

Metal hyperaccumulation in plants: mechanisms of defence against insect herbivores

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Summary

1. To determine the mechanisms by which metal hyperaccumulation in plants could provide a chemical defence against insect herbivores, the feeding behaviour and performance of the desert locust, *Schistocerca gregaria* (Forskål), was investigated on plants of *Thlaspi caerulescens* J. & C. Presl containing different zinc concentrations, as well as on artificial food differing only in Zn content.

2. In choice experiments, locusts preferred *T. caerulescens* plants containing low foliar Zn concentrations (mean value 0.42 mg g⁻¹ dry biomass) compared with intermediate (1.50 mg g⁻¹) or high (5.77 mg g⁻¹) Zn concentrations. In no-choice experiments, there was no difference in the duration of the first meal, suggesting that locusts could not taste Zn directly, but total time spent feeding, amount of foliage eaten, and locust growth rate all decreased as foliar Zn concentration increased.

3. When presented with artificial food containing Zn concentrations of 0.5, 1.5 or 5.5 mg g⁻¹, locusts fed at a slower rate on intermediate- and high-Zn diets, and their development time from fourth to fifth stadium was proportionately longer. The total amount of food eaten over the fourth stadium was similar for the different treatments, but locust mass gain was inversely related to Zn concentration in the diet. This was reflected in lower nutrient-utilization efficiencies in locusts on a high-Zn diet.

4. In choice experiments with artificial food, locusts showed very similar feeding behaviour to that on plants, consuming more of the low-Zn food. Experiments using food flavoured with coumarin, provided with or without Zn, showed that the locusts' aversion to Zn develops by a post-ingestive feedback mechanism involving associative learning.

5. These results support the notion that metal hyperaccumulation in plants can provide a defence against insect herbivores, and demonstrate that metal concentration *per se* can account for the deterrent effect of high-Zn foods. Aversion responses were evoked by Zn concentrations in the range 0.5–5 mg g⁻¹, an order of magnitude lower than those commonly observed in the foliage of *T. caerulescens*. This suggests that comparatively small elevations in Zn concentration could confer an adaptive advantage on plants by influencing the feeding behaviour of polyphagous insect herbivores, thereby contributing to evolution of the metal-hyperaccumulation trait.

Key-words: herbivory, insect–plant interactions, *Schistocerca gregaria*, *Thlaspi caerulescens*, zinc hyperaccumulation

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Introduction

Recent years have witnessed a growing interest in plants that accumulate unusually high concentrations

of metals in their above-ground tissues. Work on these so-called metal-hyperaccumulating plants has been spurred by their potential use in cleaning up contaminated soils (phytoremediation), but there are many unresolved questions surrounding the ecology and evolution of this unusual trait (Baker & Brooks 1989; Baker *et al.* 2000; Reeves & Baker 2000; Pollard *et al.* 2002; Macnair 2003). Apart from the biochemical problem of how these plants tolerate in their tissues

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concentrations of metals that are toxic to most other organisms, it has not yet been firmly established what form of adaptive advantage (if any) may be conferred on plants by metal hyperaccumulation. Hyperaccumulator species are particularly metal-tolerant, frequently being restricted in their natural distribution to metalliferous soils. However, there are many plant species that do not hyperaccumulate metals, but which are nonetheless tolerant of high soil-metal concentrations (Antonovics, Bradshaw & Turner 1971; Macnair, Tilstone & Smith 2000; Pollard *et al.* 2002). Further, there is evidence that metal tolerance and hyperaccumulation are genetically independent traits (Macnair *et al.* 1999; Pollard 2000; Macnair 2003), so hyperaccumulation may have been subject to positive selection for reasons not directly related to tolerance of metalliferous soils.

