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Composition of alkaloids in different box tree varieties and their uptake by the box tree moth *Cydalima perspectalis*

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Abstract Larvae of the moth *Cydalima perspectalis* are specialized on box trees (*Buxus* spp.). Native to eastern Asia, the moth has been introduced to Europe in 2007 and is nowadays causing severe damage to box trees in private and public gardens, as well as in semi-natural box tree forests. Box trees contain highly toxic triterpenoid alkaloids which may be sequestered by specialized herbivores such as *C. perspectalis.* We determined the alkaloid composition in leaves of the five most common box tree varieties in Europe belonging to two *Buxus* species using liquid chromatography–mass spectrometry (LC–MS) metabolite profiling. We also examined whether larvae and moths of *C. perspectalis* accumulate alkaloids from the different box tree varieties. The differences in alkaloid composition observed between the box tree species *Buxus sempervirens* and *Buxus microphylla* were mirrored in the tissue of *C. perspectalis* larvae fed on either of the different box tree species, indicating uptake of alkaloids. The larvae stored large amounts of dibasic alkaloids in their body, while monobasic alkaloids were metabolized and/or excreted. Newly emerged adult moths contained no traces of alkaloids.

Keywords *Buxus* · Lepidoptera · Chemical defence · Triterpenoid alkaloids · Plant–insect interactions · UHPLC–QTOFMS

Introduction

Many secondary plant metabolites are produced by plants as toxins that deter herbivores, but they may also have an important effect on the next trophic level, i.e. on predators and parasitoids of herbivores (Schaffner et al. 1994). Sequestration of these chemical compounds occurs in a wide array of herbivores (Rothschild 1972; Blum 1981; Bernays and Graham 1988; Rowell-Rahier and Pasteels 1992; Opitz and Müller 2009). Alkaloids are a particularly important class of plant toxins occurring in many different plant taxa (Hegnauer 1988) and are well suited for sequestration by insect herbivores for their own defence against predators due to their high level of deterrence (Rothschild et al. 1979; Blum et al. 1981; Gfeller et al. 1995). In this study, we investigated whether the invasive box tree moth *Cydalima perspectalis* takes up alkaloids occurring in its host plant.

Cydalima perspectalis (Walker 1859) (Lepidoptera: Crambidae) (formerly *Diaphania* or *Glyphodes perspectalis*, see Mally and Nuss 2010) is a new alien species in Europe, causing severe damage to box trees (*Buxus* spp.) in private and public gardens as well as in semi-natural box tree forests in the region of Basel (Switzerland), south-western Germany, France and the Netherlands since 2007 (Krüger 2008; Feldtrauer et al. 2009, Leuthardt et al. 2010; van der Straten and Muus 2010). Native to Korea, Japan and China (Inoue 1982), this moth has rapidly spread in Europe over the past 5 years. The larvae of *C. perspectalis* feed on leaves, but can also attack the bark of the trees,

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causing them to dry out and die. The light green larvae have black stripes with white dots and hairs, which might be a form of aposematic colouring, warning of certain toxicity. The larvae hatched from one single egg cluster spread over an area of 20–25 cm diameter on a tree until pupation. This is easily visible by the feeding damage, especially when only a few egg clusters have been deposited, as well as by the conspicuous behaviour of the larvae which do not hide under the leaves or inside the tree during daytime from the third larval stage on (Figure in Online Resource 1).

The genus *Buxus* belongs to the family Buxaceae, morphologically considered as one of the most primitive angiosperms, and comprises about 90 species occurring in most tropical regions and in the Mediterranean area (Balthazar et al. 2000). More than 15 box tree species or subspecies, including *Buxus microphylla* var. *japonica* and *B. microphylla* ssp. *sinica*, occur in the native range of *C. perspectalis* (Balthazar et al. 2000). Larvae of the invasive *C. perspectalis* are specialized on *Buxus*. In their native range in Japan, they have been reported to feed on *B. microphylla* in Japan (Maruyama 1993) and on *B. microphylla* ssp. *sinica* in China (Chen et al. 1993). Larvae of *C. perspectalis* were reared on *B. microphylla* var. *japonica* and *B. microphylla* var. *insularis*, as well as on *B. sempervirens* under laboratory conditions (Maruyama 1993).

Invasive herbivores may encounter known food plants in the introduced region, but often they are also successful in adjusting their diet to new plant species, related species or varieties of the original plant (Thompson and Pellmyr 1991). In Europe, there are several horticultural breeds of *Buxus* sp. that do not occur in Eastern Asia, particularly varieties of *B. sempervirens*, the native European box tree species which does not occur in high densities in the native range of *C. perspectalis*. Box tree moth-inflicted damage has been recorded on all box tree varieties examined in Europe (Leuthardt and Baur 2013), indicating a successful adjustment of the diet of the invader from *B. microphylla* to *B. sempervirens*.

Birds were not observed to feed on *C. perspectalis*, although encounters between this potential predator (especially *Parus* spp.) and larvae have been observed in the field (Leuthardt, unpublished data). On the rare occasions where larvae were picked up by birds, they were either killed and left aside or regurgitated. Field observations indicate that bird predators avoided not only the larvae but also the adults. A possible explanation could be that the moth accumulates toxic compounds from box tree leaves. Chemical compounds of the family Buxaceae have been the subject of numerous pharmacological studies in the past decades because of their biological activity as cholinesterase inhibitors (Choudhary et al. 2003), and antibacterial (Naeem et al. 1996; Mothana and Lindequist 2004), antiviral (Mothana et al. 2006) and antileishmanial activities (Devkota et al. 2008). Alkaloids constitute one of the main classes of defence secondary compounds found in Buxaceae. *Buxus* alkaloids are a unique class of triterpenoid alkaloids possessing a cycloartenol-type skeleton in which one or two nitrogen atoms are incorporated as side chains (Dildar 1990). The diversity of *Buxus* triterpenoid alkaloids is extremely rich with more than 300 different structures reported (Devkota et al. 2008). The concentration of the main alkaloids is virtually the same in all plant organs (1–2 % dry weight, DW), increasing with the age of the box trees (Khodzhaev and Shakirov 2000). Previous studies have focused essentially on wild-growing *Buxus* species. The composition of secondary compounds in horticultural breeds of both *B. sempervirens* and *B. microphylla* has, to our knowledge, never been investigated.

