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Gut pH, redox conditions and oxygen levels in an aquatic caterpillar: Potential effects on the fate of ingested tannins

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Abstract

Larvae of the freshwater moth *Acentria ephemerella* (Pyralidae, Lepidoptera) can fully develop on *Myriophyllum spicatum*, a submerged macrophyte containing 7–10% of its dry mass as tannins. We investigated the physicochemical gut parameters of larvae fed with *M. spicatum* or *Potamogeton perfoliatus*, a food plant lacking tannins, and the chemical fate of ingested polyphenols. Microelectrode studies revealed that larval midguts were slightly alkaline (pH 8) and had a positive redox potential. Whole guts were oxygen sinks owing to the oxygen demand of the gut contents. Oxygen penetrated the midgut up to 100 μm, but the centres of the foregut and midgut were always anoxic. The physicochemical parameters of the guts did not change with the food plant. The major tannin from *M. spicatum*, tellimagrandin II, was significantly depleted in the midgut and was not detected in faeces. *In vitro* studies indicated that tellimagrandin II is rapidly depleted mainly through oxidation, and hydrolysis might also occur. Our findings for *A. ephemerella* are compared with those for terrestrial Lepidoptera, and possible mechanisms for adaptations to tannin-rich food plants are discussed.

Keywords: Chemical defence; Freshwater plant–herbivore interaction; Gut redox conditions; Hydrolysable tannin

1. Introduction

Herbivory in freshwater systems may have a substantial impact on submerged macrophytes (Lodge, 1991; Newman, 1991). Although more than 500 species of aquatic invertebrates—80% of them insects—feed on macrophytes (Gaevskaya, 1969), chemical defences of freshwater macrophytes against aquatic insects have only recently received increased attention. Studies indicate that aquatic plant–herbivore interactions are influenced by allelochemicals, as are those interactions in terrestrial systems (Jeffries, 1990; Newman et al., 1996; Prusak et al., 2005).

The submerged macrophyte Eurasian watermilfoil, *Myriophyllum spicatum* L., contains remarkably high concentrations of hydrolysable tannins of 7–10% (dry mass) in whole plants and up to 30% (dry mass) in apical meristems, i.e., in concentrations much higher than in other submerged plants (Gross et al., 1996; Smolders et al., 2000). The major tannin, tellimagrandin II (β-1,2,3-tri-*O*-galloyl-4,6-hexahydroxydiphenoyl-(*S*)-glucose; TII), can represent up to 25% of all tannins in leaves and apices. It is highly algicidal and cyanobactericidal and inhibits exoenzymes and photosynthesis of other primary producers (Leu et al., 2002). In terrestrial plants, tannins are widespread and may deter herbivores. However, some insects are adapted to food plants containing high levels of tannins (Schultz and Lechowicz, 1986; De Veau and Schultz, 1992; Barbehenn et al., 2003a, b, 2005).

The larvae of the freshwater moth *Acentria ephemerella* (Pyralidae, Lepidoptera) cause severe damage to several freshwater macrophytes, including *M. spicatum* and pondweeds (Johnson et al., 1998; Gross et al., 2001). Their preferred feeding sites are the apical shoots (Gross et al., 2001, 2002). Although larvae can fully develop on *M. spicatum*, they grow slower than on the tannin-free *Potamogeton perfoliatus* (Choi et al., 2002). Some bacteria isolated from the gut of larvae fed either *P. perfoliatus*

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or *M. spicatum* are inhibited by TII and other polyphenols *in vitro* (Walenciak et al., 2002). Other bacteria found in the biofilm on *M. spicatum* or the surrounding water can degrade such polyphenols (Müller et al., 2007).

The mechanisms of insect adaptation to tannins are diverse and still not completely understood. The mostly alkaline pH of the gut of lepidopteran larvae promotes autoxidation of tannins, and the formation of detrimental metabolites (Appel, 1993). Tannin-tolerant larvae might be protected from toxic oxidation products by antioxidative mechanisms (Summers and Felton, 1996; Barbehenn, 2001), but little is known about the chemical fate of food-derived tannins in the gut of tannin-tolerant lepidopteran larvae (Table 1). In larvae of the white-marked tussock moth *Orgyia leucostigma*, tannic acid in an artificial diet is egested without being processed (Barbehenn and Martin, 1992, 1994), whereas in larvae of the gypsy moth *Lymantria dispar*, oak leaf tannins are apparently oxidized (Henn, 1999a, b).

Oxidation of polyphenols in the presence of oxygen generates reactive oxygen species (ROS; Barbehenn et al., 2006), which are harmful to cell membranes. In some polyphenol-sensitive lepidopteran species, ingestion of polyphenols leads to lesions in the gut epithelium (Steinly and Berenbaum, 1985; Lindroth and Peterson, 1988), and even tannin-tolerant species show elevated levels of oxidative damage of lipids in midgut tissues (Summers and Felton, 1994; Bi and Felton, 1995). Consequently, Barbehenn and Martin (1994) have suggested that the lower oxygen levels in the gut of tannin-tolerant caterpillars compared to that in tannin-sensitive species might protect against oxidative stress. Berenbaum (1980) indicated that a higher pH in the lepidopteran larval midgut might be an adaptation to deal with tannin-rich food plants. Several other factors may contribute to tolerance or susceptibility of the larvae, among them antioxidant enzymes (Krishnan and Kodrik, 2006), low molecular weight antioxidants (Barbehenn et al., 2003a, b), the peritrophic envelope (Barbehenn, 2001), and surfactants (De Veau and Schultz, 1992).

