

## Quantitative Effects of Cyanogenesis on an Adapted Herbivore

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Received: 31 July 2007 / Revised: 24 September 2007 / Accepted: 1 October 2007 /

Published online: 7 November 2007

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**Abstract** Plant cyanogenesis means the release of gaseous hydrogen cyanide (HCN) in response to cell damage and is considered as an effective defense against generalist herbivores. In contrast, specialists are generally believed not to be affected negatively by this trait. However, quantitative data on long-term effects of cyanogenesis on specialists are rare. In this study, we used lima bean accessions (Fabaceae: *Phaseolus lunatus* L.) with high quantitative variability of cyanogenic features comprising cyanogenic potential (HCNp; concentration of cyanogenic precursors) and cyanogenic capacities (HCNc; release of gaseous HCN per unit time). In feeding trials, we analyzed performance of herbivorous Mexican bean beetle (Coleoptera: Coccinellidae: *Epilachna varivestis* Mulsant) on selected lines characterized by high (HC-plants) and low HCNp (LC-plants). Larval and adult stages of this herbivore feed on a narrow range of legumes and prefer cyanogenic lima bean as host plant. Nevertheless, we found that performance of beetles (larval weight gain per time and body mass of adult beetles) was significantly affected by lima bean HCNp: Body weight decreased and developmental period of larvae and pupae increased on HC-plants during the first generation of beetles and then remained constant for four consecutive generations. In addition, we found continuously decreasing numbers of eggs and larval hatching as inter-generational effects on HC-plants. In contrast to HC-plants, constantly high performance was observed among four generations on LC-plants. Our results demonstrate that Mexican bean beetle, although preferentially feeding on lima bean, is quantitatively affected by the HCNp of its host plant. Effects can only be detected when

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considering more than one generation. Thus, cyanide-containing precursors can have negative effects even on herbivores adapted to feed on cyanogenic plants.

**Keywords** Herbivory · Chemical defense · Plant–herbivore interaction · Generalist · Specialist · Lima bean · *Phaseolus lunatus* · Cyanogenic glycosides · Mexican bean beetle · *Epilachna varivestis*

## Introduction

Plant cyanogenesis is considered a direct plant defense against herbivores and means the release of hydrogen cyanide (HCN) from damaged plant tissues (Poulton 1990). More than 2,500 species of vascular plant taxa belonging to over 130 families are cyanogenic (Møller and Seigler 1999). As HCN is toxic to the plant itself, nontoxic cyanide-containing precursors are synthesized and stored in compartments, spatially separated from specific enzymes ( $\beta$ -glucosidases and  $\alpha$ -hydroxynitrile lyases) required for their degradation and the following release of HCN (Selmar et al. 1989). In lima bean, the glycosidic precursors linamarin and lotaustralin accumulate in the vacuoles (Frehner and Conn 1987). Specific  $\beta$ -glucosidases are located in the apoplast, whereas  $\alpha$ -hydroxynitrile lyases are membrane-associated (Conn 1981; Selmar et al. 1987, 1989).

The release of HCN occurs only in disintegrated tissue. Although cyanogenesis is generally regarded as a constitutive mechanism, recent studies on lima bean have demonstrated an induced component:  $\beta$ -glucosidases involved in the release of HCN from cyanide-containing compounds were activated in response to herbivore damage (Ballhorn et al. 2006).

There is increasing evidence that plant cyanogenesis or cyanogenic glycosides deter generalist, but not specialist, herbivores (Jones 1988; Patton et al. 1997; Ballhorn et al. 2005). Strongly cyanogenic plants are protected from generalists (Viète et al. 2000; Ballhorn et al. 2005) but nevertheless can be attacked by specialists (Benson et al. 1976; Davis and Nahrstedt 1987). Generally, specialist or oligophagous herbivores with restricted host ranges are able to tolerate or to detoxify defensive compounds of their respective host plants because of physiological or behavioral adaptations (Rausher 1996; Agrawal and Kurashige 2003; Agrawal and Van Zandt 2003). In some cases, they even use the toxic plant compounds for their own defense (Bowers and Stamp 1997; Dobler et al. 1998), and other cases are known, in which cyanogenic glycosides act as phagostimulants for herbivores adapted to cyanogenic plants (Lapidus et al. 1963).

Studies conducted so far on specialists that live on cyanogenic plants have shown that, in principle, they can develop on such plants (Beesley et al. 1985; Engler et al. 2000; Urbńska et al. 2002). Nevertheless, whether or not specialists are quantitatively affected by cyanogenic features of their hosts remains elusive (Miguel and Alberto 2005). Long-term studies are lacking on the quantitative dependence of parameters relevant to fitness, such as growth rates, developmental period, and success of reproduction itself, as quantitatively depending on cyanogenic features of host plants.

The present study was conducted to fill this gap in functional understanding of distinct cyanogenic components. We studied quantitative effects of cyanogenic precursor concentration (HCN<sub>p</sub>, cyanogenic potential) and cyanogenic capacity (HCN<sub>c</sub>; release of gaseous HCN per unit time) on Mexican bean beetle (Coleoptera: Coccinellidae: *Epilachna varivestis* Mulsant).

We analyzed several fitness-relevant parameters over four consecutive generations of beetles (the performance of all four larval stages, the duration of pre-pupation and pupal

stages, and the body mass at emergence, and total reproduction) in different experimental settings by using selected plants with well-defined cyanogenic properties (Ballhorn et al. 2005, 2006, Ballhorn and Lieberei 2006). Based on significant differences in their HC<sub>Np</sub>, we grouped lima bean accessions into high cyanogenic (HC) and low cyanogenic (LC) plants. This set of experimental plants allowed us to quantify effects of plant cyanogenic features on the performance of beetles.

