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Comparing responses of generalist and specialist herbivores to various cyanogenic plant features

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Abstract

Plants are obliged to defend themselves against multiple generalist and specialist herbivores. Whereas plant cyanogenesis is considered an efficient defence against generalists, it is thought to affect specialists less. In the present study, we analysed the function of various cyanogenic features of lima bean [*Phaseolus lunatus* L. (Fabaceae)] during interaction with different herbivores. Three cyanogenic features were analysed, i.e., cyanogenic potential (HCNp; concentration of cyanogenic precursors), β-glucosidase activity, and cyanogenic capacity (HCNc; release of cyanide per unit time). In no-choice and free-choice feeding trials, five lima bean accessions were offered to generalist desert locust [*Schistocerca gregaria* Forskål (Orthoptera: Acrididae)] and specialist Mexican bean beetle [*Epilachna varivestis* Mulsant (Coleoptera: Coccinellidae)]. The HCNc was the most important parameter determining host plant selection by generalists, whereas choice behaviour of specialists was strongly affected by HCNp. Although locusts were effectively repelled by high HCNc, this cue was misleading for the detection of suitable host plants, as extensive consumption of low HCNc plant material resulted in strong intoxication of locusts. Balancing cyanide in consumed leaf area, the quantitative release of gaseous cyanide during feeding, and cyanide in faeces suggested that specialists metabolized significantly lower rates of cyanide per consumed leaf material than generalists. We hypothesize that specialists are able to avoid toxic concentrations of cyanide by using HCNp rather than HCNc as a cue for host plant quality, and that they exhibit mechanisms that reduce incorporation of host plant cyanide.

Introduction

Insect herbivores represent the most important group of animals consuming living plant tissues. In order to cope with attack by their multiple enemies, plants express a broad range of defensive chemical and physical traits (Walling, 2000). Among higher plants, synthesis of cyanide-containing compounds is a widely distributed direct defence. Over 3 000 species, representing more than 550 genera and 130 families, produce and accumulate cyanide-containing compounds (Møller & Seigler, 1999; Webber & Miller, 2008). Cyanogenesis means the enzymatically accelerated release of toxic hydrogen cyanide from inactive cyanide-containing components in response to cell damage.

Toxic effects of plant cyanogenesis on several herbivore species including vertebrates, molluscs and insects have frequently been reported (e.g., Compton & Jones, 1985). The main toxicity of cyanide arises from inhibition of the mitochondrial respiration pathway by blocking the cytochrome a/a3-dependent oxidase (Solomonson, 1981). Consequently, all eukaryotic organisms should be negatively affected by plant cyanogenesis. However, there are still gaps in understanding the function of cyanogenesis as a defence against multiple herbivore species (e.g., Zagrobelny et al., 2008). In numerous studies, cyanogenic glycosides have little or no effect on herbivores (e.g.,

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Ferreira et al., 1997; Struempf et al., 1999), or, in some cases, plant cyanogenic compounds function as a phagostimulant rather than an inhibitor (Brattsten et al., 1983; Mowat & Clawson, 1996).

A range of factors can potentially explain differences in efficiency of cyanogenesis as a herbivore deterrent (reviewed by Gleadow & Woodrow, 2002). For example, concentration of cyanogenic precursors can be lower than threshold toxicity, or concentration of cyanide in food can be actively reduced by the herbivore when cyanogenic plant material is consumed as part of a mixed diet (Provenza et al., 1992). Furthermore, the degree of specialization of the herbivore to cyanogenic food plants determines the defensive efficiency of cyanogenesis (Ballhorn et al., 2009a). Defences mediated by secondary plant compounds are generally believed not to affect specialist herbivores because of their capacity to tolerate or to detoxify defensive compounds of their hosts (e.g., Compton & Jones, 1985; Nahrstedt, 1985; Zagrobelny et al., 2008). In this line of vision, the specialist herbivore paradigm predicts that adapted herbivores – including oligophagous herbivores with restricted host range – are less affected by a given chemical defence than generalists (van der Meijden, 1996; but see Agrawal & Kurashige, 2003; Ballhorn et al., 2007, 2009b).

On the plant's side, the variability of defensive traits strongly affects the outcome of herbivore*plant interactions (Underwood & Rausher, 2000; Kaitaniemi & Ruohomaki, 2001). It is well documented that many cyanogenic plant species are polymorphic for cyanogenesis or at least show substantial quantitative variability of cyanogenic traits depending on genotype (Schappert & Shore, 1995; Miller et al., 2006). Beyond genotypic variability, plants generally show substantial phenotypic plasticity in the expression of defensive traits depending on plant organ, age of plants or plant parts, and environmental conditions (Schlichting & Pigliucci, 1998; Webber & Woodrow, 2009). However, an additional source of variation in defensive action of cyanogenesis is widely underestimated in analysis of herbivore*plant interactions: the functional differentiation of cyanogenic features in a static and a kinetic component (Miguel & Alberto, 2005). Cyanogenesis is based on two components – the concentration of cyanogenic precursors and the activity of specific β-glucosidases. The cyanogenic potential (HCNp) represents the maximum amount of cyanide that can be released from a given plant tissue and corresponds to the amount of cyanogenic precursors (Loyd & Gray, 1970). Contrary to this fixed trait, the cyanogenic capacity (HCNc) is a kinetic parameter and means the release of gaseous hydrogen cyanide per unit time following cell disruption (Lieberei, 1988; Ballhorn et al., 2005). Thus, HCNc is a product of HCNp and the activity of β-glucosidases. Until now, there have been no comparative studies considering both sources of variation determining the overall trait 'cyanogenesis' in interaction with specialist and generalist herbivores.

Beyond the defensive traits themselves, analysing plant defences against herbivores requires the consideration of another source of variability; that is, the variation of nutritive parameters such as protein concentration or digestible and non-digestible carbohydrates (e.g., Ball et al., 2000; Awmack & Leather, 2002). With a focus on cyanogenesis, the quantitative relationship of cyanide to protein – especially to proteins with high amounts of the sulphur-containing amino acids cysteine and methionine – is an important measure determining plant's overall food quality, as these amino acids are required for enzymatic detoxification of cyanide by rhodanese or β-cyanoalanine synthase (e.g., Nahrstedt, 1985; Urbańska et al., 2002).

To contribute to understanding the complex function of plant cyanogenesis in interaction with differently adapted herbivores, we analysed the impact of three cyanogenic features (HCNp, β-glucosidase activity, and HCNc) on a specialist and a generalist insect herbivore. In our study, we used lima bean [*Phaseolus lunatus* L. (Fabaceae)] genotypes as experimental plants, while the generalist desert locust [*Schistocerca gregaria* Forskål (Orthoptera: Acrididae)] and the specialist Mexican bean beetle [*Epilachna varivestis* Mulsant (Coleoptera: Coccinellidae)] served as herbivores. The desert locust is among the most dangerous locust pests because it builds up swarms of hundreds of millions of individuals that fly rapidly across great distances in the migratory phase and threaten many regions of Africa, the Middle East and Asia (Mainguet et al., 2000). In contrast to desert locusts, the Mexican bean beetle is an oligophagous insect that feeds on a range of legumes (Barrigossi et al., 2001) but with a distinct preference for *Phaseolus* species (Dover et al., 1988) and especially lima bean (Lapidus et al., 1963; Flanders, 1984). The Mexican bean beetle is native to southern Mexico, as are lima beans of the Mesoamerican gene pool. In their introduced range in parts of the USA, these beetles can be a serious pest in bean cultures (Biddle et al., 1992; Capinera, 2001).

This set of experimental organisms – plants with different but defined cyanogenic features and herbivores with different degrees of specialization – provides an ideal system to study quantitative effects of variable plant traits on herbivores. Results of this study contribute to understanding the complex action of plant cyanogenesis in plant*herbivore interactions.