Here we studied whether gut redox conditions and the fate of plant polyphenols in *A. ephemerella* guts determine the performance of the larvae. We tested whether plant-derived tannins are oxidized or hydrolysed in the larval gut. We chose a polyphenol-free (*P. perfoliatus* L.) and a polyphenol-containing (*M. spicatum* L.) aquatic angiosperm as food plants. Gut physicochemical properties (pH, redox potential, oxygen partial pressure) were measured with microelectrodes, and the fate of polyphenols in guts was followed by microextraction of gut sections and faecal pellets under anoxic conditions and subsequent HPLC analysis. To verify changes observed in gut sections, we measured polyphenol degradation *in vitro* in crude extracts of *M. spicatum* under oxic and anoxic conditions.

2. Materials and methods

2.1. Insects and diets

Larvae of *A. ephemerella* were collected in late summer in Lower Lake Constance, the shallow basin of Lake Constance, Germany. The food plants *M. spicatum* (tannin-containing) and *P. perfoliatus* (tannin-free) were sampled by snorkelling or scuba diving. Samples were stored in coolers and transferred to a 4 °C cooling chamber at the institute. Only larvae of the two last instar stages (5th or 6th instar; Gross et al., 2002) were picked from the plants the next day and kept in 1 l vessels filled with chlorine-free tap water. They were fed with the respective plant at least 1 week prior to use.

2.2. Extraction procedure

To prevent oxidation of tannins, larval guts were dissected under a N_2 atmosphere in an anaerobic glovebox. Foreguts and midguts, still including most of their gut content, were separated and collected individually in 200 µl tubes containing 100 µl of 70% aqueous acetone (v/v) pre-incubated in the glovebox for several hours. No intact hindguts could be prepared during the complex handling procedure.

Faecal pellets were collected for 10 h from each set of 10–12 larvae. To prevent autoxidation of polyphenols, faeces were sampled every 15 min and stored under a stream of N_2 until extraction. Plant extracts were made from two leaflets of *M. spicatum*. Faeces and plant samples were each extracted with 100 µl of 70% acetone; the mixtures were sonicated for 10 s, shaken for 1 h in an Eppendorf thermomixer (15 °C, 750 rpm), and centrifuged (5 min, 13,600 × *g*, 4 °C). The collected supernatants were analysed by HPLC. The high UV absorption of acetone close to the retention time (Rt) of gallic acid did not allow the detection of gallic acid. Methanol could not be used as an alternative solvent because it extracted two compounds, probably originating from the gut epithelium, that co-eluted with gallic acid and because extraction of hydrolysable polyphenols with methanol might lead to methanolysis of compounds. We decided not to re-dissolve the acetone extracts in methanol to avoid the loss of polyphenols in this microextraction procedure. The small size of the larvae precluded the accurate determination of the dry mass of dissected gut sections, which were each below 100 µg fresh mass. We therefore developed a ratio-based index to evaluate changes in polyphenol composition of plant material through gut passage (RA_{280}, see below).

2.3. Oxic and anoxic incubation of plant extracts

M. spicatum extracts were incubated in the presence or absence of oxygen under alkaline conditions to investigate the possible oxidation of polyphenols. Extracts were prepared using slight modifications of an established

Table 1
Literature review on the fate of dietary simple phenols and polyphenols in lepidopteran larvae

Species	Susceptibility[a]	Phenols[b]	Diet	Food plant(s)[c]	Instar[d]	Effect	Fate of phenols	References[e]
Acentria ephemerella	T	HT (TII)	Leaves	*MS*	IV–V	Growth reduction	Browning, oxidation, (hydrolysis?)	This publication
Epirrita autumnata	T	GA, HT (MGG, DGG, TGG, PGG)	Leaves	*BP*	n/a	Reduced consumption, no further effects	Browning, oxidation and hydrolysis; 0–25% unprocessed	J
Helicoverpa zea	T	HT, CT, simple phenols (CA, rutin)	Leaves; artificial diets	*LE*, *GC*, *GH*, *GM*, *TI*	All	n/a	Browning; <3.5–5.0% uptake; 74–93% unprocessed, 56% oxidized/bound	C, F, G, H
Heliothis virescens	T	HT, CT	Leaves	*GC*, *GH*, *GM*, *TI*	All	n/a	Browning	H
Hyphantrea cunea	T	HT, CT	Leaves	*GC*, *GH*, *GM*, *TI*	All	n/a	Browning	I
Lymantria dispar	T	HT, CT	Artificial diet with lyophilized leaves	*QR*	IV	n/a	Oxidation, polymerization	D, E
Malacosoma disstria	S	HT (TA)	Artificial diet	–	Early instars; late instars	High mortality; reduced growth, deformations	Browning, oxidation, 4% hydrolysed	B
Operophtera brumata	T	CA, quercetin, other simple phenols	Leaves	*S*	IV	n/a	64–100% degradation	H
Orgya leucostigma	T	TA	Artificial diet	–	n/a	None	90% unprocessed, 10% hydrolysed	A, B
Pseudoplusia includens	T	HT, CT	Leaves	*GC*, *GH*, *GM*, *TI*	All	n/a	Browning	H
Spodoptera exigua	?	CA	Artificial diet, leaves	*LE*	n/a	No effect (?), growth reduction	90% unprocessed?; 50% oxidized/bound	C

