg of HCN (232 μg as N or a significant 0.5% of the larval dry body weight) must have been metabolized by *H. erato* larva. The inhibition of HCN formation in this species may be a response to tearing off the leaf material, mastication and ingestion in a fast enough manner to overcome the natural kinetics of HCN evolution by the plant tissue. Furthermore, a large size of leaf particles left after gnawing may inhibit the cyanogenic reaction. Furthermore, the inhibitory effect of plant glucosidases by proteases in mouth parts or foregut may occur.

Spodoptera frugiperda evokes the evolution of 13.28 ± 4.33 μg HCN, a much larger figure than anticipated from the plant under its own resources. A slower maceration of plant tissue in its mouthparts alone may not account for this considerable increment. Rather, glucosidases in the saliva are probably responsible (Mattiacci *et al*., 1995). This reaction finds a parallel in the the case of *S. gregaria* with particular accessions of *P. lunatus* (Ballhorn *et al*., 2005). This behavioural convergence in two generalist herbivores appears to be an adaptation strategy by which the intake of cyanide is reduced by catalysing its release during mastication for atmospheric dispersion prior to ingestion, in stark contrast with the strategy displayed by the specialist.

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