Materials and methods

Plants and insects

A set of five lima bean accessions (*P. lunatus*) with variable, but for each of the accessions constant, cyanogenic features served as experimental plants. Plant genotypes comprised four cultivars (CV 2116, CV 2357, CV 8079, and CV 8071) and one wildtype (WT 2233) that originated in Cuba. Experimental plants had different combinations of cyanogenic traits. Accessions CV 2357 and WT 2233 both had high HCNp and high HCNc and are referred to as 'HC-plants', whereas plants of the genotype CV 2116 showed high HCNp and low HCNc and were classified as '$HC^{(-)}$-plants'. Genotypes CV 8079 and CV 8071 were characterized by low HCNp and low HCNc and are considered 'LC-plants'.

All lima bean plants were cultivated under greenhouse conditions (L16:D8) with a photon flux density of 350–450 μmol s^{-1} m^{-2} at the base of the plants and 800–950 μmol s^{-1} m^{-2} on top of the plants. Additional light was provided by 400 W high-pressure sodium lamps (Son-T Agro 400; Philips®, Hamburg, Germany). To avoid the effect of hot spots under the lamps, the position of plants was changed every 3 days. Temperature was set to 25:20 °C (corresponding to light/dark period) and ambient relative air humidity to 60–70%. Lima bean plants were supplied with a nitrogen-phosphate fertilizer (Blaukorn®-Nitrophoska®-Perfekt; Compo, Münster, Germany) twice a week (3 mg per pot) and were cultivated in plant containers 18 cm in diameter, in a 1:1 ratio of standard substrate (TKS®-1-Instant; Floragard®, Oldenburg, Germany) 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.

In feeding trials, we used the generalist desert locust (*S. gregaria*) and the specialist Mexican bean beetle (*E. varivestis*). Both insect species are unable to sequester cyanogenic glycosides (DJ Ballhorn, unpubl.). Because desert locusts are extremely polyphagous (FAO, 1994), these insects were suitable for experiments on quantitative effects of cyanogenesis on generalist herbivores. Locusts were maintained on a mixed diet consisting of fruits and leaves of non-cyanogenic plants. The locusts' life cycle encompasses five nymphal stages. At the end of the incomplete metamorphosis after 30–40 days, locusts have formed complete wings. After mating, the insects produce 90–160 eggs in their solitary phase, whereas gregarious females usually lay fewer than 80 eggs. The eggs hatch after 10–20 days depending on temperature (Steedman, 1990; FAO, 1994). For detailed information on maintenance of locusts see Ballhorn et al. (2005). Locusts used for this study were derived from a permanent maintenance culture at the University of Hamburg, Biocenter Grindel and Zoological Museum, Hamburg, Germany.

Mexican bean beetles used in this study were maintained on non-cyanogenic snap bean (*Phaseolus vulgaris* cv. Saxa) to prevent them from developing preferences for any lima bean accession (Ballhorn & Lieberei 2006, 2007). The life cycle of the beetles encompasses four larval stages followed by a short pupal stage of 5–10 days depending on temperature. Females deposit eggs in clutches of 40–75 on the lower surface of host plant leaves, and both larvae and adults feed on foliage. Beetles were obtained from Prof. CPW Zebitz (Department of Applied Entomology, University of Hohenheim, Germany).

Chemical analyses

Cyanogenic potential (HCNp). To assess the quantitative impact of genotypic and ontogenetic variability on cyanogenic precursor concentration (HCNp; cyanogenic potential) in leaves of the different lima bean genotypes, defined leaf developmental stages were analysed. According to their insertion position at the stem, leaves were classified as 'young,' 'intermediate', or 'mature' (Figure 1). By definition, leaves at the apex of the stem or a side stem that were fully unfolded for at least 4 days, but no longer than 6 days were considered 'young'. Leaves located on the stem two positions below the category 'young' were defined as 'intermediate'. Leaves assigned to this category still showed a thin and delicate leaf tissue. 'Mature' leaves were located on the stem two positions below 'intermediate' leaves. These leaves were characterized by a dark green colour and a hardened midrib; they were always completely expanded (Ballhorn et al., 2005, 2006). Cyanogenic potential (HCNp) was analysed by complete enzymatic degradation of cyanogenic glycosides, and HCN released from cyanogenic precursors was spectrophotometrically quantified (at 585 nm) using the Spectroquant® cyanide test (Merck, Darmstadt, Germany) following the method by Ballhorn et al. (2005). External β-glucosidase isolated from the rubber tree [*Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg (Euphorbiaceae)] 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).

Activity of plant β-glucosidase. In order to measure β-glucosidase activity in leaves, we used the same leaf extract that was used for the determination of HCNp. Activity of β-glucosidase was measured according to Ballhorn et al. (2006) using p-NP-glucoside (Merck) as chromogenic artificial substrate (2 mmol l^{-1}). The *p*-nitrophenol released was quantified spectrophotometrically at 400 nm (Ultraspec 3000; Pharmacia Biotech, Nümbrecht,

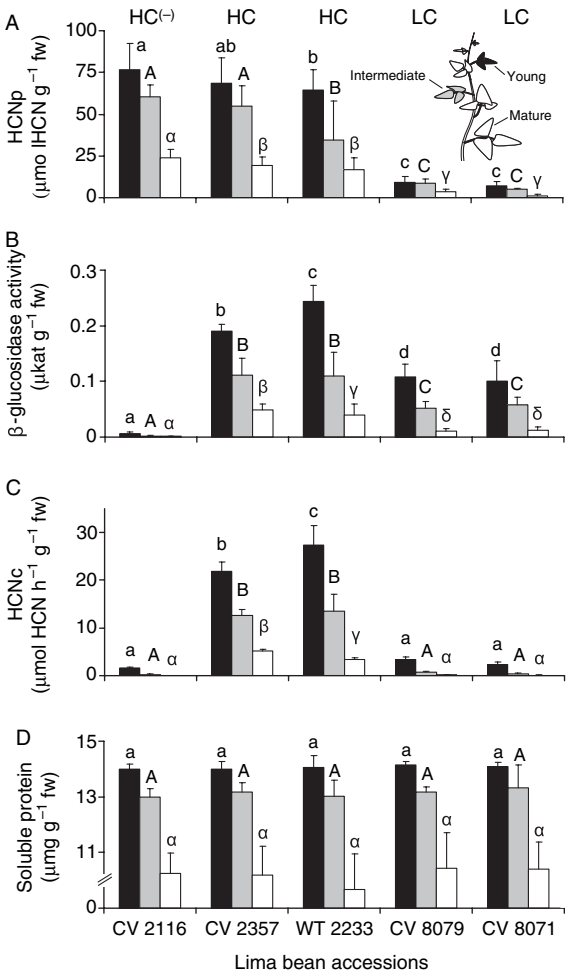

Figure 1 Cyanogenic and nutritive features of lima bean accessions. Leaf developmental stages of four cultivated (CV) and one wildtype (WT) lima bean genotypes were screened for (A) cyanogenic potential (HCNp), (B) β-glucosidase activity, (C) capacity for release of gaseous HCN (HCNc), and (D) concentration of soluble proteins. Among accessions, differences in leaf parameters were analysed separately for each leaf developmental stage and significant differences are indicated by different letters (lowercase letters for young leaves, capital letters for intermediate leaves, and greek letters for mature leaves) at the columns [post-hoc analysis (LSD: $P<0.05$) after one-way ANOVA]. Cyanogenic features of young leaves were used to group lima bean accession in different cyanogenic categories as indicated at the upper panel of the figure ($HC^{(-)}$, HC, and LC). Values given in the figure are mean + SD; n = 12 leaves per accession and developmental stage; fw, fresh weight.