[a]T, tannin tolerant; S, tannin sensitive.
[b]CA, chlorogenic acid; CT, condensed tannin; GA, gallic acid; MGG, DGG, TGG, PGG, mono-, di-, tetra-, and penta-galloylglucose; HT, hydrolysable tannin; TA, tannic acid; TII, tellimagrandin II.
[c]*BP*, *Betula pubescens*; *GC*, *Geranium carolinianum*; *GH*, *Gossypium hirsutum*; *GM*, *Glycine max*; *LE*, *Lycopersicon esculentum*; *MS*, *Myriophyllum spicatum*; *QR*, *Quercus robur*; *S*, *Salix* spp.; *TI*, *Trifolium incarnatum*.
[d]n/a, data not available.
[e]A, Barbehenn and Martin (1992); B, Barbehenn and Martin (1994); C, Felton et al. (1989); D, Henn (1999b); E, Henn (1999a); F, Isman and Duffey (1982); G, Isman and Duffey (1983); H, Johnson and Felton (1996b); I, Ruuhola et al. (2001); J, Salminen and Lempa (2002).

method (Gross et al., 1996). In brief, freeze-dried plant powder was extracted twice by shaking for 1 h at 18 °C in 50% aqueous acetone (v/v; 2 mg dry mass in 200 µl). The acetone was evaporated, and the crude extract was dissolved in 50% methanol at a final concentration of 200 µg/ml (extracted dry mass). These extracts were diluted 1:100 with Tris–HCl buffer (20 mM, pH 8) and incubated under a stream of air or N_2. Aliquots of the samples were injected into the HPLC system immediately after dilution or 1, 2, 6, or 8 h later. The experiment was carried out in triplicate.

To purify TII, crude plant extracts were fractionated with Bond Elute C18 cartridges (Varian, Phenomenex, Darmstadt, Germany) using a stepwise methanol elution with 0%, 15%, 25%, 50%, 75%, and 100% methanol (v/v) in water. The 25% fraction contained mainly TII (>95% purity determined by HPLC) and was used further. This fraction was incubated under anoxic conditions as described above.

2.4. HPLC analysis

Phenolic compounds in plant material and guts were separated by reverse-phase HPLC. Samples (50 µl) were separated on a Kromasil C18 250 × 4 mm column (5 µm) at a flow rate of 1 ml/min and using a gradient of solvent A (1% acetic acid) and B (methanol) with 5–60% B for 20 min, 60–100% B for 5 min and then isocratic at 100% B for 15 min (Gross et al., 1996). Signals were detected at 280 and 254 nm, and could be re-analysed for any other wavelength between 195 and 650 nm using photo-diode array detection. We quantified changes in the content of TII and ellagic acid (EA) using the peak area ratio (TII/EA) at 280 nm (RA_{280}). To quantify the hydrolysis products of polyphenols, we calibrated the system with pure ellagic acid (Sigma E2250), gallic acid (Sigma G7384) and HPLC-purified TII (>95%; own sources).

2.5. Microsensor measurements

Clark-type oxygen microsensors with guard cathodes (Revsbech, 1989) and tip diameters of 10–20 µm were constructed in our laboratory. They had stirring sensitivities <2% and were calibrated in air-saturated and N_2-saturated water (Brune et al., 1995). Cathodes were polarized (−700 mV) and the current was measured with a custom-made picoamperemeter (1 mV/pA) connected to a strip chart recorder.

Platinum redox microelectrodes with tip diameters of 20–30 µm were constructed as previously described (Ebert and Brune, 1997) and connected to a high-impedance electrometer amplifier ($>10^{14}\,\Omega$) via a low-noise coaxial cable. Electrode potentials were measured against a reference electrode consisting of an AgCl-coated silver wire in agarose containing 3 M KCl, which was in contact with the agar-filled measuring chamber (see below) through a KCl-filled agar bridge. Redox electrodes were calibrated with saturated quinhydrone solutions in commercial pH standard buffer solutions (pH 4.0–6.0). The measured standard reduction potential E_h was used to calculate the electron availability pe using the Nernst equation ($pe = E_h/59.2$). With pe and pH, the redox parameter pe + pH can be calculated to characterize the redox status of a complex aqueous system (Lindsay, 1979).

Glass pH microelectrodes with tip diameters from 15 to 30 µm were purchased from Unisense, Denmark. Electrode potentials were measured with a high-impedance pH meter against a saturated calomel electrode (Ref 401, Radiometer, Copenhagen), which was connected to the measuring chamber through a KCl-filled agar bridge. Potentials were recorded by means of a high-impedance ($>10^{14}\,\Omega$) electrometer amplifier. Electrodes were calibrated with commercial pH standard buffer solutions (pH 4.0–9.0).

Microelectrode measurements were conducted according to Ebert and Brune (1997). In brief, the guts from well-fed larvae (four to seven guts for each parameter) were dissected under air and rapidly (<1 min) embedded within a glass microchamber in agarose (0.5% upper layer, 1% lower layer) made up in insect Ringers solution (Brune et al., 1995) to prevent desiccation. The agar-embedded gut was exposed to the air only at the top of the chamber. All microelectrodes were positioned with a manual micromanipulator (MM33; Märzhäuser, Wetzlar, Germany). Allowing about 10 min for the oxygen gradients to reach steady state, each series of measurements was completed within 20–30 min after embedding the gut. The setup was calibrated before and after each series to ensure that electrode response remained constant. Since the electrode tips caused a deformation of the gut wall before penetration, the whole gut was first pierced with the electrodes through its entire radius, and then measurements were made as the electrode was retracted. The minimum step increment used was 50 µm; progress of the tip was observed with a horizontally mounted stereomicroscope. All measurements were performed at room temperature.