There is mounting evidence that metal hyperaccumulation by plants might be involved in mediating biotic interactions with other organisms, in a manner analogous to plant-produced allelochemicals. In particular, metal hyperaccumulation may represent a form of chemical defence against herbivores and pathogens (Reeves, Brooks & Macfarlane 1981; Boyd & Martens 1992; Boyd 1998). Several experimental studies have provided support for a defensive role of foliar-accumulated metals against insect herbivores (Boyd & Martens 1994; Martens & Boyd 1994, 2002; Pollard & Baker 1997; Boyd & Moar 1999; Jhee *et al.* 1999), as well as against fungal and bacterial pathogens (Boyd, Shaw & Martens 1994; Ghaderian, Lyon & Baker 2000). Results from experiments using other herbivores, such as molluscs, have been less consistent, suggesting that the effectiveness of the defensive function may depend on the specific herbivore concerned, in addition to characteristics of both the plant and the metal accumulated (Pollard & Baker 1997; Boyd *et al.* 2002; Hanson *et al.* 2003; Huitson & Macnair 2003). Unlike the extensive literature on allelochemicals, however, there exists virtually no information on the precise mode of action of plant-accumulated metals on invertebrate herbivores, let alone the impact of metals on feeding behaviour.

Locusts are effective models for studying the ways in which invertebrate herbivores can be influenced by various plant constituents (Simpson & Raubenheimer 2000; Behmer, Simpson & Raubenheimer 2002; Behmer & Nes 2003). With respect to metals, Pollard & Baker (1997) demonstrated that the desert locust, *Schistocerca gregaria*, showed a feeding preference for low- over high-zinc plants of *Thlaspi caerulescens*. As yet, however, it is not clear which mechanisms are responsible for insect discrimination between plants with different foliar Zn concentrations. Indeed, it has not yet been conclusively demonstrated that the accumulated metal itself is responsible for deterring herbivores, as opposed to some other plant constituent that covaries with metal concentration. Information of this type would help in assessing whether chemical defence against herbivores is a plausible explanation for the

evolutionary origins of the metal-hyperaccumulation trait (Boyd 1998; Macnair 2003).

Our aim in the current study was twofold. First, we wanted to investigate in detail the feeding behaviour of *S. gregaria* in response to different Zn concentrations, particularly to determine whether locusts are able to taste Zn directly, or whether aversion to Zn-containing foods develops post-ingestively (cf. Lee & Bernays 1988). Second, we wanted to explore the post-ingestive effects of different Zn concentrations on locust performance and nutrient utilization. We examined both issues by feeding locusts either plants with different foliar Zn concentrations or synthetic diets containing different concentrations of Zn.

Materials and methods

INSECTS

Throughout this study we used gregarious-phase nymphs of *Schistocerca gregaria* (Forskål) (Orthoptera: Acrididae) from a culture maintained on a diet of seedling wheat and wheat germ at the Department of Zoology, University of Oxford. Locusts were removed from the culture immediately after ecdysis to either the fourth or fifth stadium (depending on the experiment), weighed, and transferred to experimental arenas containing plants or synthetic foods.

PLANTS

Plants of *Thlaspi caerulescens* J. & C. Presl, a European member of the Brassicaceae, were grown from seeds collected from the site of a disused Zn and cadmium smelter in Prayon, Belgium (Denaeyer-De Smet & Duvigneaud 1974). Seeds were germinated on sterile, moist sand. Ten days after germination, seedlings were transferred to 1.2 l polystyrene boxes (10 plants per box) containing modified 0.1-strength Hoagland solution (composition given by Roosens *et al.* 2003), with Zn supplied as ZnSO₄ at a concentration of 2.0 µM. Plants were cultivated in a glasshouse with ambient light supplemented by sodium-vapour lamps to give a photoperiod of 16 h day⁻¹. Night temperature was maintained at a minimum of 15 °C and day temperature at a minimum of 24 °C. The culture solution was aerated continuously and replaced every 7 days.

After 3 weeks of precultivation on standard nutrient solution, seedlings were separated into three Zn-treatment groups. The three groups continued to receive the same Hoagland solution modified to contain either 0.04, 2.0 or 200 µM ZnSO₄. Plants were cultivated for a further 3 weeks before being used in experiments.

To determine foliar Zn concentrations, leaves were harvested at the end of the 6 week cultivation period on nutrient solution, and rinsed thoroughly with deionized water. Leaf material was oven-dried at 80 °C for 48 h and then ground to a fine powder. Weighed subsamples of the leaf material were digested in

concentrated (69%, v/v) HNO₃ using a microwave digestion system (Model MDS 2000: CEM Microwave Technology Ltd, Buckingham, UK). After appropriate dilution, Zn was measured in the clear samples by atomic absorption spectrophotometry in an air–acetylene flame using a double-beam optical system with deuterium arc background correction (AAAnalyst 100: Perkin-Elmer, Beaconsfield, UK).