Germany). The β-glucosidase activity was calculated per gram leaf dry weight as katal (kat). An enzyme activity of 1 kat is defined as a substrate conversion rate of 1 mol substrate per second under standard temperature and pressure. Enzyme activity was calculated using a coefficient of extinction for *p*-nitrophenol (400 nm = 16 159 l mol^{-1} cm; Voß, 2001).

Cyanogenic capacity (HCNc). The kinetic analysis of HCN release from experimentally treated lima bean leaves was carried out using an airflow system according to Ballhorn et al. (2005). This vessel system was passed by a constant airflow adjusted to 7 l h^{-1}. The leaflets were treated with chloroform (250 µl per leaflet) to achieve complete tissue disintegration at the cellular level and consequently, the release of gaseous hydrogen cyanide from the accumulated cyanogenic precursors. At the discharge opening of the equipment, the air together with the gaseous HCN was led into a test tube containing 0.1 mol l^{-1} NaOH solution. In this solution, cyanide was fixed as NaCN and then, as with HCNp quantification, was spectrophotometrically quantified at 585 nm as a polymethine dye formed using the Spectroquant® cyanide test.

Soluble protein concentration. Co-variation of cyanogenic and nutritive plant traits may strongly determine the overall attractiveness or resistance of plants to herbivores. Thus, in addition to cyanogenic traits, we considered leaf soluble protein as an important nutritive trait (Ganzhorn, 1992). 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 at 13 000 ***g*** (4 °C), and the supernatant was filtered over NAP™ columns containing Sephadex™ G-25 DNA-Grade (GE Healthcare, München, Germany). Subsequently, 5 µl of the eluate were pipetted on microplates (96-well Microplates, F-bottom; Greiner Bio-One, Frickenhausen, Germany), and 250 µl Bradford reagent (diluted with deionized water in the ratio 1:4) were added. Protein concentration of samples was spectrophotometrically quantified at 595 nm. Bovine serum albumin solutions (Merck) in the range between 10 and 600 µg ml^{-1} served as standard.

Feeding trials. In feeding experiments with both insect herbivores, leaves which had been individually analysed for their cyanogenic (HCNp, β-glucosidase activity and HCNc) and nutritive features (soluble protein concentration) were used. Feeding experiments were conducted in the air-flow system for cyanide detection under the same greenhouse conditions that were adjusted for plant cultivation. Leaf material was exposed to the insects for 2 h.

For locusts and beetles, we conducted no-choice and free-choice experiments. In all experiments, we used leaf material of the young developmental stage, as these leaves showed most similar physical leaf parameters (leaf texture and trichome density) and per accession high constancy of

chemical traits. In no-choice experiments, leaves of a single accession were offered, whereas in choice assays the insect could select between leaves of all five lima bean genotypes. For feeding trials and quantification of chemical leaf traits, individual trifoliate leaves were dissected. One leaflet was randomly selected for chemical analyses of HCNp and soluble protein, while the other two leaflets were used in the different feeding trials. Chemical leaf traits were assumed to be similar among the three leaflets of individual leaves, as previous studies have shown distinct homogeneity of traits in individual trifoliate leaves (Ballhorn et al., 2006, 2007, 2009b). Before and after the feeding experiments, leaves used for feeding trials were digitally photographed on a scale (Canon, EOS 40D; 10 000 pixels) and missing leaf area was computer-based determined using the analySIS software (Olympus, Hamburg, Germany).

To guarantee a similar physiological stage of insects in feeding experiments with locusts, only nymphs of the same age (third instar), similar size and body weight (287.3 ± 38.4 mg; mean ± SD; n = 24) were chosen. We selected locust nymphs that had molted approximately 24 h previously. Before the feeding trials, the insects were kept for 24 h with ad libitum access to leaf material of annual blue grass [*Poa annua* L. (Poaceae)]; a favoured food plant) but they were food deprived for 2 h prior to the bioassays.

For feeding trials with specialist Mexican bean beetles, adult insects in natural sex-ratios (n = 9 females, n = 15 males) were used that had molted at least 24 h but no longer than 5 days prior to the experiment and had similar body weight within each sex (females: 56.4 ± 1.3, males: 44.6 ± 1.7 mg). Beetles were also food deprived for 2 h prior to the experiment.

Behaviour of insects. Insect behaviour was observed during feeding trials to evaluate potential symptoms of intoxication. According to Ballhorn et al. (2005), behaviour of insects was assigned to different categories representing different levels of intoxication. Behavioural patterns were defined as follows: 0, 'normal' behaviour (similar to behaviour after consumption of leaf material used in maintenance cultures of insects); 1, reduced and slowed movements; 2, like 1 but no cleaning of the mouthparts, antennae, and legs, and in case of locusts, loss of flight reaction; 3, like 2, but, in addition, unnatural posture of antennae and legs and complete akinesia (i.e., complete loss of movement). Incidents of death were included in category 3.

Cyanide recovery. All feeding experiments were conducted in an air-flow system to detect gaseous cyanide released from leaves in response to feeding (according to Ballhorn et al., 2005). By additionally calculating consumed leaf area and cyanide concentration in the faeces, we were able to exactly quantify the intake of cyanogenic precursors and release of hydrogen cyanide to the air during feeding.

The amount of intact cyanogenic precursors in the faeces of locusts and Mexican bean beetles was determined after each feeding trial. Leaves were removed from the experimental vessel connected to the air-flow system and faeces excreted for time periods of 2 h after the experiment were included in the analysis. While faeces of locusts could easily be collected and analysed for cyanide following the procedure for HCNp determination of leaf material, Mexican bean beetles produce liquid faeces. Faeces of beetles on the surfaces of the experimental vessel were removed by washing with deionized water at 4 °C. Extracts were concentrated by freeze-drying before analysis. Faeces deposited on the leaf material, however, were not included in the analysis to avoid 'contamination' by cyanogenic precursors potentially leaking from the damaged leaf.

Activity of insect β-glucosidases. Insects used for analysis of β-glucosidase activity were maintained on leaves of the low cyanogenic lima bean genotype (CV 2441) with characteristically low endogenous β-glucosidase activity in leaves for 3 weeks (Ballhorn et al., 2005, 2006, 2007, 2010) to reduce the risk of measuring plant derived instead of insect β-glucosidase activity. In addition, insects were food deprived for an extended time period (4 h) prior sampling regurgitate to further reduce 'contamination' of regurgitate with plant derived enzymes.

For analysis of β-glucosidase activity in regurgitates of locusts and beetles, insects were carefully picked with squeezers and regurgitated droplets were collected with micro capillaries (5 µl, Blaubrand®; Brand, Wertheim, Germany), which allowed for simultaneous determination of the volume. The regurgitate of individual insects was added to 1 ml substrate solution (*p*-NP-glucoside, 2 mmol l^{-1}) and *p*-nitrophenol released was spectrophotometrically quantified after 3 min of incubation. The enzymatic activity in katal was calculated per µl regurgitate.

Statistical analysis. To test for significant differences of plant parameters (HCNp, β-glucosidase activity, HCNc and soluble protein) we applied post-hoc analyses (LSD) after one-way ANOVA using the respective plant feature as variable and 'accession' as factor. Analyses were carried out separately for each leaf stage, as otherwise differences between accessions would have been masked by ontogenetic variability of traits. Using a defined leaf developmental stage (i.e., young leaves) in the feeding trials, we tested

for differences in leaf consumption, cyanide present in consumed leaf area (no-choice and free-choice experiments), differences in release of HCN during feeding, HCN in faeces and cyanide recovery (no-choice experiments) using post-hoc analyses after one-way ANOVA. In the same way, we calculated differences in insect behaviour among lima bean accession in no-choice experiments. Because in free-choice experiments with locusts and beetles the overall release of HCN during feeding, HCN in faeces, rates of recovered cyanide and insect behaviour could not be related to specific lima bean accessions, we applied Mann–Whitney U-tests to test for differences between both insect species. Mann–Whitney U-tests were also applied to analyse for differences in β-glucosidase activities in regurgitates of locusts and beetles (Figure 9) that both had been feeding on the same lima bean accession (CV 2441). General linear models (GLM) were applied to test for effects of 'accession', 'leaf stage' and 'accession*leaf stage' on HCNp, β-glucosidase activity, HCNc and amount of soluble protein (Table 1) and the effects of 'accession', 'herbivore species' and 'accession*herbivore species' on HCN release by feeding, HCN in faeces, and percent of HCN recovered. All statistical analyses were carried out using SPSS 17 (SPSS for Windows; SPSS, Chicago, IL, USA).