2.6. Statistical analyses

The slope of oxygen consumption in different gut segments was analysed using the Friedman analysis of variance. To determine differences in gut oxygen consumption from different food treatments, we used Kruskal–Wallis ANOVA. Distinct points in the gut, i.e., gut wall and gut lumen, were compared using the Mann–Whitney *U*-test or the Wilcoxon matched pair test. Differences in the peak area ratio between TII and ellagic acid (RA_{280}) were analysed based on arcsin-transformed data (proportional data) using (a) Kruskal–Wallis ANOVA for the comparison between gut compartments and food and (b) ANCOVA using time as covariate for the comparison of autoxidation of plant extracts in the presence or absence of oxygen. All analyses were run with STATISTICA 5.5 (StatSoft, Inc.) or JMP IN 5.1 (SAS).

3. Results

3.1. Autoxidation of polyphenols in M. spicatum extracts

A typical chromatogram of *M. spicatum* extract in Tris-buffered medium, pH 8.0 at time zero is shown in Fig. 1A. The most prominent peak (Rt = 13.7 min) was TII. The second prominent peak, peak II, eluted shortly after TII (Rt = 14.1 min). Peak I (Rt = 11.1 min) might be a degradation product of TII since the peak area increased in all hydrolysis experiments. Gallic acid and ellagic acid were identified by their retention times (6.4 and 21.2 min) and UV spectra. Under anoxic conditions, the TII peak increased slightly (3%/h; Fig. 1B), presumably because of the enhanced solubility of this compound in the alkaline buffer over time. The slightly alkaline conditions of the medium led to a strong decline of TII (12%/h) over 6 h in the presence of oxygen (Fig. 1C). Peak II disappeared completely under oxic conditions after 1 h, and under anoxic conditions after 3 h.

Under anoxic conditions, the peak areas of gallic acid and ellagic acid increased by 418% and 76%, respectively. When purified TII was incubated under anoxic conditions, 10.0 ± 0.3 mmol/h gallic acid formed per mol of TII; with crude extract, 57.0 ± 0.2 mmol/h gallic acid formed. These results indicated that gallic acid was formed also by the hydrolysis of other polyphenols. The peak area ratio (TII/EA) at 280 nm (RA_{280}) decreased almost linearly from 8.7 ± 0.3 to 6.6 ± 0.3 after 6 h, and to 6.0 ± 0.1 after 8 h under anoxic conditions (Fig. 3A). Under oxic conditions, the RA_{280} reached 4.6 ± 0.6 already after 2 h and decreased further to 0.5 ± 0.3 and 0.3 ± 0.2 after 6 and 8 h, respectively. The slopes of the two curves differed significantly (ANCOVA, $P < 0.001$). The RA_{280} was lower under oxic conditions than under anoxic conditions already after 1 h.

3.2. Gut extracts

In gut extracts under anoxic conditions, the amount of TII in the midgut was far below that in extracts of *M. spicatum* leaflets, whereas substantial amounts of TII were detected in the foregut (Fig. 2A–C). Since we used a new HPLC column in this experiment, the retention times of TII and ellagic acid (here Rt = 13.3 and 20.0 min,

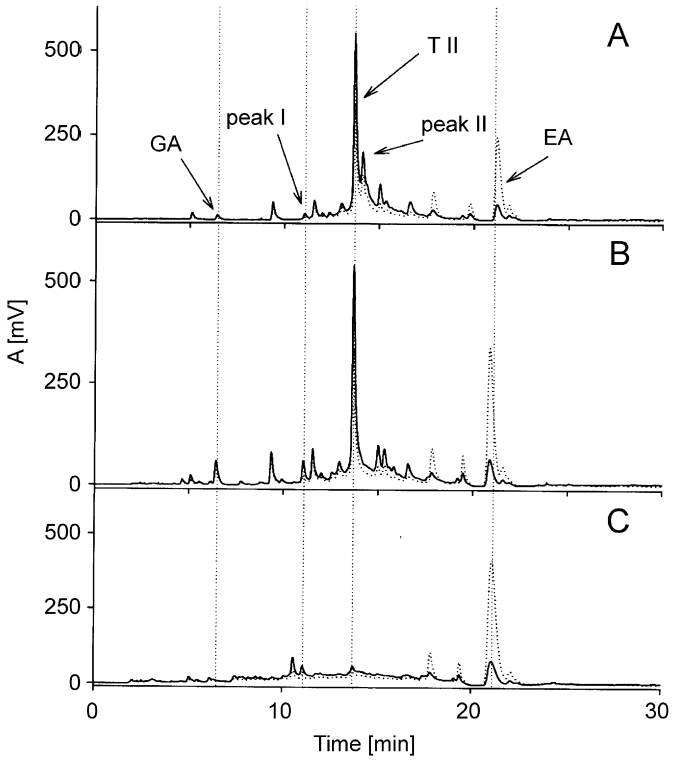

Fig. 1. HPLC separation of crude *M. spicatum* extract incubated in alkaline medium, pH 8: (A) time 0 h; (B) 6 h incubation under anoxic conditions; and (C) 6 h incubation under oxic conditions. Solid line: $A_{280\,nm}$; dotted line: $A_{254\,nm}$. EA, ellagic acid; GA, gallic acid; TII, tellimagrandin II. Peaks I and II are unidentified hydrolysable polyphenols.