## Materials and Methods

**Plants and Insects** The lima bean (Fabaceae: *Phaseolus lunatus* L.) is an obligate cyanogenic plant of Mesoamerican and Andean origin (Baudoin et al. 1991). We used lima beans of 12 accessions (11 domesticated lines and 1 wild-type native to Cuba) characterized by different cyanogenic features. Seeds were provided by the Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben, Germany. For further description of plant material, see Ballhorn et al. (2006). In addition, snap bean *Phaseolus vulgaris* cv. Saxa (Carl Sperling, Lüneburg, Germany, stock number 40.176), hereafter referred to as “Saxa,” was used as a non-cyanogenic control.

The Mexican bean beetle (Coccinellidae: *E. varivestis* Muls.) is an oligophagous insect that feeds on a range of legumes (Barrigossi et al. 2001) but with distinct preference for *Phaseolus* species (Dover et al. 1988) and especially lima bean (Flanders 1984; Baudoin et al. 1991). As feeding of the beetle is stimulated by cyanogenic glycosides even in artificial diets, an adaptation to diets containing these compounds can be assumed (Lapidus et al. 1963). Mexican bean beetle is native to southern Mexico as are lima beans of the Mesoamerican gene pool. The life cycle of the beetles encompasses four larval stages followed by a short pupal stage of 5–10 days. Females deposit eggs in clutches of 40–75 on the lower surface of host plant leaves, and both larvae and adults feed on foliage.

**Rearing of Plants and Insects** Plants were cultivated under greenhouse conditions (16:8 L/D with a photon flux density of 350–450  $\mu\text{mol s}^{-1} \text{m}^{-2}$  at the plant container and 850–950  $\mu\text{mol s}^{-1} \text{m}^{-2}$  on top of the plants depending on natural radiation). Additional light was provided by 400 W high-pressure sodium lamps (Son-Targo 400, Philips®). To avoid effects by hot spots under the lamps, position of plants was changed every 3 days. Temperature (25/20°C) and ambient relative air humidity (60–70%) were controlled by Integro® software. Plants were fertilized with a nitrogen–phosphate fertilizer (Blaukorn®-Nitrophoska®-Perfekt, Compo GmbH) twice a week (3 mg per pot) and were cultivated in plant containers 18 cm diameter in a 1:1 ratio of standard substrate (TKS®-1-Instant, Floragard®) and sand (grain size 0.5 – 2.0 mm). Plants ( $N=12$  per accession) were grown from seeds obtained by self-fertilization of a single mother plant.

Beetles were maintained on “Saxa” to prevent them from developing preferences for any particular *P. lunatus* accession. In addition, large numbers of beetles (>250 per accession) were maintained on plants of all 12 lima bean accessions used in the feeding trials. Ambient conditions adjusted for both maintenance cultures of insects and feeding experiments were identical.

**Analysis of Leaf Quality** Individual leaves of all accessions were used for analysis of cyanogenic features (HC<sub>Np</sub>,  $\beta$ -glucosidase activity, and HC<sub>Nc</sub>) and nutritive traits (soluble

protein and total phenolics). Young trifoliolate leaves ( $N=12$  leaves per accession,  $N=1$  leaf per plant) fully unfolded for 5–7 days were selected for the experiments as described in Ballhorn et al. (2006). One randomly selected leaflet of each trifoliolate leaf was cut along the midrib. One half was used for HCNp analysis, the other half for the determination of soluble protein concentration and  $\beta$ -glucosidase activity. The remaining two leaflets were used for quantification of HCNC and total phenolics.

Cyanogenic potential (HCNp) was analyzed by complete enzymatic degradation of cyanogenic glycosides, and HCN released from cyanogenic precursors was spectrophotometrically quantified (585 nm) by using the Spectroquant<sup>®</sup> cyanide test (Merck, Darmstadt, Germany) following Ballhorn et al. (2005). External  $\beta$ -glucosidase isolated from rubber tree (Euphorbiaceae: *Hevea brasiliensis*) was added to ensure complete degradation of cyanogenic precursors. This enzyme showed high affinity to cyanogenic glycosides in lima bean and was isolated following Ballhorn et al. (2006).

Cyanogenic capacity (HCNC) was measured as gaseous HCN released into the atmosphere per unit time after leaves were treated with chloroform. We used an air-flow system ( $7\text{ l air min}^{-1}$ ) for detection of the HCNC as described in Ballhorn et al. (2005).

Soluble protein concentration was quantified according to Bradford (1976). Leaf material was homogenized in ice-cold sodium acetate buffer (pH 5.0). Leaf extracts were centrifuged (Heraeus<sup>®</sup>Biofuge Fresco, 13,000 rpm, 4°C), and the supernatant was filtered over NAP<sup>™</sup> columns containing Sephadex<sup>™</sup> G-25 DNA-Grade (GE Healthcare Bio-Sciences AB). Subsequently, 5  $\mu\text{l}$  of the eluate were pipetted on microplates (96-well Microplates, PS, F-bottom, Greiner Bio-One GmbH, Maybachstr. 2, 72638 Frickenhausen, Germany), and 250  $\mu\text{l}$  Bradford reagent (diluted with aqua dest. in the ratio 1:4) were added. Protein concentration of samples was spectrophotometrically quantified at 595 nm (Spectra MAX 190 PC, Molecular Devices). BSA solutions (Merck, Darmstadt, Germany) in the range between 10 and 300  $\mu\text{g ml}^{-1}$  served as standard.