To determine whether the specialization of *C. perspectalis* on *Buxus* species is linked to the potential defence system of the larvae against vertebrate predators, we investigated the alkaloid content of *Buxus* leaves and *C. perspectalis* larvae using liquid chromatography–mass spectrometry (LC–MS). We addressed the following questions: (1) Do five common box tree varieties found in central Europe differ in their alkaloid composition? (2) Do larvae of *C. perspectalis* store *Buxus* alkaloids and are the variety-specific alkaloid compositions in box tree leaves mirrored in the tissue of larvae and moths fed on either of those box tree varieties?

Methods and materials

Plant and insect material

Five varieties of box trees belonging to two species were examined: *B. sempervirens* var. "Sempervirens" (further referred to as SE), *B. sempervirens* var. "Rotundifolia" (RO), *B. sempervirens* var. "Argenteovariegata" (AR), *B. sempervirens* var. "Aureovariegata" (AU) and *B. microphylla* var. "Faulkner" (FA). These varieties are the most frequently planted in public and private gardens in northwestern Switzerland. One 10- to 15-year-old tree of each variety (1–1.8 m high) growing outside the distribution area of *C. perspectalis* until 2010 (Lyss, 60 km SW of Basel) and not treated with phyto-pharmaceutical substances (pesticides, herbicides, fungicides or other substances) was used. Replicate trees were not available because they were already treated with chemicals against *C. perspectalis*. The experimental trees were kept outdoors and protected with nets against oviposition by *C. perspectalis* from 2010 to 2011. From each variety, we collected four samples of 1-year-old leaves from different branches, each consisting of five to six first-year leaves

representing 200–300 mg fresh mass in May 2011. To compare young and old leaves, we collected one leaf sample of 2- to 3-year-old leaves on the cultivated *B. sempervirens* var. "Sempervirens".

Newly hatched larvae of *C. perspectalis* were obtained from a culture maintained in an outdoor cage (1 m × 1 m × 2 m, wooden structure covered with netting) containing a wild *B. sempervirens* tree of 1.2 m height. The founders of the culture were captured with a light trap positioned near a box tree hedge in Riehen, 5 km NE of Basel, Switzerland (47°33′45″N, 7°38′27″E) between 23 June and 21 July 2010. Larvae were kept in groups of three to five individuals in 90 mm Petri dishes lined with moist filter paper to prevent the food from drying out. Each group was fed with only one of the five examined box tree varieties from the time of hatching. We used a sibling-split design, equally distributing larvae from the same egg cluster on all five varieties to minimize potential genetic influences. In total, ca. 100 larvae from five different females were obtained with this culture. Equal proportions of young and old leaves cut from the corresponding box tree variety were offered ad libitum as food. Food was replaced every day.

Extraction of alkaloids

We examined four larvae of the sixth larval stage kept on each of the five box tree varieties as well as eight larvae of the fourth larval stage kept on *B. sempervirens* var. "Sempervirens". Individuals were dissected and their gut was removed to exclude any influence of remaining plant material. We also examined the faeces of larvae growing on *B. sempervirens* var. "Sempervirens". Similarly, eight newly emerged adult moths were collected and analysed shortly after hatching. Each of the four replicates of plant and larvae samples as well as the unique sample of faeces and moth were lyophilized and ground into a fine powder using a mortar and pestle. 25 mg of dry powder was transferred to a microcentrifuge tube and extracted in 1 mL of a mixture of methanol:water:formic acid (70:29.5:0.5, v/v) using Eppendorf Thermomixer 5436 (Eppendorf, Hamburg, Germany). After centrifugation at 16,000 rpm for 3 min (Eppendorf 5415 R), the supernatant was collected and diluted ten times with the same extraction solvent.

Mass spectrometry analyses

Chemical analyses were carried out by ultra-high pressure liquid chromatography–quadrupole time-of-flight mass spectrometry (UHPLC–QTOFMS) in the electrospray positive ionization mode using an Acquity UPLC system (Waters, Milford, USA) and a Synapt G2 QTOF mass spectrometer (Waters). The separation was performed on an Acquity BEH C18 column (50 × 2.1 mm i.d., 1.7 μm particle size; Waters) at 400 μL/min under the following conditions: solvent A, 0.05 % formic acid in water; solvent B, 0.05 % formic acid in acetonitrile; 2–40 % B in 5 min, 40–100 % B in 2 min, 100 % B for 1 min, re-equilibration at 2 % B for 1 min. The temperature of the column was maintained at 25 °C and that of the autosampler at 15 °C. The injection volume was 2.5 μL. QTOFMS parameters were: electrospray capillary voltage +2.8 kV, cone voltage +25 V, desolvation gas temperature 350 °C, desolvation gas flow 800 L/h, mass range 85–1,200 Da, scan time 0.2 s. The analyses were carried out in the MS^E mode using alternating scans at low (4 eV) and high (10–30 eV ramp) collision energies. Data were recorded using Masslynx 4.1 (Waters).