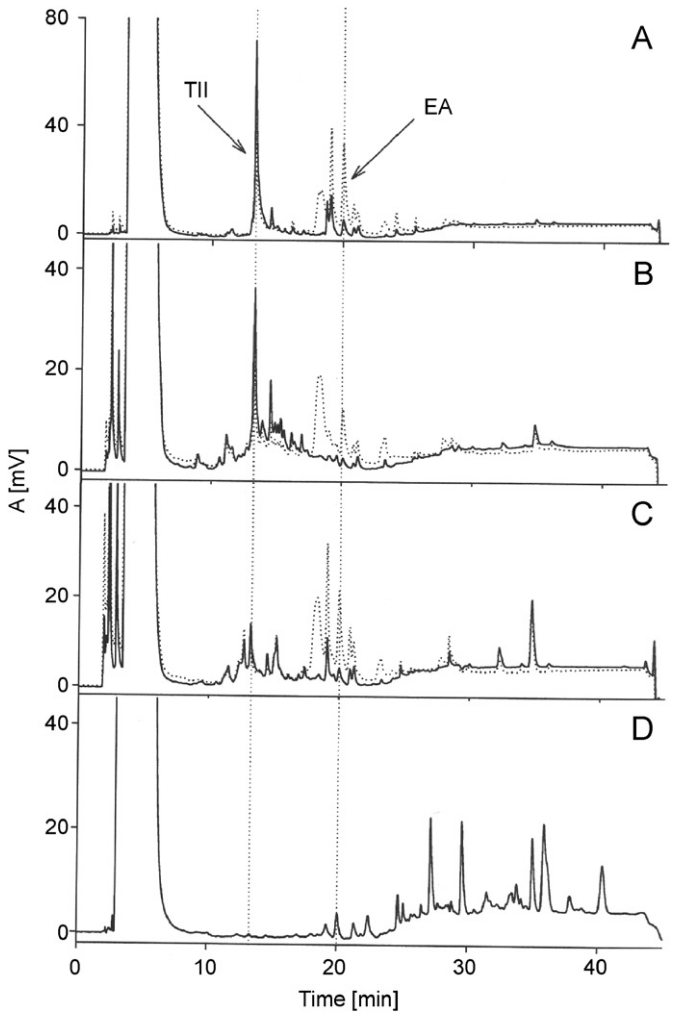

Fig. 2. HPLC separation of extracts from *M. spicatum* and gut and faeces of *A. ephemerella* larvae fed with *M. spicatum*: (A) *M. spicatum* foliage; (B) *A. ephemerella* foregut; (C) *A. ephemerella* midgut; and (D) faeces. Solid line: $A_{280\,nm}$; dotted line: $A_{254\,nm}$. EA, ellagic acid; TII, tellimagrandin II. Retention times of TII and EA were at 13.3 and 20.0 min, respectively.

respectively) slightly differed from those in the autoxidation experiments. The RA_{280} decreased from 11.6 ± 0.8 in *M. spicatum* leaflets (mean $\pm$ 1 S.E.M., range 9.6–13.9; $n = 6$) to 2.5 ± 0.5 in midguts (range 1.4–3.9; $n = 5$; Fig. 3B). These differences were significant ($P = 0.019$, Kruskall–Wallis ANOVA). The RA_{280} of foreguts was highly variable, ranging from 1.6 to 15.6 (8.3 ± 2.7; $n = 5$) and did not significantly differ from the RA_{280} of *M. spicatum* leaflets or that of midguts. In faeces, TII and ellagic acid were detected only in one sample, resulting in an RA_{280} of 0.1. In all four faeces samples, new, unidentified peaks appeared with retention times above 25 min (Fig. 2D). The gut contents of larvae fed *M. spicatum* remained green during gut passage, but faeces turned brownish after a few hours. To detect signals originating from compounds from the larval gut wall, we also investigated gut extracts from larvae fed *P. perfoliatus*. In these extracts, no peaks with retention times similar to TII or ellagic acid were detected (data not shown).

3.3. Oxygen profiles, redox status, and pH of larval guts

Using microsensors, we determined radial oxygen profiles for the different gut regions of *A. ephemerella* larvae fed with *M. spicatum.* Redox potentials and pH were measured only in the midgut because the tips of the pH and redox microelectrodes were too large to penetrate the cuticle of the foregut and hindgut. Oxygen profiles and redox conditions were compared to those obtained with larvae fed with *P. perfoliatus*.

Irrespective of the feeding regime, oxygen concentrations decreased linearly from the agar surface towards the centre of all three gut segments, identifying the entire gut as an oxygen sink (Fig. 4). The slopes of the oxygen profiles above the gut varied, most probably caused by different distances between the gut and the agar surface.

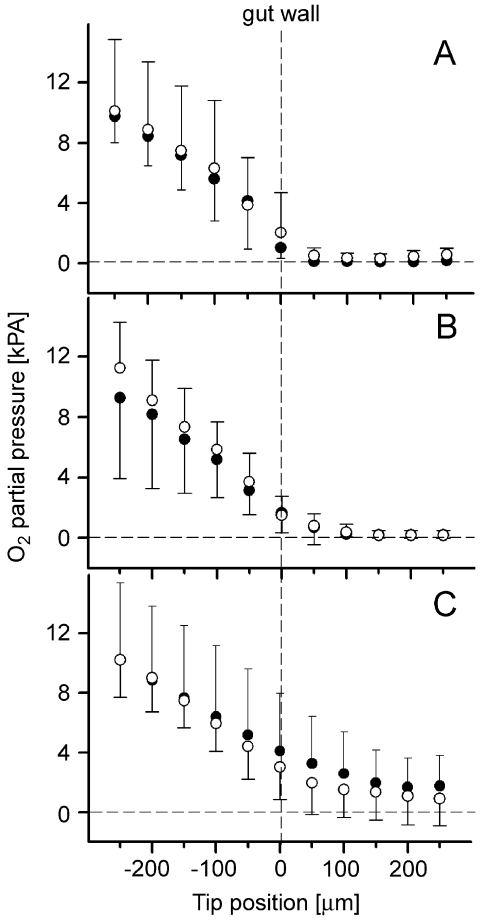

Fig. 4. Radial profiles of oxygen partial pressure around and within agarose-embedded guts of *A. ephemerella* larvae: (A) foregut; (B) midgut; and (C) hindgut. Electrode tip position is given relative to the gut wall. Filled circles: larvae fed with *P. perfoliatus*; open circles: larvae fed with *M. spicatum*. Mean $\pm$ 1 S.D., $n = 6$.