Activity of  $\beta$ -glucosidase was measured according to Ballhorn et al. (2006). We used *p*-NP-glucoside (Merck) as chromogenic artificial substrate. The released *p*-nitrophenol was quantified spectrophotometrically at 400 nm (Pharmacia Biotech, Ultraspec 3000).

Total phenolics were analyzed following Tikkanen and Julkunen-Tiitto (2003). Homogenates of leaves were extracted three times for 15 min while stirring in 50-ml acetone diluted with aqua dest. (60:40). After each extraction, samples were incubated in an ultra sonic bath (3 min) and were finally centrifuged for 10 min at  $5,000\times g$ . The supernatant was transferred in 2-ml concentrated acetic acid (Merck), acetone was removed in vacuum (60 mbar) at 40°C, and the residue was quantitatively transferred by using aqua dest. Samples were diluted with 2.5% acetic acid, and 1 ml of this solution was mixed with 0.5 ml Folin–Ciocalteus phenol reagent (Merck). After adding 2 ml 20%  $\text{Na}_2\text{CO}_3$ , the solution was made up to 10 ml with aqua dest. Samples were incubated at 70°C and, after cooling, spectrophotometrically quantified at 730 nm against a blank containing water instead of sample. Epicatechine (Sigma, Deisenhofen, Germany) served as standard.

*Feeding Trials* Beetles' performance on leaves of *P. lunatus* accessions was analyzed in no-choice long-term feeding experiments. Males and females were considered separately due to inter-sex variability in weight gain. These experiments included four consecutive generations. Freshly hatched larvae ( $N=11$  per generation) were individually placed on single detached leaves supplied with water (perforated Eppendorf tubes). Feeding trials were carried out in petri dishes (9.5 cm) lined with slightly moist filter paper to avoid wilting of leaf material under controlled conditions (25/20°C, photon flux density of

200  $\mu\text{mol s}^{-1} \text{m}^{-2}$  in a 16:8 L/D period). Leaf material was renewed daily, and larvae were weighed daily until pupation. At the end of the assays, body weight and sex of the freshly emerged beetles were documented.

In these long-term experiments, trans-generational effects were also considered. The reproduction of beetles on different bean accessions was analyzed by pooling freshly emerged female beetles in a cage that contained four intact plants of the respective accession. Male beetles that emerged in the same experiment were immediately exchanged against freshly emerged males from large maintenance populations of Mexican bean beetles (>250 beetles per accession). These populations were kept for the same time period on the same lima bean accessions that were used in the feeding trials. These “external” males were added to the females in a 1:1 sex ratio. Number of males added depended on number of females that emerged in the experiment. By this procedure, we avoided creating a family bias on the different accessions, which otherwise might have led to artificial effects of development of different beetle genotypes. Eggs produced by the beetles were removed from plants by cutting leaf discs around the attached egg clutches. Eggs were incubated until hatching under identical ambient conditions that were adjusted for long-term feeding trials. The number of eggs and of hatched larvae was documented for all egg clutches. Because females started oviposition simultaneously and there were constant intervals of 8–9 days between productions of egg clutches per female, these clutches were assigned as consecutive clutches (1, 2, and 3) in this pooled experimental setup. A part of the progeny ( $N=11$  randomly selected larvae per accession) was used for the next generation again, which was studied under the same experimental conditions as mentioned above.

In these long-term experiments, detached leaves were used. Detached leaves are sensitive to changes in leaf biochemistry depending on degradation processes of cell components. These processes can lead to a spontaneous release of gaseous HCN from leaf material, which complicates quantitative analysis of cyanogenesis and its impact on herbivore performance. Therefore, to test for losses of HCN during the experimental period, water-supplied leaves were placed in an air-flow system according to Ballhorn et al. (2005) under ambient conditions identical to those used for feeding experiments, and released HCN from leaf material was measured quantitatively. No loss of HCN from leaves was detected.

*Larval Efficiency of Food Utilization* We calculated three parameters of food utilization efficiency (Farrar et al. 1989; Anaya et al. 2003; Jallow et al. 2003). Relative growth rate (RGR) was calculated as: biomass gained (mg fresh weight)/[duration of feeding period (days)  $\times$  mean fresh weight (mg) of larvae during feeding period]. The relative consumption rate (RCR) estimates the weight of food consumed relative to time and mean weight of the insect during the feeding period and was calculated as: food ingested (mg dry weight)/[duration of feeding period (days)  $\times$  mean fresh weight (mg) of larvae during feeding period]. The efficiency of conversion of ingested food (ECI) estimates the percentage of ingested food that is converted to biomass. It was calculated as: [biomass gained (mg fresh weight)/food ingested (mg dry weight)]  $\times$  100.

The experiments were carried out in Petri dishes (9.5 cm). We used larvae of the third instars between days 6 and nine from hatching and leaf discs (2.73 mm diameter), which were exchanged daily. Two discs per setup were cut from two randomly selected leaflets of one trifoliate leaf, each from one leaflet. Positions on the lamina at which the discs were removed were the same for both leaflets. One disc was used for the feeding trial; the other was dried at 45°C to constant weight for determination of dry matter and leaf mass per unit

leaf area (LMA). Leaf discs cut for the feeding trials were put on moist filter paper and then were placed in the air-flow system according to Ballhorn et al. (2005) before the experiment. Leaf discs were used for feeding trials when no more gaseous HCN was released from the cutting edge (5–10 min after cutting). Release of gaseous HCN from leaf discs by wilting during the feeding trial was avoided by placing leaf discs on moist filter paper in the Petri dishes. We tested for spontaneous loss of HCN over the experimental period from leaf discs in the same way as described for long-term feeding trials with intact detached leaves.