SYNTHETIC FOODS

Dry, granular, chemically defined foods were prepared in a manner similar to those developed by Dadd (1961) and modified by Simpson & Abisgold (1985). The foods were identical except for their Zn content, which was based on Zn concentrations measured in plants from the three experimental Zn treatments. In total there were four Zn treatments: low (0.5 mg g⁻¹); intermediate (1.5 mg g⁻¹); high (5.5 mg g⁻¹); and a control (0.0 mg g⁻¹). All foods contained 21% protein and 21% carbohydrate (p21 : c21) by dry mass, which previous work has shown to be near optimal for late-stadium *S. gregaria* nymphs (Simpson *et al.* 2002), together with cellulose (54%), Wesson salt mixture (0.5%), linoleic acid (0.3%), L-ascorbic acid (0.3%) and vitamin mix (0.2%).

LOCUSTS FEEDING ON PLANTS

Food selection among plants containing different zinc concentrations (experiment 1)

The first experiment was a choice experiment designed to explore whether locusts responded differentially to plants with different Zn concentrations. After 6 weeks of growth on nutrient solution, plants were removed from their containers, the root systems gently blotted dry, weighed and transferred to individual plastic containers (7 × 4 × 2 cm high, with two 1.5 cm holes in the top). The roots of the plants were fed through one of the holes and immersed in the same nutrient solution in which they had been growing previously. The other hole was sealed using Sellotape to prevent the locust from drinking the nutrient solution. Next, one plant from each of the three Zn-cultivation treatments was placed inside a circular Plexiglass arena 23 cm in diameter and 16 cm high. The three plants were arranged in an equidistant, symmetrical pattern just on the inside of the arena's perimeter; directly behind each plant, an aluminium perch was provided as an additional place for the locusts to roost. The experiment, which used newly moulted fourth-stadium nymphs, was conducted in a constant temperature room at 26 °C with a 12 h light/12 h dark (L12 : D12) photoregime, and allowed to run for 7 days. If, at any time during the experiment, one plant was entirely consumed or significantly damaged, all three plants were simultaneously replaced. This ensured the locusts were always presented with plants of similar quality and size. When plants were removed

they were reweighed, including any leaves that had been clipped but not eaten (this was considered the most accurate method of measuring the quantity of plant material consumed). In total, 10 arenas were set up and equal numbers of male and female locusts were used.

Feeding behaviour and growth on plants containing different zinc concentrations (experiment 2)

This experiment was designed to examine in greater detail the feeding behaviour of naïve locusts presented with *T. caerulea* plants containing low, intermediate or high concentrations of Zn in their foliage. Again, newly moulted fourth-stadium nymphs were used, but in this instance they were housed in small Plexiglass arenas (17 × 12 × 6 cm) and fed on wheat for the first 2 days of the stadium. On the morning of the third day the wheat was removed and replaced with a single *T. caerulea* plant (low, intermediate or high Zn concentration). The locust's feeding behaviour was then observed and recorded continually over a 4 h period with a laptop computer using the software program OBSERVER 3.0 (Noldus Information Technology Ltd, Wageningen, the Netherlands). The recorded behaviours were: (1) on the plant and eating; (2) on the plant but not eating; (3) off the plant. During the 4 h period most individuals took at least three meals (meals were defined as the sum of all feeding bouts not separated by more than a 5 min non-feeding gap; Simpson 1995). In total, 12 locusts were observed on each treatment (approximately equal numbers of males and females) over two sessions on consecutive days.

The first group of locusts was maintained on the same Zn treatment for an additional 6 days after completing the observations (18 locusts in total, six on each treatment). On the morning of day 6, all plants that had been introduced for initial behavioural observation were removed and replaced with fresh material; the locusts were left on these new plants for an additional 4 days (by the end of this time much of the plants had been consumed). Locusts were then removed from their arenas and weighed. All stages of these experiments were conducted in a constant-temperature room maintained at 32 °C with an L12 : D12 photoregime.