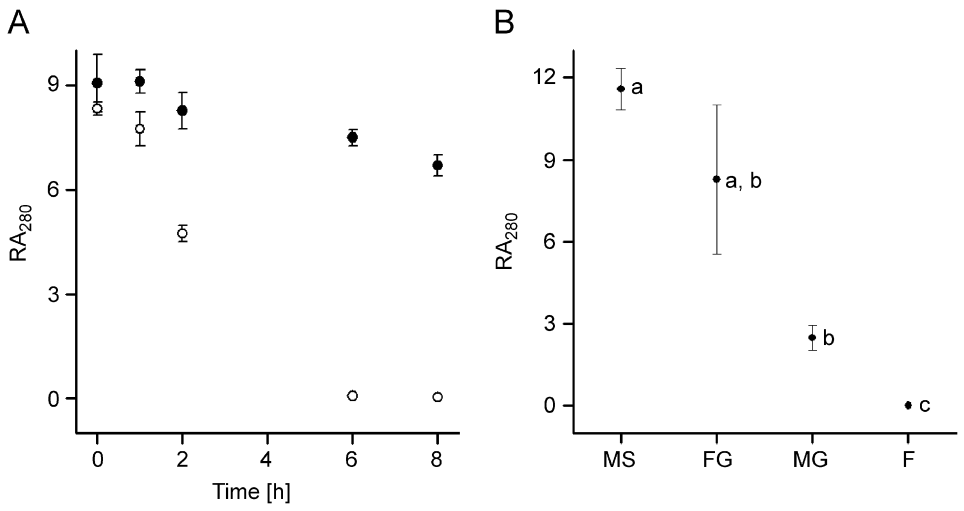

Fig. 3. Change in the peak area ratio tellimagrandin II/ellagic acid (RA_{280}). (A) Change in RA_{280} in crude extracts of *M. spicatum* incubated in alkaline medium, pH 8. Open circles: anoxic conditions; filled circles: oxic conditions. (B) Change in RA_{280} during gut passage. Note the different *y*-axis scaling in the different panels. Data are means $\pm$ 1 S.E.M. MS, *Myriophyllum spicatum*; FG, foregut; MG, midgut; F, faeces.

Table 2
Oxygen partial pressure at gut wall and in lumen in different gut regions of *Acentria ephemerella* larvae fed either with *Potamogeton perfoliatus* or *Myriophyllum spicatum*

Gut section	Site	*Potamogeton perfoliatus*			*Myriophyllum spicatum*		
		Mean ± S.D. (kPa)	Range (kPa)	N	Mean ± S.D. (kPa)	Range (kPa)	N
Foregut	Gut wall	2.0 ± 2.7	0–5.7	6	1.1 ± 0.6	0–1.8	6
	Lumen	0 ± 0	0	6	0 ± 0	0	6
Midgut	Gut wall	1.7 ± 1.3	0.2–3.2	6	1.5 ± 1.3	0–3.2	6
	Lumen	0 ± 0	0	6	0 ± 0	0	6
Hindgut	Gut wall	4.1 ± 3.9	0.2–8.7	4	3.0 ± 2.2	0.4–5.4	5
	Lumen	1.8 ± 2.0	0.2–4.2	4	1.1 ± 1.6	0–3.6	5

Only the interior of the foregut and the midgut differed significantly from other sites. No difference between larvae fed with either host plant was observed. Mean ± 1 S.D.

In foreguts of larvae fed with either plant, oxygen was almost completely depleted within 50 μm beyond the gut wall (0.5 ± 0.5 kPa, $n = 12$). Also in midguts, oxygen penetrated only up to 100 μm deep into the gut lumen. The situation was different in hindguts. Those of larvae fed with *P. perfoliatus* were never anoxic, whereas oxygen was completely depleted within 50 and 250 μm distance from the gut wall in 3 of 5 hindguts of larvae fed with *M. spicatum* (Table 2).

The decline of oxygen partial pressure from the gut wall to the lumen was always significant (Friedman ANOVA; $P < 0.05$). There was no significant influence of the food plant. The lack of a significant change of slope between the gradients above and below the gut wall documents that the respiratory activity of the gut tissue did not create a significant oxygen sink. Gut contractions during the measurements disturbed the gradients in the surrounding agarose, but oxygen partial pressures returned to a steady state within a few seconds.

The redox potential in the midgut lumen was always positive, ranging from 240 to 280 mV (260 ± 20 mV, mean ± S.D., $n = 6$) in larvae fed with *M. spicatum* and from 210 to 320 mV (270 ± 40 mV, mean ± S.D., $n = 6$) in those fed with *P. perfoliatus*. This corresponds to pe values of 4.4 ± 0.3 and 4.5 ± 0.6. No significant effects of food plants were found.

The pH in midguts of larvae fed with *M. spicatum* was slightly alkaline, ranging from 7.7 to 8.5 (8.0 ± 0.3, mean ± S.D., $n = 7$). The corresponding redox parameter (pe + pH) was 12.4 ± 0.6 (mean ± S.D.). Unfortunately, larvae fed with *P. perfoliatus* could not be measured because no intact electrodes were available when insects were on hand. Since the pH of the food plant does not greatly affect the gut pH of Lepidoptera (see discussion below), we decided to use only the measurements for *M. spicatum*.