After the feeding trial, remaining leaf material was dried at 45°C and weighed to the nearest 0.001 mg. Dry weight equivalents of food consumed by individual larvae were obtained by multiplying the daily wet weight of food consumed by percentage dry matter in each diet (Waldbauer 1968). The remaining third leaflet of the leaf was used for analysis of cyanogenic precursor concentration (HCN<sub>p</sub>) to check for constancy in precursor concentrations of leaves actually used in feeding trials, as compared to leaves that were used for analysis of leaf quality of the respective accessions before.

*Statistical Analysis* Differences in HCN<sub>p</sub>, HCN<sub>c</sub>, and nutritive components, i.e., soluble protein concentration and total phenolics among lima bean accessions and differences in beetles' performance on different accessions, were analyzed by one-way analysis of variance (ANOVA) and *post hoc* comparisons (HSD). The effects that different accessions of lima bean, i.e., plants with different HCN<sub>p</sub>, had on egg production were consequently analyzed with a repeated measures ANOVA design on a balanced subset of data created by randomly selecting four females per generation/accession/clutch number-combination after testing the assumption of sphericity with Mauchly's test. Data comprised four consecutive generations of beetles and three consecutively laid egg clutches per generation. Statistical analyses were carried out by use of Statistical Package for the Social Sciences (SPSS) 13.0 (SPSS for Windows, SPSS, Chicago, IL, USA).

## Results

*Leaf Quality* Lima bean accessions showed distinct differences in leaf HCN<sub>p</sub> (Table 1). The three accessions with the highest (high cyanogenic, HC) and the lowest concentration of cyanogenic precursors (low cyanogenic, LC) were used (Table 1).

The difference in HCN<sub>p</sub> between HC- and LC-plants was significant (one-way ANOVA;  $F_{1,73}=500.57$ ,  $P<0.001$ ). In addition to HCN<sub>p</sub>, HCN<sub>c</sub> was highly variable among the accessions that were used for feeding experiments, depending on cyanogenic precursor concentration and activity of specific endogenous  $\beta$ -glucosidases. For instance, accession 2116 was characterized by the highest concentration of cyanogenic precursors, yet showed low HCN<sub>c</sub> due to a low activity of  $\beta$ -glucosidase (Table 1).

In contrast to cyanogenic features, total phenolics and concentration of soluble protein in leaves and LMA did not differ significantly among the accessions (Table 1).

*Body Mass of Beetles* Substantial inter-sex variability in beetles' body mass was observed among HC- and LC-plants. Females showed higher body mass compared to males independent of plant cyanogenic features (HC:  $F_{1,12}=102.56$ ,  $P<0.001$ ; LC:  $F_{1,13}=82.18$ ,  $P<0.001$ ; "Saxa":  $F_{1,44}=69.31$ ,  $P<0.001$ ). Thus, male and female beetles were considered

**Table 1** Cyanogenic and nutritive traits of leaves

Cyanogenic Traits		Nutritive Traits					
Accessions	Cyanogenic Status [High (HC) and Low (LC)]	Cyanogenic Potential HCNp ( $\mu\text{mol HCN g}^{-1}\text{ fw}$ )	Cyanogenic Capacity HCNc ( $\mu\text{mol HCN g}^{-1}\text{ fw min}^{-1}$ )	$\beta$ -Glucosidase Activity ( $\mu\text{kat g}^{-1}\text{ dw}$ )	Total Phenolics ( $\text{mg g}^{-1}\text{ dw}$ )	Soluble Protein ( $\text{mg g}^{-1}\text{ dw}$ )	Leaf Mass per Area LMA ( $\text{mg cm}^{-2}$ ) <sup>a</sup>
2116 <sup>b</sup>	HC	76.5±15.3 (a)	0.10±0.03 (g)	0.06±0.04 (h)	1.92±0.30 (a)	140.55±8.70 (a)	0.80±0.11 (a)
2357 <sup>b</sup>	HC	68.4±14.8 (ab)	0.58±0.07 (ab)	1.91±0.26 (b)	2.11±0.41 (a)	140.04±2.59 (a)	0.80±0.12 (a)
WT <sup>b,c</sup>	HC	64.7±11.4 (ab)	0.64±0.07 (a)	2.85±0.33 (a)	2.34±0.42 (ab)	144.07±4.12 (a)	0.83±0.13 (a)
8078	–	61.8±6.0 (ab)	0.56±0.05 (bc)	1.90±0.22 (bc)	2.18±0.41 (ab)	139.80±6.91 (a)	0.81±0.09 (a)
1315	–	55.9±11.4 (bc)	0.49±0.08 (c)	1.64±0.25 (cd)	2.40±0.40 (ab)	144.23±3.95 (a)	0.79±0.10 (a)
1738	–	40.1±12.7 (cd)	0.29±0.08 (d)	1.42±0.23 (def)	2.21±0.53 (ab)	137.31±12.64 (a)	0.82±0.11 (a)
8151	–	35.0±5.9 (de)	0.27±0.06 (de)	1.34±0.18 (ef)	2.32±0.51 (ab)	140.77±2.60 (a)	0.82±0.09 (a)
8073	–	28.6±7.2 (def)	0.20±0.06 (ef)	1.53±0.25 (de)	2.72±0.50 (ab)	137.24±5.41 (a)	0.81±0.12 (a)
2441	–	19.8±4.8 (efg)	0.08±0.01 (g)	0.07±0.04 (h)	1.94±0.24 (a)	141.16±2.51 (a)	0.80±0.07 (a)
8067 <sup>b</sup>	LC	18.5±4.2 (fg)	0.15±0.06 (fg)	1.25±0.11 (fg)	2.53±0.69 (ab)	138.50±9.93 (a)	0.81±0.10 (a)
8079 <sup>b</sup>	LC	9.3±3.2 (fgh)	0.14±0.03 (fg)	1.01±0.12 (g)	2.64±0.41 (ab)	142.95±3.71 (a)	0.80±0.12 (a)
8071 <sup>b</sup>	LC	7.3±2.1 (gh)	0.13±0.04 (g)	1.22±0.15 (fg)	2.81±0.42 (ab)	140.41±5.79 (a)	0.82±0.10 (a)
Saxa <sup>b,d</sup>	Non-cyanogenic	0.0±0.0 (h)	0.00±0.00 (h)	0.01±0.01 (h)	3.16±0.22 (b)	142.02±4.27 (a)	0.83±0.10 (a)