Except for cyclovirobuxine D, whose identification was ascertained by comparison with an authentic standard (Chengdu Biopurify Phytochemicals LTD, Chengdu, China) run under identical conditions, we tentatively identified the alkaloids based on high-resolution mass spectra of pseudo-molecular and fragment ions obtained by QTOFMS in the absence of available standards. For the determination of elemental compositions, the following elements were included: C (max. 500), H (max. 1,000), O (max. 200), N (max. 5), S (max. 6), P (max. 1) and Na (max. 1). Mass accuracy tolerance was set to 3 ppm, ring plus double bond equivalents limits were −1.5 to 50 and the nitrogen rule was applied. Accurate measurements of masses and isotopic ratios provided unique elemental compositions for all ions investigated with an excellent degree of confidence. Compound databases including the *Dictionary of Natural Products* (Chapman & Hall, CRC Informa, London; version 20.2) were investigated to match the found elemental compositions with potential alkaloids. MS/MS spectra obtained by collision-induced dissociation (CID) were interpreted for additional structural information.

We measured the concentration of cyclovirobuxine D in *Buxus* leaves and *C. perspectalis* larvae relative to the initial dry mass weighed for the respective tissues before extraction (i.e. 25 mg of dried tissue for both leaves and caterpillars) using external calibration. The concentrations of the calibration points were 0.02, 0.2, 1.0, 2.0, and 5.0 μg/mL. For other alkaloids, relative concentrations were measured.

Peak picking of LC–MS data was performed by Markerlynx XS (Waters) using the following parameters: initial and final retention times 0.5–7.2 min, low and high masses 85 and 1,200 Da, mass window 0.03 Da, retention time window 0.1 min, intensity threshold 1,000 counts, automatic peak width measurement, automatic peak-to-peak baseline noise, noise elimination disabled, deisotoping

function enabled. This yielded a list of 'features' (i.e. variables characterized by mass and retention time). The obtained data were then normalized to the total ion intensity, Pareto scaled, and subjected to multivariate analysis using EZinfo (Umetrics, Umea, Sweden).

Results

Determination of alkaloids in plant tissues

UHPLC–QTOFMS analyses revealed that numerous alkaloids were present in the four varieties of *B. sempervirens* (Fig. 1a–d) as well as in *B. microphylla* (Fig. 1e). A list of the main alkaloids tentatively identified in both species is presented in Table 1. Compound **1**, which was present only in *B. sempervirens* but not in *B. microphylla*, was identified as cyclovirobuxine D based on an $(M + H)^+$ ion at *m/z* 403.3687 ($C_{26}H_{47}N_2O$) and a doubly charged ion at *m/z* 202.1886 typical of alkaloids containing two basic moieties (Fig. 2a). In the MS/MS spectrum, the loss of $-NH_2CH_3$ gave an intense fragment at *m/z* 372.3269 ($C_{25}H_{42}NO$). Another typical fragment at *m/z* 330.2795 ($C_{22}H_{36}NO$) resulted from the concomitant losses of $-CHCH_3NHCH_3$ and $-CH_3$ from the singly charged pseudo-molecular ion. The structure of cyclovirobuxine D (Fig. 3) was confirmed by injecting a pure standard under identical conditions. Fig. 2b, c shows the almost identical MS/MS spectra of compound **1** and the cyclovirobuxine D authentic standard. Following a similar scheme, compound **2** was putatively identified as either buxaminol E or cyclobuxine B, based on an $(M + H)^+$ ion at *m/z* 401.3524 ($C_{26}H_{45}N_2O$) and a doubly charged ion at *m/z* 201.1801 (Fig. 4a). The characteristic fragment at *m/z* 384.3257 ($C_{26}H_{42}NO$) in the MS/MS spectrum resulting from a loss of $-NH_3$ (primary amine) suggested the presence of buxaminol E, which contains both primary and tertiary amines (Figs. 3, 4b), rather than cyclobuxine B, which contains secondary and tertiary amines. Compound **10** was annotated as cyclobuxophylline O (Fig. 3) based on a pseudo-molecular ion at *m/z* 356.2951 corresponding to the molecular formula $C_{24}H_{38}NO$ (Fig. 4c), and a characteristic fragment ion at *m/z* 339.2685 ($C_{24}H_{35}O$) in the MS/MS spectrum resulting from the loss of $-NH_3$ (Fig. 4d). Compound **11** (*m/z* 370.3114, $C_{25}H_{40}NO$) was putatively identified as cyclobuxophylline M, an *N*-methyl derivative of **10** (Fig. 3): the fragment at *m/z* 339.2685 resulting from a loss of $-NH_2CH_3$ also occurred in the corresponding MS/MS spectrum.