4. Discussion

We demonstrated that the major hydrolysable polyphenol from *M. spicatum*, tellimagrandin II (TII), is completely depleted during gut passage in larvae of the aquatic moth *A. ephemerella*. We showed that especially the foregut and midgut are oxygen sinks, being anoxic in the centre and possessing an oxic periphery. Oxygen consumption was independent of the food source. Midguts of larvae fed with *M. spicatum* were slightly alkaline and had positive redox potentials. *In vitro* studies with *M. spicatum* extracts confirmed the depletion of TII at gut pH after 4–6 h in the presence of oxygen. TII remained stable under anoxic conditions. However, the increase of gallic acid suggests that other galloylglycosides or gallotannins were hydrolysed at pH 8. HPLC results suggested that plant polyphenols were oxidized and/or hydrolysed in the gut of *A. ephemerella* larvae. New peaks appearing in the faeces indicated further processing of polyphenols by hydrolysis, oxidation and possibly also polymerization, similar to findings in *L. dispar* (Henn, 1999a, b).

Tannins may have diverse effects on terrestrial herbivores, ranging from beneficial to toxic. The underlying mechanisms are still not fully understood, even though knowledge of the chemical fate of ingested tannins is crucial for the appreciation of their effects on herbivores. The stability of polyphenols strongly depends on the physicochemical environment. Gut contents of *A. ephemerella* larvae were slightly alkaline, with a pH of 8. Polyphenols de-protonate and are readily oxidized under such conditions (Appel, 1993). Polyphenols generally turn brownish after oxidation, and browning of gut contents has been described for several Lepidoptera (Table 1). The gut content of *A. ephemerella* larvae remained green during active feeding, yet the faeces turned brownish after a few hours (Gross et al., 2001; this study). Thus, initially we expected that *A. ephemerella* larvae have antioxidative mechanisms in the gut, which allow them to egest polyphenols unprocessed, as was shown for *O. leucostigma* (Barbehenn and Martin, 1992).

Several authors report even gut pH values of 10–12 in *Manduca sexta* (Dow, 1992), *L. dispar* and other lepidopteran larvae (Gringorten et al., 1993). The pH of the food plant foliage is considered to have little influence on midgut pH (Appel and Maines, 1995; Johnson and Felton,

1996a, b). Yet, Skibbe et al. (1996) stated that high alkaline conditions prevail throughout the gut lumen (radial section) only if the food has a high pH, but declines if the food is acidic to neutral. The pH of our food plants, *P. perfoliatus* and *M. spicatum,* ranged from pH 6.5 to 7.0 (data not shown). Thus, we cannot exclude the occurrence close to the gut epithelium of even higher pH values than the pH 8 measured in the lumen.

We used the ratio of the TII and ellagic acid (TII/EA: RA_{280}) peak areas as a proxy to follow changes in polyphenol content during gut passage. RA_{280} was significantly lower in the midgut than in *M. spicatum* foliage, and TII was almost completely depleted in the faeces. TII also was not present in whole body homogenates of *A. ephemerella* larvae according to HPLC analyses (data not shown). These results indicated processing of TII during gut passage, which would occur by assimilation, hydrolysis or oxidation.

Our results indicate that TII is not assimilated during gut passage. A first barrier to assimilation in the gut of insects is the peritrophic envelope. It can form a barrier to the uptake of polyphenols by adsorption, by ultra-filtration or as an anion-exchange filter, or it might even possess antioxidant functions (Barbehenn, 2001). TII, with a molecular weight of 938, probably would pass the peritrophic envelope by ultra-filtration only if it remains a monomer and does not complex with proteins (Barbehenn, 2001). Tannic acid (MW 1700) is retained by the peritrophic envelope in *O. leucostigma* (Barbehenn and Martin, 1992). If TII is able to pass the peritrophic envelope, one would also expect smaller molecules such as ellagic acid to pass this barrier. Consequently, the RA_{280} would not have changed or would have increased, which was not the case.

Our results rather indicate that TII is oxidized during gut passage. In Lepidoptera, the food bolus remains in the foregut only for minutes, not hours; hence, the decrease in RA_{280} from the foregut to the midgut is rapid. Such a rapid decline in our *in vitro* experiments occurred only in the presence of oxygen. Under anoxic conditions, the RA_{280} in the midgut never reached such low values, even after 8 h. We therefore hypothesize that oxidation of TII caused the decrease in RA_{280}. Although larval guts were always anoxic at the centre, oxygen penetrated up to 100 μm into the midgut lumen. Given a maximum midgut diameter of 1000 μm and treating the gut as an endless cylinder, it can be estimated that less than 65% of the gut content would be anoxic, allowing oxidation of polyphenols to occur primarily in the gut periphery, close to the epithelium. The higher susceptibility of neonate larvae in tannin-adapted species would be explained by the smaller gut diameter, which—given the same penetration depth—would result in an increased proportion of oxic gut lumen.

A. ephemerella and *L. dispar* guts had the highest electron availability among the Lepidoptera tested (Table 3); both species are adapted to tannins. Guts of *L. dispar* larvae are oxic even at the gut centre (Johnson and Barbehenn, 2000); therefore, oxygen partial pressures in the gut periphery should be at least equally high, and consequently should cause autoxidation of phenols.