<sup>a</sup> Values shown for leaf characteristics are means (±SD; N= 12 individual plants per accessions). Significant differences between accessions were calculated by a *post hoc* test (Tukey's HSD; *P*<0.001) after one-way ANOVA and are indicated by different letters in parentheses.

<sup>b</sup> Accessions used for feeding trials (data for HCNp presented also in Fig. 1)

<sup>c</sup> Accession WT represents lima bean, *P. lunatus*, wild type (Cuba) with small seeds and leaves.

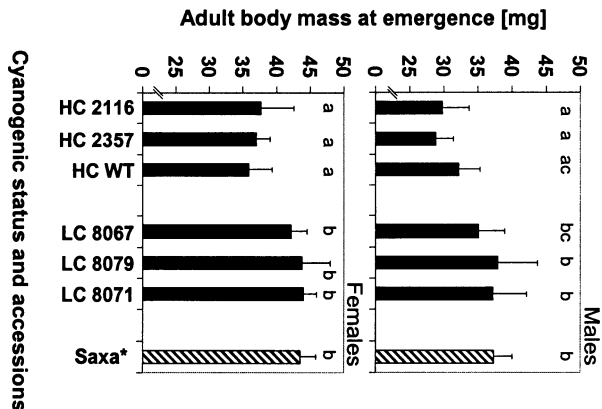
<sup>d</sup> Saxa represents *P. vulgaris* cv. Saxa

separately (Fig. 1). Male and female adults had significantly lower body mass at emergence when larvae had developed on HC-plants as compared to LC-plants and “Saxa”, whereas beetles that had developed on LC-plants showed no significant differences in body mass compared to beetles whose larvae had fed on “Saxa” (Fig. 1). Body mass of male and female beetles on plants of the same accessions did not vary significantly among four consecutive generations. In contrast to clear effects of high HCNp on beetles’ body mass, variation of HCNc in the group of high HCNp plants had no significant effect on adult weight. Beetles feeding on the accession 2116 (high HCNp, low HCNc) showed no differences in body mass compared to the other two HC-plants, accession 2357, and to lima bean wildtype (WT), which were characterized by high HCNp and HCNc (males:  $F_{2,611}=2.23$ ,  $P=0.117$ ; females:  $F_{2,512}=0.734$ ,  $P=0.485$ ).

**Development of Beetles** The developmental period of individual beetles was affected quantitatively by HCNp concentrations. Among HC-plants, larval feeding period, pre-pupation phase (larvae attached to leaves but not yet pupated), and pupal dormancy were significantly prolonged on HC-plants as compared to LC-plants and non-cyanogenic “Saxa” (Table 2). We found no significant differences in phase length between LC-plants and “Saxa” in any of the beetles’ life cycle phases. In contrast to strong quantitative effects of HCNp, no difference in performance and reproduction of beetles among HC-plants depending on quantitative variation of HCNc were observed. Females produced at least three egg clutches in intervals of 8–9 days. Females kept laying eggs after 27 days, but these eggs were often deformed and were not positioned in distinct clutches. Thus, only the first three egg clutches were included in analyses of reproduction.

Egg clutch sizes, i.e., the numbers of eggs per clutch, significantly depended on generation and clutch number and on plant accession (Table 3). Interactions between all these factors indicate that the intergenerational effect depended on the accession on which beetles developed and that the strength of the intergenerational effect was different among the consecutively produced egg clusters (Table 3). In fact, the differences between HC- and LC-plants were already visible in the first generation but became more prominent in the following generations (Fig. 2). In particular, the third clutches became consecutively smaller in beetles reared on HC-plants, whereas no such effect was visible in beetles reared on LC-plants, or “Saxa”. Consequently, *post hoc* analysis (LSD) grouped the three HC-accessions in one group that was significantly different from the three LC-accessions and

**Fig. 1** Adult body mass at emergence. Body mass of male and female beetles, which had developed on lima beans with different HCNp (HC high cyanogenic, LC low cyanogenic, see Table 1) was measured. Values are means ( $\pm$ SD) among beetles of four consecutive generations (for number of replications see Table 2). Significant differences between groups were calculated by a *post hoc* test (Tukey’s HSD;  $P<0.001$ ) following one-way ANOVA and are indicated by different letters. Saxa\* (= *P. vulgaris*), no HCN detected