Results

Plant features

Cyanogenic potential (HCNp). Lima bean genotypes showed substantial differences in cyanogenic potential (HCNp). 'Accession' significantly affected HCNp of all leaf stages investigated. The cyanogenic potential of both HC-accessions (CV 2357 and WT 2233) and the $HC^{(-)}$-accession CV 2116 was significantly higher than that of the LC-accessions (CV 8079 and CV 8071) (according to post-hoc analysis (LSD: $P<0.05$) after one-way ANOVA; Figure 1A). For all lima bean accessions, 'leaf stage' was a significant source of variation (Table 1). Young leaves exhibited a higher HCNp than intermediate leaves, while HCNp in intermediate leaves was higher than in mature leaves (Figure 1A). Consequently, 'accession', 'leaf stage' as well as the interaction 'accession*leaf stage' were significant sources of variation using the GLM (Table 1).

β-glucosidase activity. The HC-accessions CV 2357 and WT 2233 showed significantly higher β-glucosidase activities than both LC-accessions. The $HC^{(-)}$-genotype CV 2116 exhibited only minimal enzymatic activity, significantly lower than all other accessions [according to post-hoc analysis (LSD: $P<0.05$) after one-way ANOVA]. For

Table 1 Effects of 'accession', 'leaf stage', and 'accession*leaf stage' on HCNp, β-glucosidase activity, HCNc, and amount of soluble protein

Source	Dependent variable	SS	d.f.	F	P-value
Corrected model	HCNp	123231.999	14	87.502	<0.001
	β-glucosidase	86.685	14	138.657	<0.001
	HCNc	12668.842	14	372.366	<0.001
	Protein	49568.644	14	71.331	<0.001
Accession	HCNp	75662.256	4	188.036	<0.001
	β-glucosidase	38.438	4	215.190	<0.001
	HCNc	7397.211	4	760.972	<0.001
	Protein	296.033	4	1.491	0.207
Leaf stage	HCNp	31880.319	2	158.458	<0.001
	β-glucosidase	34.878	2	390.527	<0.001
	HCNc	2763.216	2	568.520	<0.001
	Protein	48997.644	2	493.566	<0.001
Accession*leaf stage	HCNp	1569.424	8	19.496	<0.001
	β-glucosidase	13.369	8	37.423	<0.001
	HCNc	2508.414	8	129.024	<0.001
	Protein	274.967	8	0.692	0.698
Total	HCNp	305120.891	180		
	β-glucosidase	189.625	180		
	HCNc	19933.446	180		
	Protein	2849796.000	180		

Results were obtained using the GLM analysis of variance after a multivariate design with HCNp, β-glucosidase activity, HCNc, and amount of soluble protein as variables. 'Accession' and 'leaf stage' were set as fixed factors.

all genotypes, β-glucosidase activity significantly decreased with leaf age (Figure 1B; Table 1). As observed for HCNp, 'accession', 'leaf stage' as well as the term 'accession*leaf stage' were significant sources of variation affecting β-glucosidase activity in plants (GLM; Table 1).

Cyanogenic capacity (HCNc). The total amount of cyanide released within 60 min after chloroform treatment differed significantly among the five accessions within each leaf developmental stage [post-hoc analysis (LSD: $P<0.05$) after one-way ANOVA; Figure 2]. Lima bean plants that had the highest HCNc (Figure 1C) expressed both high concentration of cyanogenic precursors (Figure 1A) and high enzymatic activity (Figure 1B). Consequently, plants of the genotype CV 2116, despite their high HCNp, exhibited minimal HCNc because of their extremely low β-glucosidase activity (Figure 1C). The HCNc of different leaf developmental stages revealed a decrease of cyanogenic capacity depending on leaf age for all HC- and LC-accessions (Figures 1C and 2; Table 1). Young and intermediate leaves of high cyanogenic genotypes were characterized by a rapid release of HCN as well as a rapid decrease of measurable hydrogen cyanide within 50 min after chloroform treatment (Figure 2). Mature leaves of these genotypes showed a lower and more continuous release of HCN that lasted as long as 60 min (Figure 2). Gaseous hydrogen cyanide was also released from leaves of all developmental stages of the low cyanogenic lima bean genotypes analysed, but the release of HCN especially from mature leaves was close to zero (Figure 2). The total release of hydrogen cyanide was significantly affected by 'accession' and 'leaf stage' as well as the interaction 'accession*leaf stage' (GLM; Table 1).

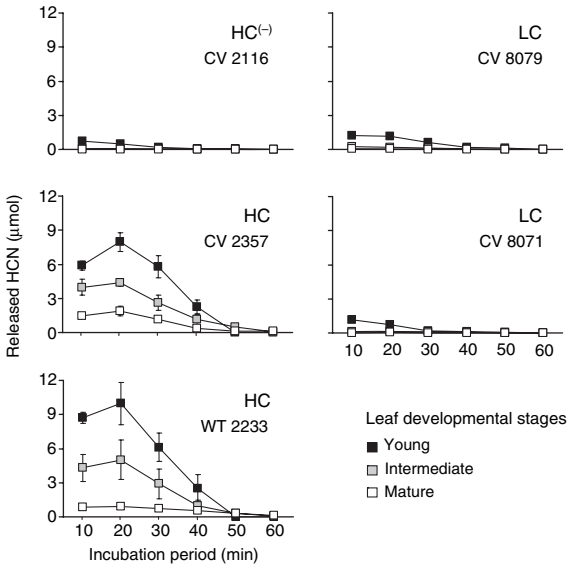

Figure 2 Cyanogenic capacity. Kinetics of cyanide release in response to chemical tissue disintegration after chloroform treatment are shown for leaves of defined developmental stages (young, intermediate, and mature) of lima bean accessions with different cyanogenic features ($HC^{(-)}$, HC, and LC). The experiments covered time periods of 60 min. Values given in the figure are mean ± SD; n = 12 leaves per accession and developmental stage.

Soluble protein concentration. Analyses of soluble protein revealed similar concentrations among the different genotypes for each respective leaf stage (Figure 1D). 'Accession' was not a significant source of variation (Table 1). Focussing on ontogenetic differences in protein concentration, 'leaf stage' significantly affected soluble protein concentration (GLM; Table 1). For each genotype, young leaves were always characterized by the highest protein concentration (140.53 ± 2.56 µg mg^{-1}), followed by intermediate (131.27 ± 5.05 µg mg^{-1}), and mature (101.83 ± 10.79 µg mg^{-1}) leaf developmental stages. 'Accession' and the interaction 'accession*leaf stage' were not significant sources of variation (GLM; Table 1).

Insect responses

Leaf consumption and cyanide incorporation in no-choice feeding trials. In no-choice feeding experiments, locusts consumed between 16.00 ± 8.81 (CV 2357) and 416.75 ± 113.82 mm^2 leaf area (CV 8071). Total leaf consumption of locusts was significantly higher on leaves of the $HC^{(-)}$- and LC-plants than on HC-plants (Figure 3A). A comparison of $HC^{(-)}$-plants and LC-plants revealed no significant differences in leaf consumption [post-hoc analysis (LSD: $P<0.05$) after one-way ANOVA]. Mexican bean beetles consumed between 130.08 ± 76.33 (CV 2357) and 143.67 ± 33.27 mm^2 leaf area (CV 8079) in no-choice feeding trials. Among lima bean accessions, leaf consumption by beetles showed no significant differences [post-hoc analysis (LSD: $P<0.05$) after one-way ANOVA; Figure 3A].