Table 3
Comparison of *Acentria ephemerella* larval gut parameters with those of other terrestrial lepidoptera

	Sensitivity[a]	Gut segment[b]	Oxygen[c] (%)	Eh[d,e,f,g] (mV)	pH[d,e,f,g]	pe	pe + pH
Acentria ephemerella (this study)	T	MG	0.0±0.0	259	8.0	4.4	12.4
Danaus plexippus[c,d]	S*[h]	FG	0.0±0.0	127	7.5	2.1	9.6
		AMG	0.2±0.0	77	8.6	1.3	9.9
		PMG	0.1±0.0	91	8.1	1.5	9.6
Helicoverpa zea[c,f]	T	MG	0.1±0.1	50	9.6	0.8	10.4
Lymantria dispar[c,d]	T	FG	4.4±0.6	236	6.4	4.0	10.4
		AMG	0.2±0.0	238	8.2	4.0	12.2
		PMG	0.7±0.1	218	7.9	3.7	11.6
Malacosoma disstria[c,e]	S	FG	3.8±0.2	96	6.6	1.6	8.2
		AMG	0.5±0.1	−30	10.0	−0.5	9.5
Manduca sexta[c,d]	T	AMG	0.2±0.0	−133	8.0	−2.2	5.8
		PMG	0.0±0.0	−88	8.2	−1.5	6.7
Orgyia leucostigma[c,d]	T	AMG	0.8±0.1	−3	9.8	−0.1	9.7
Papilio glaucus[c,d]	T	FG	0.0±0.0	122	8.9	2.1	11.0
		AMG	0.0±0.0	17	10.1	0.3	10.4
		PMG	0.0±0.0	57	9.8	1.0	10.8

Pe and redox parameters were calculated as described in the methods section.
[a]T, tannin tolerant; S, tannin sensitive.
[b]FG, foregut; MG, midgut; AMG, anterior midgut; PMG, posterior midgut.
[c]Oxygen levels: Johnson and Barbehenn (2000).
[d]pH values: Appel and Martin (1990).
[e]pH values: Barbehenn and Martin (1994).
[f]pH values: Johnson and Felton (1996b).
[g]For other reports on pH values in lepidoptera, see Berenbaum (1980) and text.
[h]S*, specialist on milkweeds.

Apparently, adaptation to tannins does not necessarily avoid the autoxidation of polyphenols. Rather, tannin adaptation should depend on the effective quenching of ROS at or near the epithelium because the epithelium is considered the main target of ROS.

The alkaline conditions prevalent in guts of lepidopteran larvae should promote ROS formation and result in deleterious effects. Among various tannins, ellagitannins have the highest oxidative activities and form the highest concentrations of semiquinone radicals (Barbehenn et al., 2006). It is possible that TII and other, not yet identified ellagitannins in *M. spicatum* might cause oxidative stress in *A. ephemerella* guts, resulting in the poorer performance when fed this plant (Choi et al., 2002).

Hydrolysis of tannins has been reported for the isopod *Porcellio scaber* (Zimmer, 1999) and the tannin-adapted locusts *Schistocerca gregaria* and *Anacridium melanorhodon* (Bernays, 1978). In Lepidoptera, hydrolysis of hydrolysable tannin, measured as the formation of gallic acid, seems to be of minor importance (Barbehenn and Martin, 1992, 1994). However, gallic acid formed by hydrolysis of galloylglucose was not recovered in faeces of larvae of the autumnal moth *Epirrita autumnata*, possibly because of oxidation or assimilation (Salminen and Lempa, 2002). This means that the amount of gallic acid detected in guts or faeces cannot be used to calculate the level of hydrolysed gallotannin in caterpillar guts since this would possibly underestimate hydrolysis.

Our results indicate that TII might be hydrolysed during gut passage in *A. ephemerella*. *In vitro* experiments indicated hydrolysis of TII and other polyphenols in extracts of *M. spicatum* under alkaline conditions. The definite degradation of TII could not be determined because its solubility in Tris buffer at pH 8 increased with time. Our calculations based on ellagic acid and gallic acid formed during incubation of purified TII revealed that under anoxic conditions, only about 2.5% of TII was degraded per hour (data not shown). Compared with purified TII, substantially more gallic acid was formed per mol TII in crude extracts, which indicated that other polyphenols, possibly galloylglucoses or gallotannins, were hydrolysed. Peak II, which rapidly decreased, might represent such a compound.

We conclude that the physicochemical conditions of *A. ephemerella* larval guts promote the oxidation of *M. spicatum* hydrolysable tannins and their hydrolysis products, as well as the partial hydrolysis of hydrolysable tannins. These processes might be enhanced by ingested plant enzymes. A survey of the literature revealed surprisingly few studies investigating the fate of ingested polyphenols in larval guts (Table 1). The published studies revealed, however, that almost all of the investigated Lepidoptera showed signs of oxidation and hydrolysis of ingested polyphenols, regardless whether they were tannin adapted or sensitive. The freshwater moth *A. ephemerella* resembled most closely *L. dispar* with regard to tannin adaptation, physicochemical gut conditions, and transformation of ingested polyphenols. It is not clear yet whether other, non-physicochemical antioxidant defences, such as antioxidant enzymes and low molecular weight antioxidants, are also activated in *A. ephemerella*.

Based on the present measurements with these aquatic moth larvae, we postulate that the difference between tannin-adapted and tannin-sensitive caterpillars can be explained by (1) a higher defence potential and better repair mechanisms in the gut tissue to cope with ROS produced during polyphenol oxidation, as suggested for grasshoppers (Barbehenn et al., 2003a, b), and (2) a better protection of tissues against low molecular weight polyphenols entering the hemolymph (Johnson, 2005).

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