LOCUSTS FEEDING ON SYNTHETIC FOODS

Effects of zinc on performance and nutrient utilization (experiment 3)

This experiment measured the direct effects of Zn on locust performance and consumption patterns. Immediately after moulting to the fourth stadium, locusts were weighed to the nearest 0.1 mg and placed in a Plexiglass arena (17 × 12 × 6 cm) containing a single dish of p21 : c21 food with one of four Zn concentrations: control (0.0 mg g⁻¹); low (0.5 mg g⁻¹); intermediate (1.5 mg g⁻¹); or high (5.5 mg g⁻¹). This food dish, designed to minimize food spillage (Raubenheimer &

Simpson 1990), was placed at one end of the arena; directly behind it was an aluminum perch for the locusts to roost. At the opposite end of the arena was a small plastic container ($7 \times 4 \times 2$ cm high, with two 1.5 cm holes in the top) containing water for drinking.

Experimental food was placed in the appropriate dishes 1 day before the start of the experiment and allowed to equilibrate to the room's ambient humidity. The next day (day 0 of the experiment), diets were weighed to the nearest 0.1 mg and the dish was placed in the appropriate arena. On day 3, after the locusts had been allowed to feed for 72 h, the original dish of food was removed from each box and replaced with a fresh dish of identical food. The original dish of food was placed on top of the arena from which it had been taken, and was allowed to equilibrate to room humidities for 24 h before reweighing. From these values, consumption over the first 72 h of the experiment – referred to as time period 1 (TP1) – could be calculated. Foods were replaced again on day 5 after they had been in the arenas for 48 h, referred to as TP2. Finally, foods were removed once the locusts had moulted to the fifth stadium, or on day 16, whichever came first (TP3). The experiment was terminated on day 16, as this was twice the time it normally takes locusts to complete development of the fourth stadium. Any faecal pellets (frass) that had collected in the food dishes during any of the three periods was removed and returned to the arena.

This experiment was performed in a constant temperature room maintained at 30 °C with an L12 : D12 photoregime. Locusts were checked daily for deaths and moults, and when necessary the water was topped up. On moulting or death, locusts were collected, weighed and their sex determined. At the end of the experiment, frass pellets were collected from the arenas, dried for 10 days in a drying oven at 70 °C, and weighed to allow calculation of various utilization efficiencies (Waldbauer 1968; Raubenheimer & Simpson 1992; Raubenheimer 1995).

Choice among foods containing different zinc concentrations (experiment 4)

This experiment was designed to explore how locusts distribute their feeding among two foods with different Zn concentrations. The protocol was similar to the no-choice experiment, except that each locust had access to two diets, a low-Zn (0.5 mg g^{-1}) diet and either an intermediate- (1.5 mg g^{-1}) or high-Zn (5.5 mg g^{-1}) diet. In total there were 24 arenas, with each treatment replicated 12 times. Food dishes were colour-coded on the bottom of the container and maintained in the same positions in the arena throughout the experiment. To eliminate any positional effect, half the boxes had diets positioned at one end and half at the opposite end. Each arena also held a water container for drinking, located between the two food dishes, and aluminium perches were positioned behind each food dish.

Freshly moulted, weighed fourth-stadium locusts were used for the experiment. The foods were also weighed and replaced every 2 days. By day 11 all but one of the locusts had moulted and the experiment was terminated. Frass was not collected for this choice experiment, as it was not possible to quantify frass production from each diet.

Time course of deterrent response (experiment 5)

This experiment tested whether locusts are deterred by the presence of Zn in the synthetic diet on first encounter, or whether prior experience of Zn is necessary, by observing the feeding behaviour of 2- or 3-day-old locusts presented with control (0.0 mg g^{-1}) and high-Zn (5.5 mg g^{-1}) synthetic diets. Before the experiment, 40 newly moulted fourth-instar locusts were transferred individually to arenas containing a single dish of control food (p21 : c21, with no Zn) for 2 days. These arenas were similar to those described above, except that they did not contain perches. During this 2-day period, locusts were maintained at 30 °C with an L12 : D12 photoregime. After 2 days the food was removed and locusts were deprived of food for 4 h. From the original 40 individuals collected, 24 locusts were selected on the basis of frass production, the presence of frass indicating that food was being eaten.

Locusts were then placed individually into new arenas that contained a single dish of food (either the control or a 5.5 mg g^{-1} Zn concentration) and a perch for roosting. Locusts were visually isolated from one another by placing a white partition between each arena. Locusts were observed continually as described above. The behaviours recorded were: (1) on the food and eating; (2) on the food but not eating; (3) off the food. Equal numbers of males and females were used for each treatment, and the experiment was conducted in a constant-temperature room maintained at 30 °C with an L12 : D12 photoregime.