**Table 2** Effects of host plant cyanogenic status on developmental phases in beetles' life cycle

Accessions	Cyanogenic Status	Sex	N	Larval Feeding Period (day)	Pre-pupation Period (d)	Pupal Dormancy (d)
2116	HC	♂	23	18.1±1.0 <sup>ab</sup>	3.0±1.0 <sup>a</sup>	8.3±0.8 <sup>b</sup>
2357	HC	♂	27	19.0±0.4 <sup>a</sup>	3.1±0.3 <sup>a</sup>	7.4±0.8 <sup>ab</sup>
WT	HC	♂	25	19.0±1.1 <sup>a</sup>	2.8±0.7 <sup>ab</sup>	7.5±1.1 <sup>ab</sup>
8067	LC	♂	22	17.5±1.0 <sup>bc</sup>	1.8±0.6 <sup>c</sup>	6.3±0.6 <sup>de</sup>
8079	LC	♂	20	16.9±0.9 <sup>bc</sup>	2.0±0.4 <sup>bc</sup>	5.6±0.8 <sup>cd</sup>
8071	LC	♂	22	16.6±1.0 <sup>c</sup>	2.2±0.6 <sup>bc</sup>	5.3±0.5 <sup>c</sup>
Saxa	Non-cyanogenic	♂	21	16.8±1.0 <sup>c</sup>	2.0±0.6 <sup>c</sup>	6.7±0.7 <sup>ac</sup>
2116	HC	♀	21	18.1±0.6 <sup>a</sup>	3.0±0.4 <sup>a</sup>	8.1±0.6 <sup>a</sup>
2357	HC	♀	15	18.6±0.5 <sup>a</sup>	3.1±0.4 <sup>a</sup>	6.7±0.5 <sup>b</sup>
WT	HC	♀	18	18.8±1.1 <sup>a</sup>	2.5±0.6 <sup>ab</sup>	7.8±0.9 <sup>a</sup>
8067	LC	♀	21	17.9±1.1 <sup>ac</sup>	2.0±0.7 <sup>b</sup>	6.1±0.8 <sup>bc</sup>
8079	LC	♀	24	17.0±0.6 <sup>bc</sup>	1.9±0.5 <sup>b</sup>	5.6±0.6 <sup>c</sup>
8071	LC	♀	20	16.4±0.9 <sup>b</sup>	2.0±0.6 <sup>b</sup>	5.3±0.7 <sup>c</sup>
Saxa	Non-cyanogenic	♀	22	17.1±0.5 <sup>bc</sup>	2.2±0.4 <sup>b</sup>	6.5±0.6 <sup>b</sup>

Differences in performance of beetles depending on host plant accessions characterized by different HCNp (HC high cyanogenic, LC low cyanogenic) were tested using a *post hoc* test (Tukey's HSD) following one-way ANOVA. Different letters indicate significant differences in developmental period among beetles of four consecutive generations ( $P<0.001$ ). Values shown for developmental periods are means (±SD).

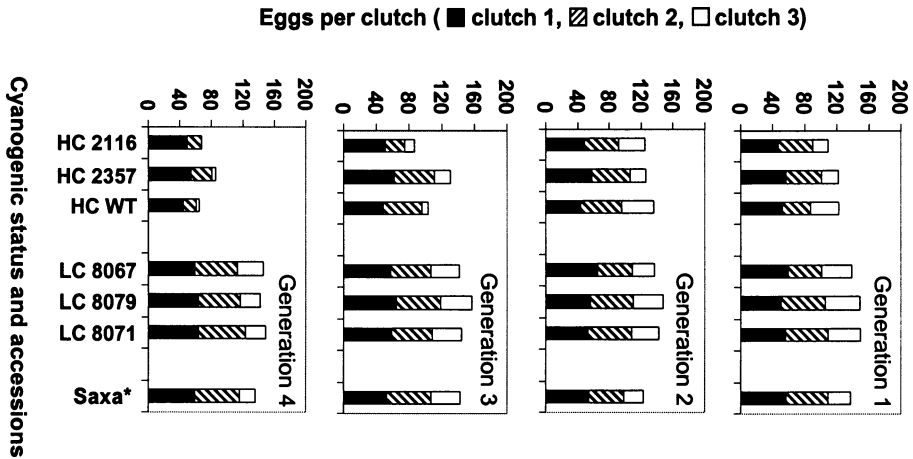
“Saxa”. Over four generations, larval hatching was also negatively affected by high host plant HCNp (Fig. 3).

*Efficiency of Larval Food Utilization* Indices of food utilization differed significantly among beetles on beans with different HCNp (Table 4). Larvae reared on HC-plants ( $N=33$  larvae) showed an increased consumption rate and a reduced ECI into insect biomass compared to larvae reared on LC-plants (RCR:  $F_{1,63}=19.126$ ,  $P<0.001$ ; ECI:  $F_{1,63}=83.820$ ,  $P<0.001$ ;  $N=55$ ). The RGR was reduced on HC-plants compared to LC-plants ( $F_{1,63}=117.67$ ,  $P<0.001$ ). We found no significant differences in all three parameters between the LC-plants and “Saxa” ( $N=11$  larvae). The variation of HCNc in the group of HC-plants had no effect on beetles' food utilization.