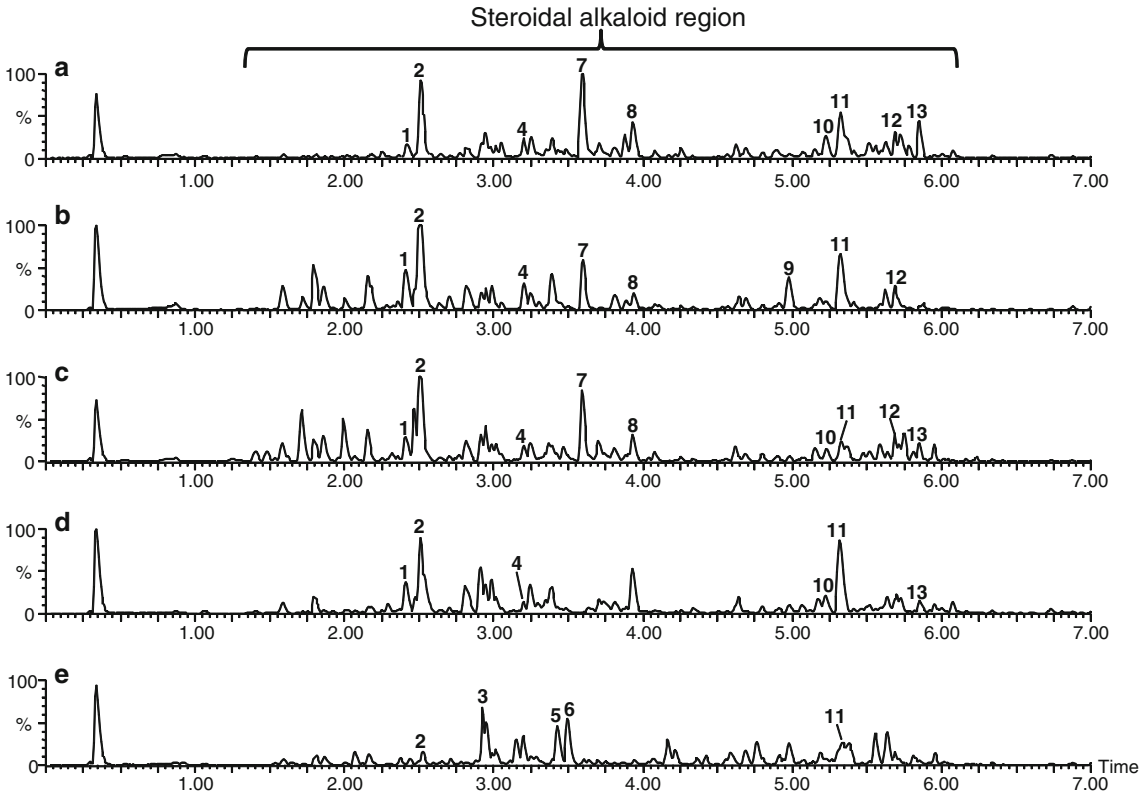

Fig. 1 Chromatograms of leaves of the five most common box tree varieties in the region of Basel, Switzerland. **a** *Buxus sempervirens* var. "Sempervirens"; **b** *B. sempervirens* var. "Rotundifolia"; **c** *B. sempervirens* var. "Argenteovariegata"; **d** *B. sempervirens* var. "Aureovariegata"; **e** *B. microphylla* var. "Faulkner"

Uptake of alkaloids by *C. perspectalis*

To determine whether *C. perspectalis* larvae feeding on box tree store alkaloids, we analysed their tissue and excrements using the same profiling approach as used for the plant material. We compared the alkaloid profile of young leaves of all five examined varieties with those of sixth instar larvae ($n = 4$) fed on either of these varieties. To receive more detailed information on the variability among samples than we could derive from visual inspection of individual chromatograms, chemometric tools were employed. A list of more than 3,000 'features' was obtained by peak picking and condensed using principal component analysis (PCA). The PCA separated the profiles of *Buxus* spp. leaves from those of larvae fed on the corresponding leaves along the first PC axis (variance explained 37 %, Fig. 5). Furthermore, the alkaloid profiles of larvae fed on either *B. sempervirens* or *B. microphylla* were separated along the second PC axis (variance explained 17 %). Interestingly, even the subtle differences in alkaloid profiles between the four *B. sempervirens* varieties were reflected in larval profiles (inset in Fig. 5). In the loadings plot, the four most contributive features to the discrimination between *B. microphylla* and *B. sempervirens* leaves were compounds **2** and **7** (typical for *B. sempervirens*) and **3** and **6** (typical for *B. microphylla*) (see loadings in Online Resource 2). However, this difference was only partly maintained in larval profiles. Indeed, although compounds **2** and **7** were still among the main contributive features to larvae feeding on *B. sempervirens*, three less dominant alkaloids from *B. microphylla* leaves became the most characteristic features of larvae feeding on *B. microphylla* (see loadings in Online Resource 2). One was tentatively identified as dihydrocyclomicrophylline F ($C_{26}H_{46}N_2O_2$) based on its MS/MS spectrum, but the two others ($C_{28}H_{48}N_2O_2$ and $C_{28}H_{48}N_2O$) could not be fully identified because of several possible isomeric derivatives reported for these molecular formulae in the literature. Yet, larval profiles could also be separated only based on the 13 predominant alkaloids identified in *Buxus* leaves (Online Resource 3). Corroborating these observations, cyclovirobuxine D accumulated only in caterpillars that fed on *B. sempervirens* leaves, while it was absent in those feeding on *B. microphylla*.

More generally, larvae stored only dibasic alkaloids (i.e. containing two basic amine groups), while monobasic alkaloids (i.e. containing only one amine group) were metabolized and/or eliminated in their excrements (Fig. 6). In particular, an intense peak of *m/z* 388.3209 eluting at 3.98 min was discovered in the excrements of larvae feeding on *B. sempervirens* (Fig. 6c). This compound could possibly be formed from the predominant compound **11**, tentatively identified as cyclobuxophylline M, by ketone reduction and hydroxylation.

A more detailed analysis of the concentration of dibasic cyclovirobuxine D revealed marked differences between young (340 µg/g DW) and old (2,090 µg/g DW) *B. sempervirens* leaves as well as between larvae of different instars. The cyclovirobuxine D concentrations in larvae of the fourth and sixth instar reared on a mixture of old and young leaves were 540 and 250 µg/g DW, respectively, indicating a strong accumulation of this alkaloid in body tissues. Even more spectacular was the heavy

Table 1 List of predominant alkaloids tentatively identified in *Buxus* spp.