Test for aversion learning towards zinc (experiment 6)

The final experiment was designed to test the extent to which associative learning might be used by locusts to regulate Zn intake. For this experiment, 60 newly moulted fifth-stadium nymphs were fed wheat for 24 h, and were then transferred individually for a 48 h pre-treatment period to small arenas containing two dishes of synthetic food and a container that provided drinking water (as in experiment 4). Four different pretreatment food combinations were employed. In the first instance (pretreatment 1), 20 locusts were placed individually into arenas that had one dish of p21 : c21 food (the control) and one dish of p21 : c21 food containing both Zn (at a concentration of 1.5 mg g^{-1} , chosen based on the treatments used in experiments 4 and 5) plus an added flavour (coumarin at 0.1% dry mass concentration). Coumarin at this concentration can be tasted by the locusts, but is neither a phagostimulant nor a deterrent (Bernays 1998). A second group of 20

locusts (pretreatment 2) was given two dishes of p21 : c21 food, each containing Zn plus coumarin. Finally, the remaining 20 locusts were split into two even groups, with one of these (pretreatment 3) receiving two dishes of p21 : c21 food containing coumarin while the second group (pretreatment 4) received two dishes of p21 : c21 food containing Zn. At the end of the 48 h pretreatment period, the foods were removed and replaced with two new previously weighed food dishes. Locusts from pretreatments 1 and 2 were then split into two groups, with half from each pretreatment receiving a dish of control food plus a dish of food containing coumarin. The remaining locusts from each of these two pretreatments were placed in arenas containing a dish of control food and a dish of food containing Zn. Finally, the locusts from pretreatment 3 were placed in arenas containing a dish of control food and a dish of coumarin-flavoured food, while those from pretreatment 4 were placed in arenas containing a dish of control food and a dish of food containing Zn. After 48 h, the foods were removed and weighed.

STATISTICAL ANALYSIS

Statistical analysis was performed using the statistics packages SAS 6.12 and STATVIEW (both for the Macintosh: SAS Institute Inc., Cary, NC, USA). Food consumption was analysed using ANOVA, analysis of covariance (ANCOVA), repeated-measures analysis (RMA), one-tailed *t*-tests and Mann–Whitney *U*-tests. All comparisons of food consumption were limited to those individuals that completed development or were alive at the end of experiments. In all cases, statistical assumptions were checked by inspection of raw data and residual plots; in no instance was transformation of the data required. To measure survival and moulting success, proportion tests were used as described by Zar (1996). Development times were analysed using failure-time analysis and, when significant, treatments were compared using *post hoc* contrasts (Fox 1993). Where not otherwise specified, significant differences denote $P \leq 0.05$.

Results

LOCUSTS FEEDING ON PLANTS

Food selection among plants containing different zinc concentrations (experiment 1)

Thlaspi caerulescens, precultivated on standard nutrient solution for 3 weeks and then transferred for 3 weeks to nutrient solution containing 0.04, 2.0 or 200 μM ZnSO_4 , accumulated foliar Zn concentrations of 415 ± 56 , 1502 ± 148 and 5774 ± 566 $\mu\text{g g}^{-1}$, respectively (values are means \pm SE; ANOVA: $F_{2,57} = 61.11$, $P < 0.001$). Over the 7-day feeding period, locusts given a choice between the three types of plant consumed 3.09 ± 0.36 , 1.72 ± 0.17 and 1.50 ± 0.28 mg fresh biomass from the plants with low, intermediate and

high foliar Zn concentrations, respectively. Because the design of this experiment meant that the data obtained were not independent, the results were analysed by plotting amounts eaten against Zn concentration (low, intermediate or high) for each individual and calculating a single regression coefficient. If locusts ate indiscriminately among the three Zn treatments, the average regression coefficient would approximate zero. Results gave a mean regression coefficient of -0.091 , a value significantly different from zero (one-sample *t*-test: d.f. = 9, $t = -4.021$, $P = 0.003$). The negative slope indicates that locusts preferred plants containing low Zn concentrations, and good regression fits were obtained for all locusts (R^2 values ranging from 0.678 to 1.0)

Feeding behaviour and growth on plants containing different zinc concentrations (experiment 2)

Having established that locusts show a significant preference for low-Zn plants over the long term (7 days), we investigated how rapidly aversion to plants with higher Zn concentrations could develop. When naïve locusts were presented with plants containing different foliar Zn concentrations, there was no difference in the duration of the first meal observed (ANOVA: $F_{2,27} = 0.19$, $P = 0.831$; Fig. 1a). However, when feeding over the first 4 h was examined, there was a significant difference among the three treatments (ANOVA: $F_{2,27} = 7.11$, $P = 0.003$), with feeding time being longest on the low-Zn treatment and decreasing as the Zn content of the plant biomass increased (Fig. 1a).