**Table 3** Intergenerational effects on clutch size

	Sum of Squares	df	F	P value
<b>Within-subject effects</b>				
gen	2,513.390	3	9.557	<0.001
gen × acc	7,205.881	18	4.567	<0.001
clu	39,629.720	2	160.523	<0.001
clu × acc	3,381.113	12	2.368	0.020
gen × clu × acc	3,829.994	6	5.760	0.000
	8,924.173	36	2.240	0.001
<b>Between-subject effects</b>				
Acc	15,087.619	6	9.262	<0.001

Effects of generation (*gen*) and clutch number (*clu*) as well as accession (*acc*) on clutch size (i.e., number of eggs per clutch) were tested by using a repeated measures ANOVA after confirming that there was no significant deviation from sphericity (tested with Mauchly's test). Four consecutive generations and three clutches per female were considered and generation and clutch were therefore included as within-subject variables, whereas lima bean accession served as between-subject variable.



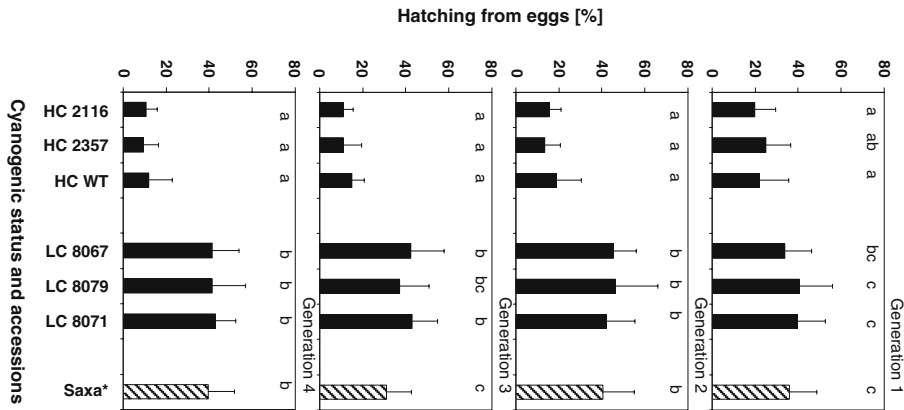
**Fig. 2** Eggs per clutch. Female beetles produced three egg clutches in each of the reproductive cycles between the feeding trials [total number of clutches per generation (gen); *gen 1*  $N=120$ ; *gen 2*  $N=99$ ; *gen 3*  $N=96$ ; *gen 4*  $N=108$ ]. Missing clutches were considered as 0. Values shown for number of eggs per clutch are means. For statistical analyses of effects on clutch size (i.e., number of eggs per clutch) such as beetle generation, clutch number, and accession of host plants see Table 3. Plants with different cyanogenic status were used for the experiments (HC high cyanogenic, LC low cyanogenic). Saxa\* (= *P. vulgaris*), no HCN detected

## Discussion

Defenses mediated by secondary plant compounds are generally believed not to affect specialist herbivores due to their capacity to tolerate or to detoxify defensive compounds of their host plants by behavioral or physiological adaptations (Rausher 1996; Zagrobelny et al. 2004). The specialist herbivore paradigm predicts that adapted herbivores—including oligophagous herbivores with restricted host range—are less affected by a given chemical defense as compared to generalists (Van Der Meijden 1996; Van Dam et al. 2000). However, the observation that a herbivore can develop successfully on a given host plant—or even that it prefers this particular host plant under artificial or natural conditions—does not necessarily mean that it is not affected negatively by some of its host plant’s chemical defenses at higher thresholds, which actually occur in nature (Agrawal and Kurashige 2003).

To study such effects, quantitatively, we used an oligophagous herbivore, the Mexican bean beetle (*E. varivestis*), and reared it on different accessions of cyanogenic lima bean (*P. lunatus*), its preferred natural host plant species. The plants significantly differed in their cyanogenic traits: Three accessions were characterized by concentrations of 64.7 to 76.5  $\mu\text{mol HCN g}^{-1}$  fw (high-cyanogenic, HC) bound in cyanogenic precursors, whereas the “low cyanogenic” (LC) accessions had 7.3 to 18.5  $\mu\text{mol HCN g}^{-1}$  fw.

In spite of its specialization on lima bean, concentration of cyanide-containing compounds significantly affected several fitness-relevant traits of the beetles. Larvae feeding on HC-leaves showed prolonged developmental times (Table 2) and gained less weight per time, although food intake rates were even higher on HC- than on LC-leaves (Table 4). Consequently, the conversion efficiency was lower on HC- than on LC-leaves (Table 4). As longer developmental times were not sufficient to compensate for lower growth rates, the average weight of adult beetles was lower on HC- than on LC-leaves (Fig. 1). Although male and female beetles differed in weight gain, both a reduced total body weight and a lowered larval and pupal performance were significant for both sexes.



**Fig. 3** Number of larvae per female beetle and generation. Hatching of larvae on different lima bean accession was observed over four consecutive generations of beetles. Values are means ( $\pm$ SD) of percentage hatching of larvae including eggs from all three clutches laid per female ( $N=141$  female beetles). Significant differences between groups were calculated by a *post hoc* test (Fishers LSD;  $P<0.001$ ) following one-way ANOVA and are indicated by different letters. Plants with different cyanogenic status were used for the experiments (HC high cyanogenic, LC low cyanogenic). Saxa\* (= *P. vulgaris*), no HCN detected

The effects of defensive compounds and/or products released from these compounds on insect growth depend strongly on the background variation in nutritive leaf traits (Slansky and Wheeler 1992). For example, compensatory feeding on low nutritive diets increases the intake of defensive compounds, and the availability of growth-limiting nutritive compounds may thus modify the effects of chemical defense (Simpson and Raubenheimer 2001; Haukioja 2003). In the present study, we compared HC- and LC-accessions of the same species and can exclude other factors that may vary strongly among different plant species. Yet, we also quantified the most important nutritive and defensive traits of the food plants, i.e., soluble protein concentration and total phenolics, as well as leaf mass per area, which means an approximate measure for crude fiber content. None of these traits differed significantly among young leaves of the accessions (Table 1). Additional analyses indicate that there was no variation of the digestible sugar concentration in leaf developmental stages among the accessions studied (unpublished data). Differences observed among beetles reared on HC- and LC-accessions resulted from differences in plants cyanogenic features.