In no-choice feeding trials with locusts, total amounts of cyanide present in consumed leaf material of $HC^{(-)}$-genotype CV 2116 were substantially higher than in all other lima bean genotypes (Figure 3B). However, feeding on LC-genotypes CV 8079 and CV 8071 also resulted in amounts of cyanide in consumed leaf tissue that were significantly higher than both HC-genotypes (CV 2357 and WT 2233). In contrast to locusts, the calculated amount of cyanide in leaf tissue consumed by beetles was significantly higher for $HC^{(-)}$- and HC-genotypes than for LC-plants [post-hoc analysis (LSD: $P<0.05$) after one-way ANOVA; Figure 3B].

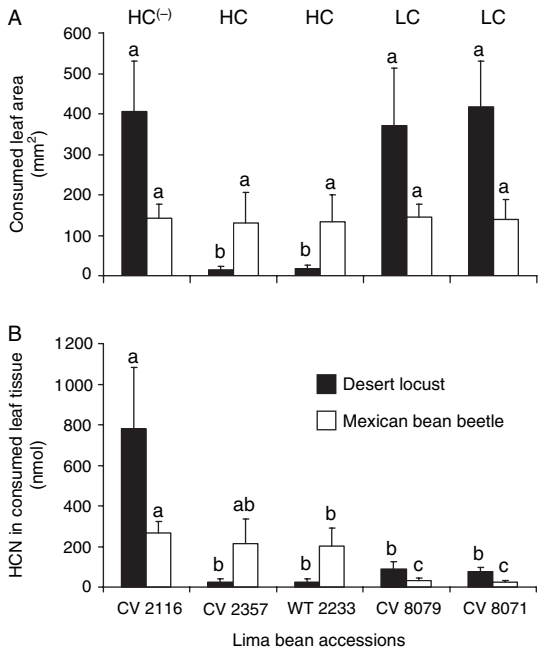

Figure 3 Leaf area and cyanide consumed in no-choice feeding trials. Experiments were carried out with generalist desert locusts (black columns) and specialist Mexican bean beetles (white columns). In no-choice setups, young leaves of different lima bean accessions were offered to individual insects. Leaf area consumption (A) was measured and amount of cyanide present in consumed leaf tissue was calculated (B). Values shown are mean + SD; n = 12 feeding trials per herbivore species. Different letters at columns within insect species indicate significant differences [post-hoc analysis (LSD: $P<0.05$) after one-way ANOVA].

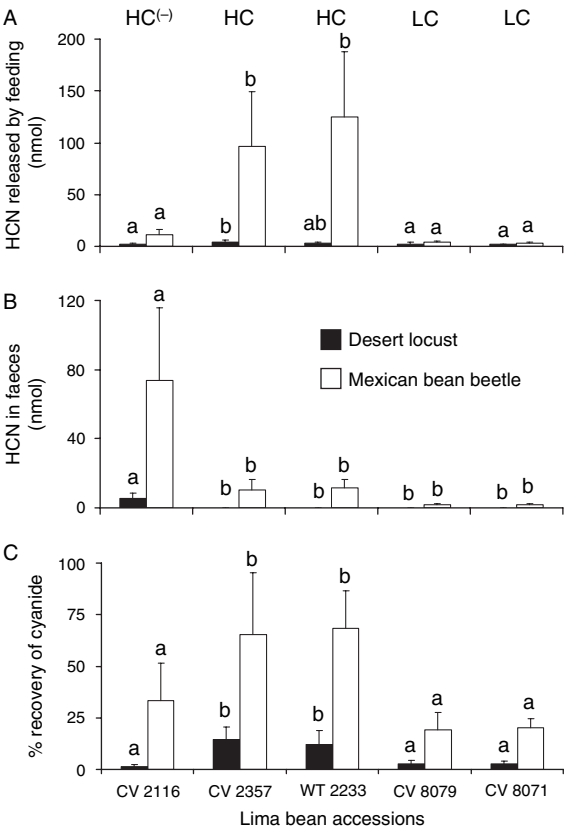

Figure 4 Cyanide balance in no-choice feeding trials. For generalist locusts and specialist beetles, the amount of HCN that was (A) released during feeding and (B) excreted after feeding on different lima bean accession with different cyanogenic features ($HC^{(-)}$, HC, and LC) was measured and the rate of recovered cyanide (%) was calculated (C). Values shown are mean + SD; n = 12 feeding trials per herbivore species. Different letters at columns within insect species represent significant differences [post-hoc analysis (LSD: $P<0.05$) after one-way ANOVA].

Cyanide balance in no-choice feeding trials. Feeding by both insect species resulted in the release of measurable amounts of gaseous cyanide (Figure 4A). Release of HCN in response to feeding by locusts was low and ranged from 1.73 ± 0.52 nmol cyanide (mean ± SD) for accession CV 8071 to 3.91 ± 1.98 nmol cyanide (CV 2357). These amounts corresponded to 2.62 ± 1.36 (CV 8071) and 14.50 ± 5.85% (CV 2357) of cyanide present in consumed leaf material. In contrast, differences in overall cyanide release in response to feeding by beetles were substantial (Figure 4A). Feeding on HC-accessions CV 2357 and WT 2233 resulted in a release of 96.61 ± 52.19 and 124.52 ± 63.28 nmol HCN, corresponding to 54.21 ± 22.76 and 61.27 ± 17.48% of the amount of cyanide accumulated in consumed leaf tissue, respectively. Feeding on the $HC^{(-)}$-accession CV 2116 resulted in considerably lower releases of gaseous cyanide (11.41 ± 4.81 nmol HCN), corresponding to 4.39 ± 1.68% of accumulated cyanide, and feeding on LC-accessions revealed even lower values. However, the percentage release of cyanide from these LC-accessions was higher than for the $HC^{(-)}$-accession CV 2116 and corresponded to 13.68 ± 6.87% (CV 8079) and 14.13 ± 3.75% (CV 8071). 'Accession' and 'herbivore species' as well as the interaction 'accession*herbivore species' were significant sources of variation for the amount of gaseous cyanide recovered from the atmosphere in the air-flow system (according to GLM; Table 2).

Quantitative analysis of cyanide in the locusts' faeces showed values near to zero for both HC- and LC-accessions (CV 2357: 0.02 ± 0.01; WT 2233: 0.01 ± 0.01; CV 8079: 0.05 ± 0.05; and CV 8071: 0.05 ± 0.04). Only in faeces of locusts that had been feeding on $HC^{(-)}$-plants did we find increased cyanide levels (5.55 ± 2.68 nmol HCN), which corresponded to 0.89 ± 0.86% of the cyanide in consumed leaf tissue (Figure 4B). The amount of cyanide in faeces of

Table 2 Effects of 'accession', 'herbivore species', and 'accession*herbivore species' on HCN release by feeding, HCN in faeces, and % HCN recovered

Source	Dependent variable	SS	d.f.	F	P-value
Corrected model	HCN release by feeding	223789.968	9	36.763	<0.001
	HCN in faeces	55689.462	9	34.505	<0.001
	HCN recovery	9.417	9	27.316	<0.001
Accession	HCN release by feeding	82893.458	4	30.639	<0.001
	HCN in faeces	26158.159	4	36.467	<0.001
	HCN recovery	3.168	4	20.673	<0.001
Herbivore species	HCN release by feeding	61839.340	1	91.429	<0.001
	HCN in faeces	10520.548	1	58.666	<0.001
	HCN recovery	4.794	1	125.162	<0.001
Accession*herbivore species	HCN release by feeding	79057.169	4	29.221	<0.001
	HCN in faeces	19010.755	4	26.503	<0.001
	HCN recovery	1.455	4	9.497	<0.001
Total	HCN release by feeding	374765.621	120		
	HCN in faeces	88641.385	120		
	HCN recovery	13.630	120		