Results from the growth part of this experiment showed that locusts on the low-Zn treatment had a significantly faster growth rate than locusts on the high-Zn treatment (one-tailed ANOVA: $F_{2,13} = 2.85$, $P = 0.047$; Fig. 1b), and that growth rate decreased linearly as the foliar Zn concentration in the plants increased (linear contrast: $F_{1,13} = 7.72$, $P = 0.016$). Additionally, of the 18 locusts used in this study, only three moulted to the fifth stadium, all of which came from the low-Zn treatment.

LOCUSTS FEEDING ON SYNTHETIC FOODS

Effects of zinc on performance and nutrient utilization (experiment 3)

To test the effects on locust feeding of diets that differed solely in Zn content, nutritionally balanced artificial foods were prepared containing low (0.5 mg g^{-1}), medium (1.5 mg g^{-1}) or high (5.5 mg g^{-1}) Zn to mimic the metal concentrations observed in *T. caerulescens* leaves. Death of locusts was observed only on the intermediate- and high-Zn treatments, although the proportion of locusts surviving on the various treatments did not differ significantly (χ^2 test: d.f. = 3, $\chi^2 = 6.43$, $P > 0.05$). There was a significant difference between treatments in numbers moulting to the fifth stadium by the end of the experiment (χ^2 test: d.f. = 3, $\chi^2 =$

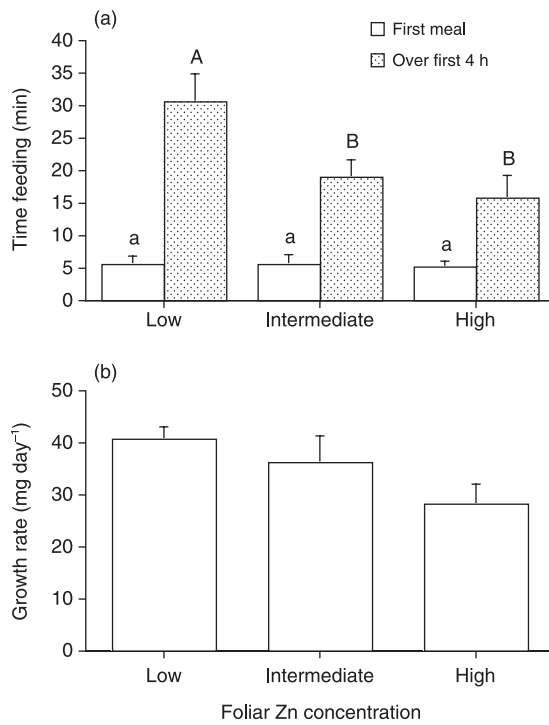


Fig. 1. Time spent feeding and growth rate of fourth-stadium locusts on *T. caerulea* plants with different foliar Zn concentrations. Locusts were reared on wheat for the first 2 days of the fourth stadium and switched to *T. caerulea* plants with one of three foliar Zn concentrations (low, intermediate or high) on the start of the third day. (a) Mean feeding time (\pm SE) of the first meal and over the first 4 h following initial contact with the test plant. Different letters above the treatments indicate differences significant at $P < 0.05$. (b) Growth rates on plants with different foliar Zn concentrations (mean \pm SE).

16.40, $P < 0.01$). All locusts moulted on the control and low-Zn treatments, but moulting success on the intermediate- and high-Zn treatments was only 82 and 50%, respectively. When development time to the fifth stadium was analysed (including those insects still alive on day 16 that had not moulted), a significant treatment effect was observed (failure-time analysis: d.f. = 3, $\chi^2 = 94.69$, $P < 0.01$). *Post hoc* comparisons using contrasts indicated that development time on the intermediate- and high-Zn treatments, but not the low-Zn treatment, was significantly longer relative to controls (Fig. 2a). For those locusts that moulted to the fifth stadium, there was a significant difference in mass gain among the treatments (ANCOVA, $F_{3,34} = 4.28$, $P = 0.015$; Fig. 2a). A Dunnett's *post hoc* test showed that mass gain in each of the Zn treatments was significantly lower than on the Zn-free diet.