The quality of resources encountered during development can have effects on individual performance and on reproductive success (Janzen 1977; Fox et al. 1995; Ojeda-Avila et al. 2003). Reproductive success is even more relevant than growth rates, as it directly determines fitness. Females that had been reared as larvae and that were feeding as adults on HC-leaves produced significantly fewer eggs than females on LC-leaves (Fig. 2; Table 3). This effect intensified with every consecutive generation. It was also observed in different episodes of oviposition within a single generation. Females of the fourth generation that had developed consecutively on HC-plants produced smaller second clutches than comparable females that had developed on LC-plants or “Saxa” plants. Fourth generation females from HC-plants rarely produced a third egg clutch (Fig. 2). The size of the first clutches, in contrast, was affected.

The quality of eggs was affected by the type of food utilized by females. Eggs laid by females that fed on HC-leaves hatched at lower rates than those laid by females that fed on

**Table 4** Indices of consumption, growth, and food utilization

Accession	Cyanogenic Status	Relative Growth rate (mg)	Relative Consumption Rate (mg)	Efficiency of Conversion of Ingested Food (mg)
2116	HC	0.26±0.09 <sup>b</sup>	0.42±0.07 <sup>a</sup>	63.20±16.98 <sup>b</sup>
2357	HC	0.28±0.07 <sup>ab</sup>	0.37±0.09 <sup>ab</sup>	78.19±26.30 <sup>ab</sup>
WT	HC	0.25±0.04 <sup>b</sup>	0.34±0.07 <sup>ab</sup>	76.24±22.25 <sup>ab</sup>
8067	LC	0.42±0.05 <sup>c</sup>	0.31±0.05 <sup>b</sup>	142.33±35.11 <sup>c</sup>
8079	LC	0.38±0.13 <sup>ac</sup>	0.29±0.07 <sup>b</sup>	136.04±26.19 <sup>c</sup>
8071	LC	0.43±0.06 <sup>c</sup>	0.29±0.08 <sup>b</sup>	160.86±53.76 <sup>c</sup>
Saxa	Non-Cyanogenic	0.39±0.06 <sup>c</sup>	0.33±0.08 <sup>b</sup>	123.14±23.39 <sup>ac</sup>

Larvae were reared on leaves of different *P. lunatus* accessions. Values shown for different performance efficiency are means (±SD). Letters indicate significant differences calculated by a *post hoc* test (Tukey's HSD;  $P < 0.001$ ) after one-way ANOVA.

LC-leaves or “Saxa” leaves (Fig. 3). This effect appeared to be cumulative and became stronger with each successive generation. So far, such long-term effects of cyanogenesis have been underestimated in analyses of herbivore–plant interactions. However, our study suggests that the long-term effects of cyanogenic features on herbivores must be considered. Future studies should reassess the potential costs arising from host plant defensive traits even on specialist herbivores.

We did not carry out a comparative study between a specialist and a generalist. Thus, we cannot exclude in this study that a generalist herbivore would be affected more severely by cyanogenic features of lima beans. However, negative long-term effects of host plant cyanogenic features on the specialized Mexican bean beetle were definitive. Although negative long-term effects of host plant cyanogenesis were established, the exact physiological mechanism by which this effect is mediated is unclear. Cyanide may interfere with P450 cytochromes involved in final steps of cell respiratory pathways or it may affect nutrient availability for the insects by inhibition of digestive enzymatic activities (Solomonson 1988). As we did not create a balance between cyanide incorporated in bound form as cyanogenic precursors and cyanide released during the feeding process of beetles, we cannot tell in what way the insects came into contact with inhibitory concentrations of cyanide. However, previous studies with the generalist herbivore *Schistocerca gregaria* (Ballhorn et al. 2005) showed that even lima beans with high concentrations of cyanide-containing precursors and highly active  $\beta$ -glucosidase—and therefore rapid and substantial release of gaseous HCN as a reaction to insect feeding—released only about 5–20% of the HCN present as precursors in the consumed leaf material. Thus, as observed in this study, feeding on all high HCNp plants should result in substantial incorporation of cyanide in bound form. The incorporation of intact precursors and the subsequent release of HCN within the gastrointestinal tract by herbivore-internal  $\beta$ -glucosidases appear to be the most harmful way to contact plant cyanide (Ballhorn et al. 2005; Amelot et al. 2006).

In summary, our results demonstrate that the Mexican bean beetle, although being an oligophagous species, is still quantitatively affected by its host plant cyanogenic features. As variation of HCNc among plants that contain high amounts of cyanogenic precursors did not result in differences in food utilization, development, or reproduction, the HCNp is the crucial cyanogenic parameter that affects performance of Mexican bean beetles.

**Acknowledgements** We thank Dr. Christoph Reisdorff and Dr. Helmut Kassner for assistance and helpful comments and thank the “Institute of Plant Genetics and Crop Plant Research (IPK)” in Gatersleben, Germany, for providing seed material of *P. lunatus*.

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