Results were obtained using the GLM analysis of variance after a multivariate design with HCN release by feeding, HCN in faeces, and % HCN recovered as variables. 'Accession' and 'herbivore species' were set as fixed factors.

beetles was higher than for locusts (Figure 4B). The percentage recovery of cyanide in faeces of beetles feeding on LC-accessions CV 8079 and CV 8071 was 5.65 ± 4.24% and 6.04 ± 2.29% of the calculated amount of cyanide in consumed leaf tissue. In faeces of beetles that had been feeding on the HC-accessions CV 2357 and WT 2233, cyanide values were considerably higher (Figure 4B). The percentage recovery, however, was similar to LC-accessions (CV 2357: 5.56 ± 1.3% and WT 2233: 7.21 ± 6.72%; Figure 4B). Compared with both HC- and LC-accessions, the amount of cyanide in faeces of beetles feeding on the $HC^{(-)}$-accession CV 2116 was substantially enhanced (Figure 4B). In faeces, 28.87 ± 18.79% of the cyanide present in consumed leaf tissue were recovered.

Balancing the total recovery of cyanide (released cyanide during feeding and cyanide in faeces) in no-choice feeding trials with generalist locusts and specialist Mexican bean beetles revealed a higher rate of cyanide recovery for beetles on all lima bean accessions (Figure 4C). 'Accession', 'herbivore species' as well as the interaction 'accession*herbivore species' were significant sources of variance (GLM; Table 2). Thus, the amount released in the insect's bodies was significantly higher for the generalist than for specialist herbivores.

Leaf consumption and cyanide incorporation in free-choice feeding trials. When having the choice between leaves of the different accessions, locusts significantly preferred $HC^{(-)}$- and LC-leaves over HC-leaves [post-hoc analysis (LSD: P<0.05) after one-way ANOVA]. In contrast, Mexican bean beetles significantly preferred LC- over $HC^{(-)}$-and HC-leaf material and the insects did not distinguish between leaves of the two HC- and the $HC^{(-)}$-plants (Figure 5A). The total amount of leaf material consumed by beetles was lower (156.17 ± 36.40 mm^2) than that consumed by locusts (240.08 ± 80.72 mm^2), which can be explained by the larger body size of the latter.

In free-choice feeding trials with locusts, the amounts of cyanide present in extensively consumed leaf material of $HC^{(-)}$-genotype CV 2116 were substantially higher than in all other lima bean genotypes (Figure 5B). Incorporated amounts of cyanide were similar among the two HC- and two LC-accessions [post-hoc analysis (LSD: P<0.05) after one-way ANOVA]. This means that the much higher amount of consumed leaf tissue of low cyanogenic plants resulted in high incorporation of cyanide by locusts. Consequently, the consumption of large amounts of leaf tissue of the $HC^{(-)}$-genotype CV 2116 resulted in incorporation of extraordinarily high amounts of cyanide. Beetles, however, incorporated equal amounts of cyanide by feeding on leaves of the different accessions [post-hoc analysis (LSD: P<0.05) after one-way ANOVA; Figure 5B].

Cyanide balance in free-choice feeding trials. Measuring overall release of gaseous cyanide during feeding in free-choice experiments revealed significantly higher rates of cyanide released in response to feeding by beetles than by locusts (Mann–Whitney U-test: $Z = -3.868$, P<0.001; Figure 6A). Amounts of detected cyanide between both herbivore species differed significantly by a factor of 3.7.

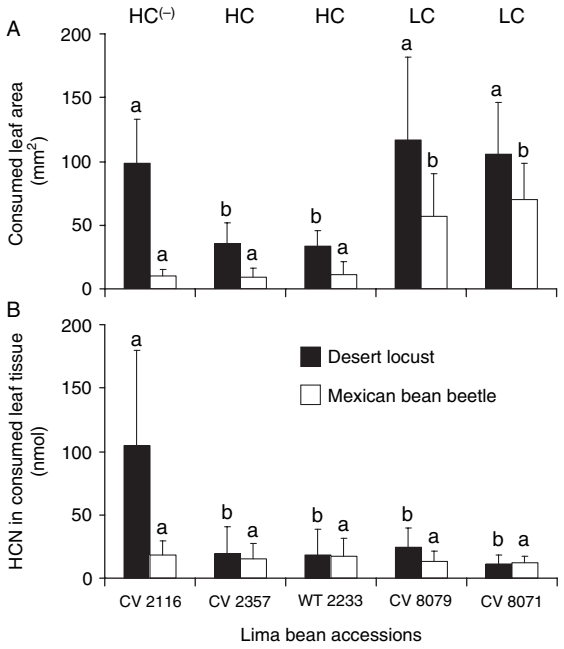

Figure 5 Leaf area and cyanide consumed in free-choice feeding trials. Leaf material of the various lima bean accessions was offered simultaneously to individual locusts and beetles. The amount of (A) consumed leaf area per accession was measured and the amount of (B) cyanide present in consumed leaf tissue of each accession was calculated. Values shown are mean + SD; n = 12 feeding trials per herbivore species. Different letters at columns within insect species represent significant differences among accessions [post-hoc analysis (LSD: $P<0.05$) after one-way ANOVA].

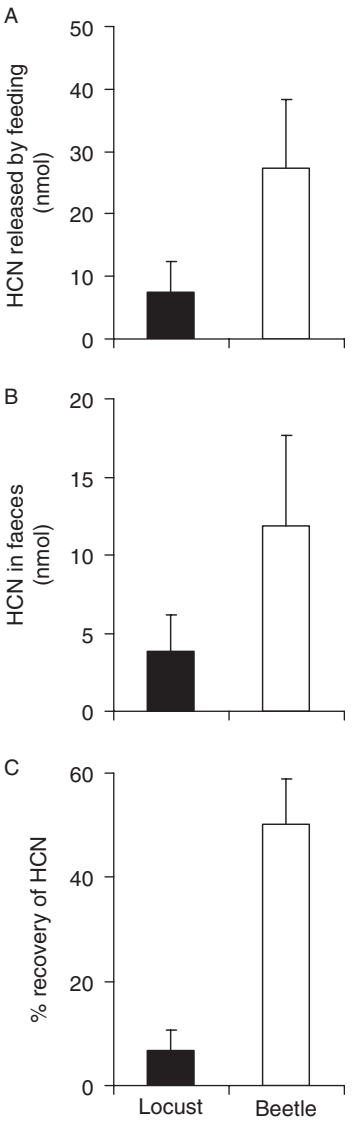

Figure 6 Cyanide balance in free-choice feeding trials. For generalist locusts and specialist beetles, the total amount of HCN that was (A) released during feeding and (B) excreted after feeding on simultaneously offered leaves of different lima bean accession was measured. The rate of recovered cyanide (%) was calculated (C). Values shown are mean + SD; n = 12 feeding trials per herbivore species. For all parameters analysed, differences between locusts and beetles were significant (Mann–Whitney U-test: $P<0.001$).

Furthermore, cyanide recovered from faeces was significantly higher for beetles than for locusts (Mann–Whitney U-test: $Z = -3.233$, $P<0.001$; Figure 6B). Amounts of cyanide detected in faeces of both herbivore species differed by a factor of 3.1.

Comparing the total recovery of gaseous HCN released during feeding and cyanide in faeces revealed significantly higher rates of recovered cyanide for beetles than for locusts (Mann–Whitney U-test: $Z = -4.157$, $P<0.001$). The proportion of recovered cyanide present in consumed leaf area in free-choice feeding trials differed by a factor of 7.6 (Figure 6C).