#	RT (min)	$(M + H)^+$	Formula	Species	Tentative identification
1	2.40	403.3690	$C_{26}H_{46}N_2O$	*semp.*	Cyclovirobuxine D[a]
2	2.51	401.3524	$C_{26}H_{44}N_2O$	*semp.*	Buxaminol E
3	2.92	599.4050	$C_{35}H_{54}N_2O_6$	*micro.*	Buxmicrophylline H
4	3.20	372.2903	$C_{24}H_{37}NO_2$	*semp.*	Buxtauine M
5	3.43	549.4046	$C_{35}H_{52}N_2O_3$	*micro.*	Cyclomicrophyllidine A
6	3.50	523.3901	$C_{33}H_{50}N_2O_3$	*micro.*	Buxepidine
7	3.63	497.4102	$C_{32}H_{52}N_2O_2$	*semp.*	O-tigloylcyclovirobuxeine B
8	3.88	519.3944	$C_{34}H_{50}N_2O_2$	*semp.*	O30-benzoyl-16-deoxybuxidienine C
9	5.00	354.2802	$C_{24}H_{35}NO$	*semp.*	Cyclosuffrobuxinine M
10	5.23	356.2951	$C_{24}H_{37}NO$	*semp.*	Cyclobuxophylline O
11	5.32	370.3114	$C_{25}H_{39}NO$	*semp.*	Cyclobuxophylline M
12	5.68	547.3899	$C_{35}H_{50}N_2O_3$	*semp.*	Buxadienine/*N*-Benzoyl-*O*-acetylbuxodienine E
13	5.85	547.3893	$C_{35}H_{50}N_2O_3$	*semp.*	Buxadienine/*N*-Benzoyl-*O*-acetylbuxodienine E

RT Retention time

semp. sempervirens, *micro. microphylla*

[a] confirmed by an authentic standard

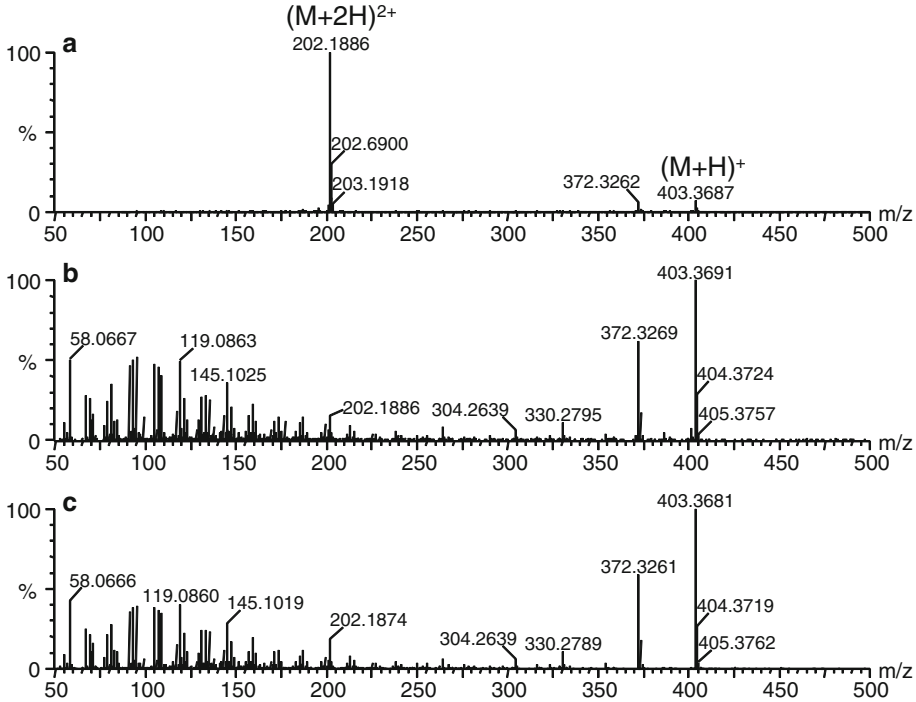

Fig. 2 High-resolution mass spectra obtained for cyclovirobuxine D in *B. sempervirens* extract and comparison with an authentic standard. **a** Mass spectrum obtained at low collision energy from *B. sempervirens* extract, **b** mass spectrum obtained at high collision energy from the same extract, **c** mass spectrum obtained at high collision energy for the authentic standard of cyclovirobuxine D