Behavioural responses in no-choice feeding trials. Observation of locusts in no-choice experiments revealed substantial differences in behaviour depending on the leaf material offered (Figure 7A). Locusts that consumed small amounts of leaves of HC-accessions showed almost no impairment (Figure 7A). Locusts that fed extensively on LC-plants showed changes in behaviour that we interpreted as symptoms of intoxication. The insects exhibited reduced movements, reduced sensitivity to external stimuli, and slowed down flight reactions. In addition, the insects showed a characteristic unnatural posture of antennae and legs (Figure 7A). Effects of intoxication were even more drastic for locusts feeding on the $HC^{(-)}$-genotype CV 2116. Here, 10 out of 12 insects died during the feeding

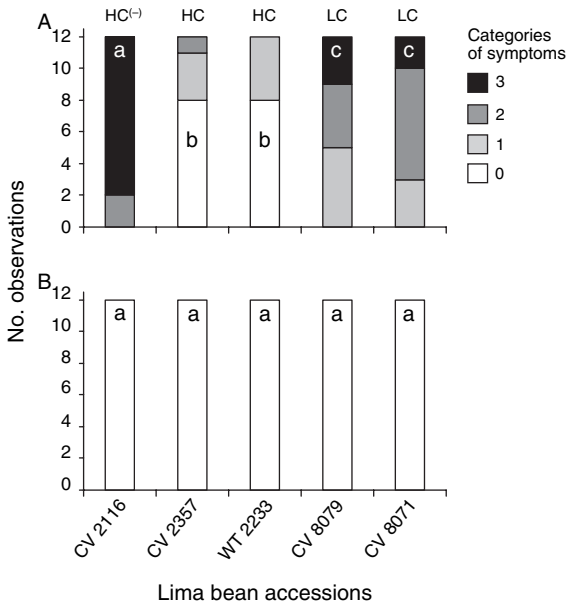

Figure 7 Behaviour of herbivores in no-choice feeding trials. Behaviour of (A) locust and (B) beetle as reaction to consumption of leaves of lima bean accessions with variable cyanogenic features ($HC^{(-)}$, HC, and LC) was documented and assigned to defined categories representing different levels of intoxication. Categories of intoxication ranged from 0 (= normal behaviour) to 3 (= strong impairment of herbivores). Letters in columns represent significant differences in behaviour among accessions [post-hoc analysis (LSD: $P<0.05$) after one-way ANOVA; n = 12 feeding trials per herbivore species].

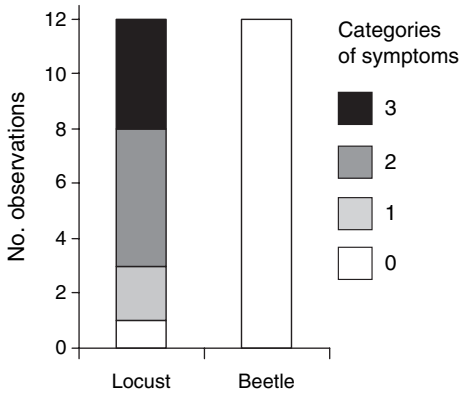

Figure 8 Behaviour of herbivores in free-choice feeding trials. Behaviour (n = 12 experiments per herbivore species) was documented and assigned to defined categories representing different levels of intoxication (0–3). The behaviour of locusts and beetles differed significantly (Mann–Whitney U-test: $P<0.001$).

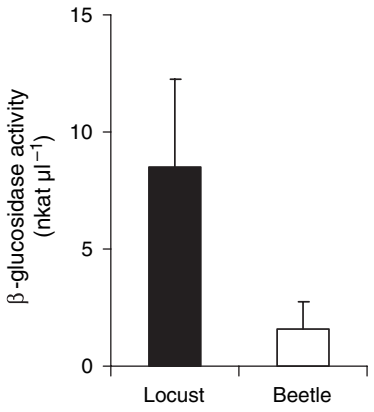

Figure 9 β-glucosidase activity in insect regurgitate. Values represent enzymatic activity per µl regurgitate (mean + SD; locusts and beetles each n = 24). The enzymatic activity of locusts and beetles differed significantly (Mann–Whitney U-test: $P<0.001$).

trials or within 60 min after the experiment. The surviving two insects showed symptoms of intoxication (category 2) that were comparable to those observed for locusts that were feeding on LC-genotypes.

Behavioural responses in free-choice feeding trials. In free-choice experiments (Figure 8), locusts consumed LC- and $HC^{(-)}$-leaf material at high rates and consequently showed similar symptoms of intoxication as observed in no-choice feeding trials (Figure 7A). In contrast to locusts, Mexican bean beetles showed no symptoms of intoxication, neither in no-choice nor in choice experiments (Figures 7B and 8). In free-choice feeding trials, behavioural responses of locusts and beetles were significantly different (Mann–Whitney U-test: $Z = -4.187$, $P<0.001$; Figure 8).

Activity of insect β-glucosidases. Locusts regurgitated 3.15 ± 0.83 µl while beetles regurgitated 0.97 ± 0.43 µl. The β-glucosidase activities in the regurgitates of locusts and beetles were significantly different (Mann–Whitney U-test: $Z = -5.724$, $P<0.001$; Figure 9). Enzymatic activity in the regurgitate of locusts was by factor 5.3 (n = 24 individuals per insect species) higher than in the regurgitate of beetles (Figure 9).

Discussion

Although common among wild and cultivated plant species (Jones, 1998; Møller & Seigler, 1999; Webber & Miller, 2008), the function of cyanogenesis in the interaction with herbivores is not fully understood (reviewed by Gleadow & Woodrow, 2002). While some herbivores are effectively repelled by plant cyanogenesis, others seem unaffected (e.g., Ferreira et al., 1997), or plant cyanide might even act

as phagostimulant rather than feeding deterrent (Fitzgerald et al., 2002). Functional analyses of cyanogenesis and plant defences are in general complicated by the variability of plant traits. In addition, feeding behaviour of herbivores with different adaptations towards cyanogenic plants is often an unknown source of variation (Burgess & Ennos, 1987; Engler et al., 2000). Here, to better understand the complex action of plant cyanogenesis in herbivore*plant interactions, we considered the variability of cyanogenic plant features and included herbivores with different degrees of specialization towards cyanogenic food plants in our experiments.

Lima bean plants used in this study exhibited distinct genotypic differences of cyanogenic features including cyanogenic potential (HCNp; concentration of cyanogenic glycosides), β-glucosidase activity, and cyanogenic capacity (HCNc; release of gaseous hydrogen cyanide per unit time). Following the predictions of the optimal defence hypothesis (McKey, 1974; Rhoades, 1979), different leaf developmental stages of each accession revealed ontogenetic variation in all cyanogenic parameters tested with highest values found in youngest leaves (Figures 1 and 2). The basic assumption of the optimal defence hypothesis is that three main factors – cost of defence, risk of attack and value of the respective plant organ – determine the allocation of defensive secondary metabolites (Rhoades, 1979; Stamp, 2003). The higher the risk of a given plant tissue being consumed by herbivores and the higher its value for plant fitness, the more energy that should be allocated to its defence (Zangerl & Bazzaz, 1992). According to the optimal defence hypothesis, within the total foliage of a plant, young leaves make a larger contribution to plant fitness than old leaves, as they have a higher potential photosynthetic value resulting from a longer expected life-time (Coley et al., 1985). In addition, younger leaves are often more nutritious and thus more attractive to herbivores and should be better defended (Anderson & Agrell, 2005). Our data on cyanogenic plant traits and on leaf soluble protein concentration, as a trait crucially affecting plant palatability to herbivores (Mattson, 1980; Ganzhorn, 1992), supported these assumptions.