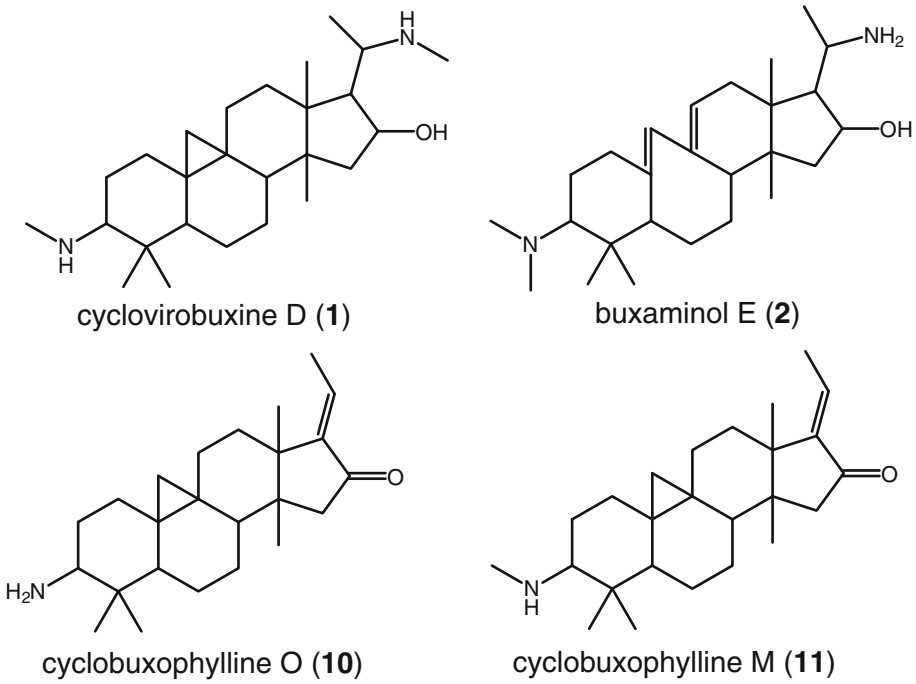

Fig. 3 Structures of triterpenoid alkaloids cyclovirobuxine D, buxaminol E, cyclobuxophylline O and cyclobuxophylline M. Numbers in *brackets indicate* the respective numbering in Table 1

sequestration of putative dihydrocyclomicrophylline F and the two other unidentified alkaloids ($C_{28}H_{48}N_2O_2$ and $C_{28}H_{48}N_2O$) whose relative amounts were 1.5 to 20 times higher in larval bodies than in *B. microphylla* (Fig. 7). In contrast, the relative amount of monobasic putative cyclobuxophylline M in larvae represented less than 0.5 % of that found in *B. sempervirens* leaves. Finally, the adult moths examined did not contain any traces of alkaloids, indicating that alkaloids do not persist after metamorphosis.

Discussion

This study is, to our knowledge, the first to investigate the uptake of *Buxus* alkaloids in the highly specialized larvae

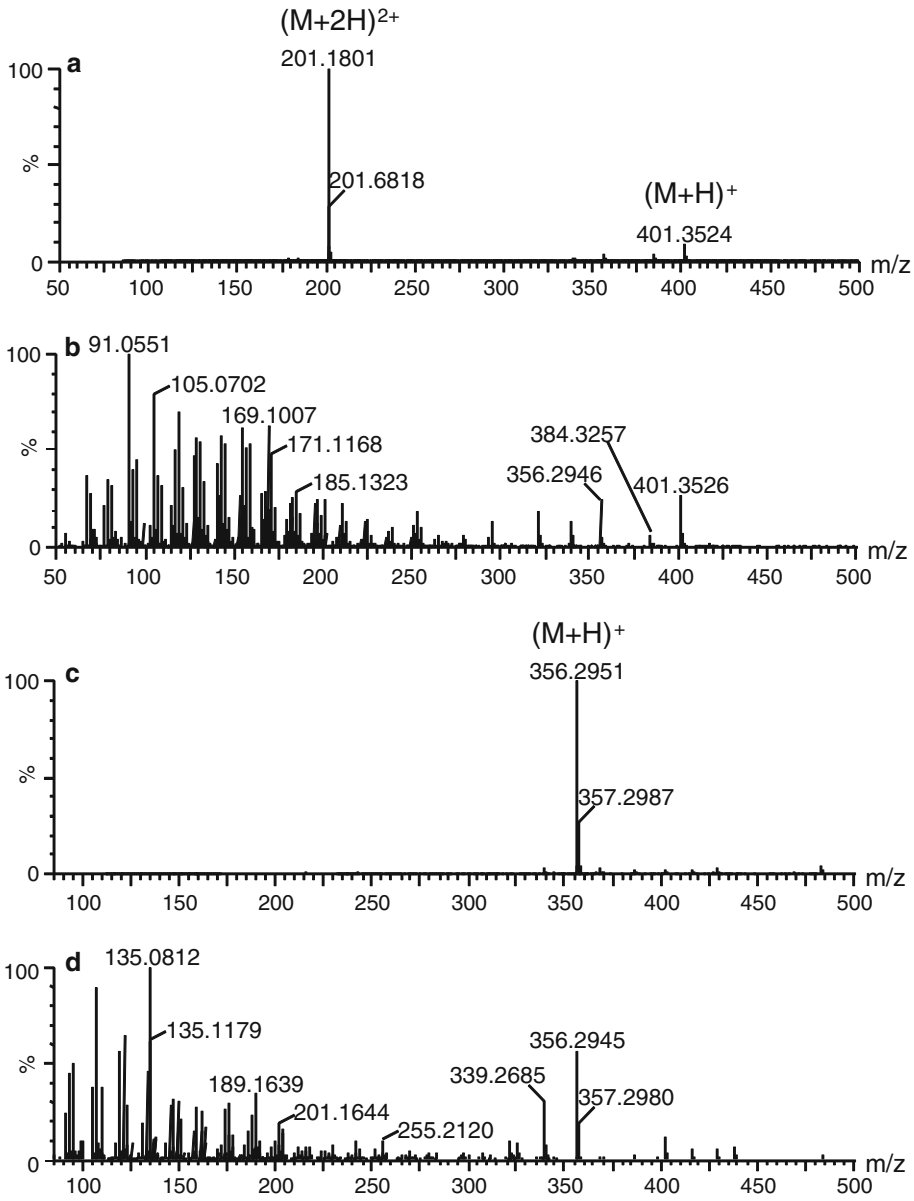

Fig. 4 Representative mass spectra of tentatively identified buxaminol E (compound **2**) and cyclobuxophylline O (compound **10**). **a** Mass spectrum of **2** obtained at low collision energy. **b** Mass spectrum of **2** obtained at high collision energy. **c** Mass spectrum of **10** obtained at low collision energy. **d** Mass spectrum of **10** obtained at high collision energy

of the moth *C. perspectalis*. The fact that alkaloid profiles of a box tree variety were mirrored in the tissue of larvae feeding on its leaves suggests that larvae of *C. perspectalis* store alkaloids. A detailed analysis of the absolute or relative amounts of specific alkaloids in *Buxus* leaves and larvae confirmed this hypothesis. This may explain why larvae of *C. perspectalis* are avoided or regurgitated by birds that commonly feed on Lepidoptera (F. Leuthardt, unpublished data). Specialized herbivores such as the sawfly *Rhadinoceraea nodicornis* are able to metabolize toxic polycyclic alkaloids and store them for their own predator defence (Schaffner et al. 1994). So far, the accumulation of triterpenoid alkaloids by Lepidoptera has not been investigated, in contrast to that of other alkaloid types such as pyrrolizidine alkaloids (PA), quinolizidine alkaloids and tropane alkaloids (Nishida 2002; Opitz and Müller 2009).

Insects sequestering plant secondary compounds often specialize on a specific group within a class of compounds (Rowell-Rahier and Pasteels 1992). The uptake of triterpenoid alkaloids by the box tree moth seems to follow this pattern. We found that the levels of certain alkaloids in larvae feeding on *B. microphylla* were up to 20 times higher than those present in the corresponding leaves. A number of possible explanations can be suggested for the selective sequestration of given alkaloids. First, there may be a trade-off between storing the compounds most effective for defence and the potential of these toxins to harm the insect itself. Indeed, feeding on suboptimal food sources such as toxic plants may reduce individual growth rate,

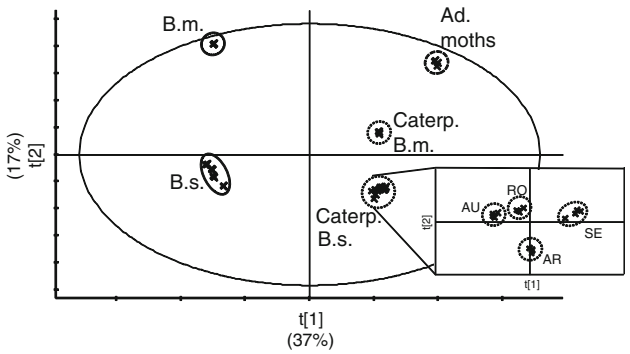

Fig. 5 Principal component analysis (PCA) of leaves of the five box tree varieties (B.s. respectively B.m.) and sixth instar caterpillars fed on the corresponding varieties (Caterp. B.s. respectively Caterp. B.m.), as well as adult moths from larvae fed on *Buxus sempervirens* var. "Sempervirens" ($n = 4$ replicates per variety). t[1] and t[2] are first and second principal components, with their percentage of variance explained. Inset: PCA based on larvae fed on the four different varieties of *B. sempervirens* (AR, *B. s.* var. "Argenteovariegata"; AU, *B. s.* var. "Aureovariegata"; RO, *B. s.* var. "Rotundifolia"; SE: *B. s.* var. "Sempervirens")

but this might be outweighed by a lower mortality due to avoidance by predators, provided that the food source presents either a protected feeding site or contains secondary compounds which can be sequestered (Damman 1987). However, comparative data on the toxicity of the box tree alkaloids for the sequestering insect and for its predators are not yet available. Second, there might be chemical and physical properties such as lipophilicity that make some compounds easier to diffuse through the gut membrane (Rowell-Rahier and Pasteels 1992). Indeed, compounds that are highly polar or highly hydrophobic cannot easily cross biological membranes passively and thus are expected to be more difficult to absorb unless they can be actively transported by specific carriers (Duffey 1980). Additionally, the pH in the insect gut may play an important role in their absorption. The pH of the midgut of Lepidoptera is usually very alkaline, exceeding 12 in certain species (Dow 1984). At such high pH, triterpenoid alkaloids are certainly deprotonated, and hence particularly hydrophobic. Dibasic *Buxus* alkaloids are typically more polar than monobasic alkaloids (as confirmed by their elution order in reverse-phase HPLC). It is therefore conceivable that, under the pH conditions in the midgut of *C. perspectalis*, dibasic alkaloids, unlike monobasic alkaloids, are within the acceptable range of polarity for passive diffusion through the cell membranes. Alternatively, it is possible that specific carrier transport favours the uptake of polar dibasic alkaloids over monobasic alkaloids.