In addition to visual cues, feeding insects recognize their food plants primarily on the basis of olfactory and gustatory cues (e.g., Fernandez & Hilker, 2007; Stenberg & Ericson, 2007). It is therefore essential to understand which cyanogenic plant features (HCNp or HCNc) are responsible for the perception of toxic food by a foraging herbivore. Results of our study clearly demonstrated that the relative importance of HCNp and HCNc as cues indicating food plant toxicity depend on the herbivore. Choice and no-choice experiments with generalist desert locusts showed that the insects used HCNc as chemical cue to identify highly cyanogenic food plants, whereas HCNp seemed to play a subordinate role, or no role at all. Plants with high HCNp but, because of minimal β-glucosidase activity, low HCNc (i.e., the $HC^{(-)}$-genotype CV 2116) were consumed extensively and seemed not to be perceived as toxic (Figures 3 and 5). In no-choice experiments, extensive consumption of these plants resulted in the death of most locusts. In contrast, plants characterized by high HCNp as well as HCNc (CV 2357 and WT 2233) showed strong repellent activity. Locusts feeding on these HC-accessions consumed only small amounts of leaf tissue and showed almost no symptoms of intoxication. Although intoxication was most apparent on $HC^{(-)}$-plants, also feeding on plants with low HCNp and low HCNc (LC-accessions) resulted in intoxication of locusts. This unexpected finding could be explained by balancing the cyanide intake of insects on the basis of HCNp, consumed leaf area, gaseous cyanide released during the feeding process, and non-cleaved cyanogenic precursors in the faeces (Figure 4A and B). The cyanide balance revealed that the amount of incorporated cyanide, i.e., cyanide that was released in the insects' bodies and, thus, lost in the balance, was significantly higher for LC-plants as for HC-plants (Figure 4C). Highest values of incorporated cyanide found for locusts feeding on $HC^{(-)}$-plants causally explained the observed patterns of intoxication. Choice experiments with locusts supported the interpretation that the insects could not identify low HCN-releasing plants as toxic. When given the choice, locusts preferred $HC^{(-)}$- and LC-plants over HC-plants and consequently showed similar symptoms of intoxication as observed in no-choice feeding trials. Our findings on the low ability of generalist desert locusts to tolerate incorporated cyanide or cyanogenic precursors are in contrast to observations by Mainguet et al. (2000), who observed a distinct tolerance of desert locusts to thioglucosides.

Feeding trials with specialist Mexican bean beetles revealed distinct differences in herbivores' responses to cyanogenic host plant features when compared with generalist locusts. In no-choice feeding trials, beetles consumed similar amounts of all five leaf materials offered (Figure 3A). The beetles obviously tolerated high cyanide concentrations, as they showed no symptoms of impairment during or after feeding on the various lima bean accessions. The tolerance of Mexican bean beetles to cyanogens in lima bean is in accordance to an earlier study by Nayar & Fraenkel (1963), in which even a phagostimulative role of cyanogenic precursors was reported.

Balancing the cyanide intake of beetles revealed that compared with locusts, significantly higher amounts of cyanide were released during the feeding process (Fig-

ure 4A). In addition, higher amounts of cyanide were excreted in faeces (Figure 4B). Actually, the amount of cyanide in faeces should be considered underestimated, as faeces deposited on leaves were not included into the analysis. Based on the cyanide balance, beetles appeared to metabolize substantially less cyanide or cyanogenic glucosides than locusts. The higher excretion rate of non-digested cyanogenic precursors by beetles compared with locusts might be explained by lower activity of endogenous β-glucosidases or conditions in the gastrointestinal tract that are unfavourable for activity of plant β-glucosidases ingested with leaf material. A central role of insects' β-glucosidase activity in determining the rate of metabolization and excretion of cyanide in the food is supported by the significantly lower β-glucosidase activity in the regurgitate of bean beetles compared with locusts (Figure 9). In other plant*herbivore systems, such physiological strategies of specialists to overcome toxic constituents of their host plants have been observed (e.g., El Sayed et al., 1996; Bernays & Minkenberg, 1997; Engler et al., 2000). Although the high excretion rate of cyanide by Mexican bean beetles could be nicely explained by their low β-glucosidase activity, the higher release of gaseous hydrogen cyanide during feeding of beetles compared with locusts seem contradictory to the low enzymatic activity in the beetle's saliva. However, this finding can be explained by the beetles' feeding mode (Gleadow & Woodrow, 2002). While beetles have small mandibles and chew leaf material intensively and thereby destroy a high percentage of cyanide-containing cells, locust nymphs consume leaves more quickly by 'cutting' leaf tissue with their relatively large mandibles rather than by chewing, and higher amounts of intact plant cells might be ingested.

In addition to physiological adaptations by the different activity of digestive enzymes, adaptive strategies to tolerate toxic plant compounds often include induced activity of detoxifying enzymes (Cohen et al., 1964; Wadleigh & Yu, 1988). In insects, different enzymatic activities involved in cyanide detoxification have been reported (Urbańska et al., 2002). Rhodanese (thiosulphate: cyanide sulphur transferase; EC 2.8.1.1) is a common enzyme that converts cyanide into the less toxic thiocyanate (Long & Brattsten, 1982; Jones, 1998). Although rhodanese may be involved in cyanide detoxification, in herbivorous insects (and plants) tolerance of insects to cyanogenic food is attributed mainly to β-cyanoalanine synthase (beta-CAS; EC 4.4.1.9) (Ahmad et al., 1986; Brattsten, 1992). This enzyme transfers cyanide into the amino acid pool by catalysing the first in a series of reactions. Another enzyme, β-cyanoalanine hydratase (EC 4.2.1.65) is also involved in this process and the sequential action of these two enzymes constitutes an efficient mechanism for detoxification of cyanide (Brattsten, 1992). In which way Mexican bean beetles exactly cope with the ingested cyanogenic β-glucosides remains elusive. Most importantly, observations in our study clearly reveal that adapted beetles are less affected by cyanide than generalist locusts.

In choice experiments, however, beetles preferred LC- over HC- and $HC^{(-)}$-plants, while they made no differentiation between HC- and $HC^{(-)}$-plants. When having the choice, Mexican bean beetles avoided food plants with high cyanogenic potential while, in contrast to locusts, the cyanogenic capacity did not serve as an essential cue for host plant selection. This appears reasonable, as long-term studies with Mexican bean revealed that fitness was reduced in beetles that had been feeding on plants with high HCNp for several generations (Ballhorn et al., 2007).

In the present study, we could demonstrate different effects of HCNp and HCNc on generalist and specialist insect herbivores. The question may arise whether the observed effects really can be attributed to variation in plant cyanogenic features or whether other plant parameters also affected the interaction of both herbivores with lima beans. In general, plants express a broad array of defensive and nutritive traits determining the overall palatability to herbivores (e.g., Walling, 2000). However, for lima beans there appear to be no other co-varying physical or chemical factors besides cyanogenic traits that could explain the observed effects (Ballhorn et al., 2007, 2010). Soluble protein concentration, for example, was highly homogeneous among accessions depending on leaf stage (Figure 1D). Other leaf parameters known to affect attractiveness to herbivores, such as total phenolics, leaf toughness and tissue hydration have been demonstrated to be similar in young leaves among lima bean genotypes used in previous studies (Ballhorn et al., 2007). Thus, attractiveness of leaves to herbivores observed in our study can most likely be attributed to variation in cyanogenic features rather than to variation of other defensive or nutritive parameters.

Understanding the basic mechanisms affecting the outcome of plant interactions with differently adapted herbivores has a key position in ecology research. Results presented in this study provide new insights in the function of cyanogenic potential and cyanogenic capacity as two parameters forming the widely distributed trait 'cyanogenesis'.

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