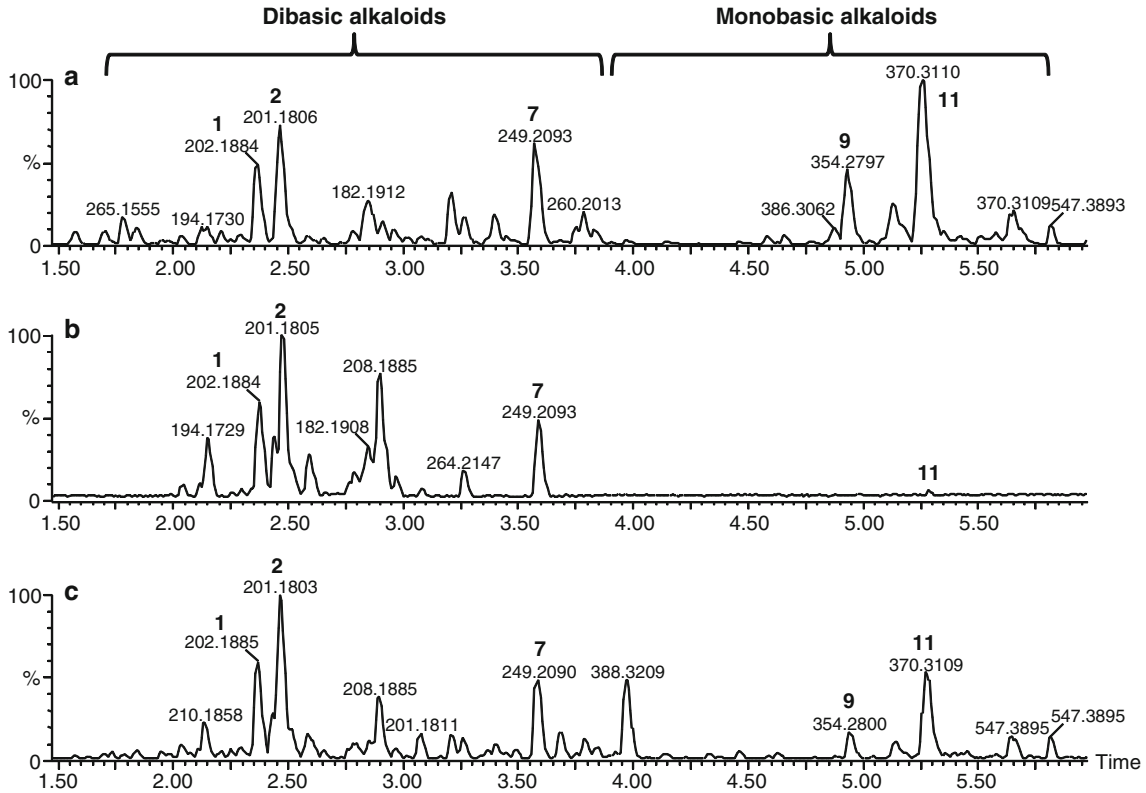

Fig. 6 Chromatograms of **a** 1-year-old leaves of *Buxus sempervirens* var. "Sempervirens", **b** larval tissue and **c** excrements of larvae. Predominant peaks are labeled according to their numbers in Table 1 and/or *m/z* base peak, either singly charged (e.g. compounds **9** and **11**) or doubly charged (e.g. compounds **1**, **2** and **7**)

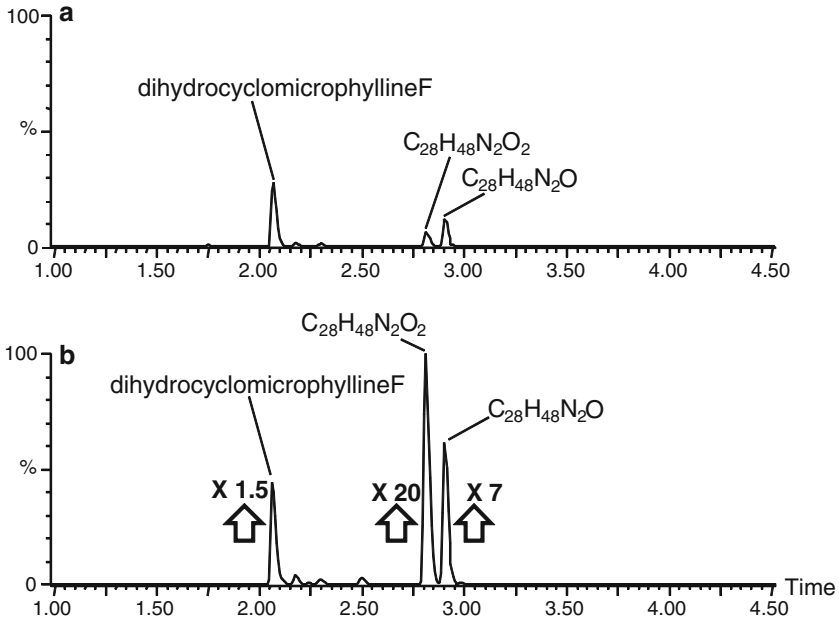

Fig. 7 Extracted ion chromatograms (EIC) showing a strong relative increase in the levels of three specific alkaloids in larvae compared to *B. microphylla* leaves. **a** EIC obtained from *B. microphylla* leaf extract, **b** EIC obtained from *C. perspectalis* extract

We observed a strong difference in cyclovirobuxine D concentration between young and old larvae and leaves: younger instars contained a twofold concentration of cyclovirobuxine D related to last-instar larvae, whereas the concentration of this alkaloid in box tree leaves was sixfold higher in 2- to 3-year-old leaves compared to 1-year-old leaves. This suggests a preference of young larvae for leaves containing a high concentration of alkaloids, i.e. older leaves. Indeed, under natural conditions, young larvae tend to feed on old leaves at the bottom of box tree plants (F. Leuthardt, unpublished data). This behaviour permits a fast uptake of large amounts of alkaloids within a short time to increase protection against predators, before maximizing the larval growth rate. However, this observation remains to be confirmed experimentally.

Newly emerged individuals prevented from any contact with box trees did not contain any traces of alkaloids. Nevertheless, field observations indicated that bird predators avoided not only the larvae, but also the adults. This suggests that adults become deterrent at a later time, which could be explained by a certain level of pharmacophagy. It is well known that several lepidopteran species sequester PA as larvae or obtain them pharmacophagously as adults (Nishida, 2002), but there are no data on this phenomenon concerning triterpenoid alkaloids. This hypothesis remains to be investigated.

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