There was a significant treatment effect on total food consumed (ANCOVA: $F_{3,34} = 5.59$, $P = 0.003$), with locusts on the low-Zn treatment eating less food (491 ± 41 mg) compared with locusts on the high-Zn treatment (610 ± 47 mg). No significant differences, however, were detected between the treatments containing Zn and the control (537 ± 15 mg consumed). With

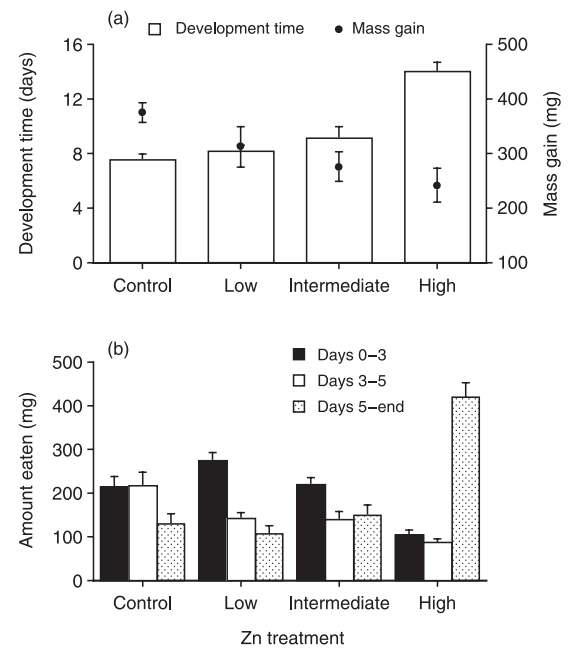


Fig. 2. Development time, mass gain and consumption patterns of fourth-stadium locusts reared on a nutritionally balanced synthetic food containing different Zn concentrations. (a) Development time and mass gain (means \pm SE) on diets containing no Zn (control) or a low, intermediate or high Zn concentration. (b) Amount of food eaten on the various treatments for each of the three time periods shown (means \pm SE). The duration of the last period was variable, depending on the time taken for the locust to moult to the fifth stadium (up to a maximum of 16 days on the high-Zn treatment).

respect to feeding rate, RMA detected a significant time-treatment interaction ($F_{6,68} = 17.22$, $P < 0.01$). Inspection of these data showed that locusts on the low- and intermediate-Zn treatments fed at rates similar to locusts on control diets. In contrast, locusts on the high-Zn diet had lower feeding rates compared with locusts on the other diets, including the control (Fig. 2b); the large amount consumed in the final period was a consequence of the longer development time of locusts on the high-Zn treatment (cf. Fig. 2a).

To determine whether Zn content influenced food utilization (*sensu* Waldbauer 1968), ANCOVA techniques were used (Raubenheimer & Simpson 1992; Raubenheimer 1995). We first looked at efficiency of conversion of ingestion (ECI = mass gain/food eaten) by treating mass gain as the dependent variable and food eaten as a covariate, and found both significant covariate (ANCOVA: $F_{1,33} = 35.15$, $P < 0.01$) and Zn (ANCOVA: $F_{3,33} = 11.95$, $P < 0.01$) effects. As shown in Fig. 3a, there was a positive correlation between food intake and mass gain, but mass gain decreased as Zn concentration in the food increased. Next we examined the efficiency of conversion of digestion (ECD = mass gain/food assimilated, where food assimilated = food eaten - food excreted). Here mass gain was treated as the dependent variable and food assimilation was used as a covariate. This analysis also revealed significant covariate (ANCOVA:

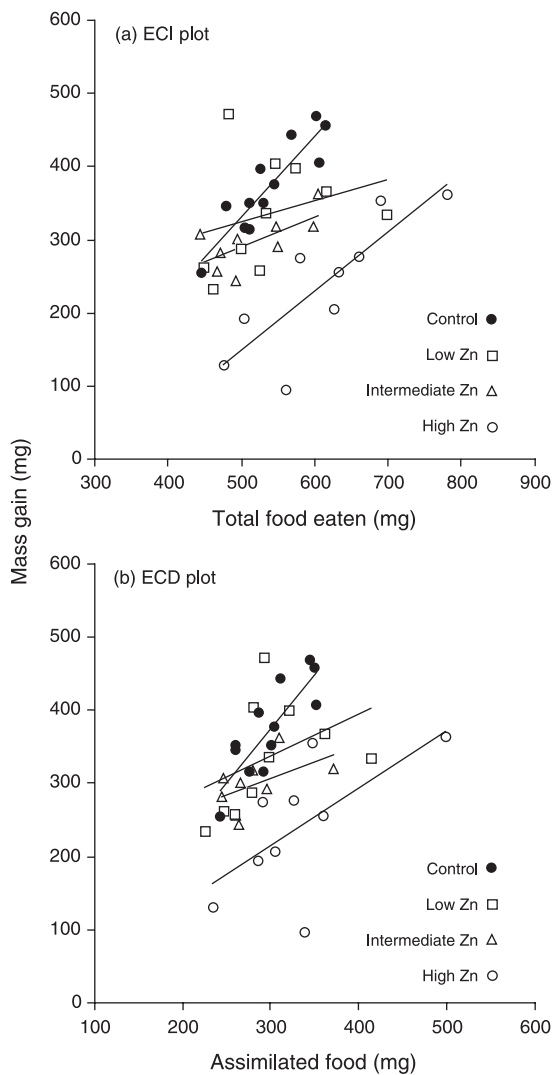


Fig. 3. Utilization plots for fourth-stadium locusts reared on nutritionally balanced synthetic foods containing four different Zn concentrations. Values refer to cumulative amounts measured during completion of the fourth stadium, or over a period of 16 days, whichever was shorter. Regression lines are shown for each treatment, as fitted by least squares. (a) Relationship between mass gain (fresh mass) and amount of food eaten (dry mass) for each food (efficiency of conversion of ingested food, ECI). (b) Relationship between mass gain and amount of food assimilated (efficiency of conversion of digested food, ECD: digested food = food eaten – food excreted).

$F_{1,33} = 26.13$, $P < 0.01$) and Zn (ANCOVA: $F_{3,33} = 8.90$, $P < 0.01$) effects. There was a positive correlation between food assimilated and mass gain, and a decrease in the average mass gain as Zn concentration in food increased (Fig. 3b). Finally, we analysed approximate digestibility (AD = food assimilated/food eaten), treating food assimilated as the dependent variable and food eaten as a covariate. Here there was a significant covariate effect (ANCOVA: $F_{1,33} = 186.17$, $P < 0.01$), but no significant Zn effect (ANCOVA: $F_{3,33} = 0.279$, $P = 0.839$). This indicated that digestibility was independent of food Zn concentration, but was positively correlated with the amount eaten. For all three analyses there was no significant interaction between the main effect

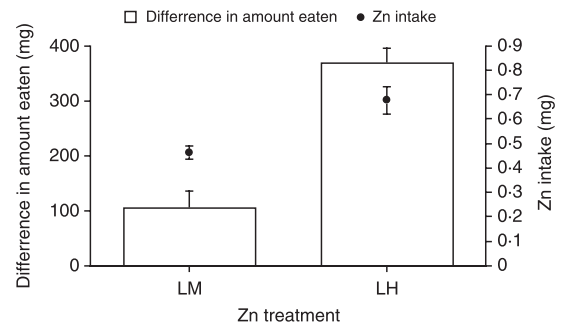


Fig. 4. Food preferences for locusts offered a choice between two foods containing different Zn concentrations. Newly moulted fourth-stadium nymphs were placed in an arena containing two dishes of nutritionally balanced synthetic food that differed in Zn concentration. Each arena contained a food dish with a low Zn concentration paired with another food dish containing either an intermediate (LM treatment) or high (LH treatment) Zn concentration. The figure shows the difference in amounts eaten between the two available food dishes (low-Zn food – higher-Zn food) and the total Zn intake (both means \pm SE). Values are for the entire fourth stadium.

(Zn concentration) and the covariate being used, satisfying a prerequisite for ANCOVA (Sahai & Ageel 2000).

Choice among foods containing different zinc concentrations (experiment 4)

In this experiment, low-Zn food was paired with either intermediate-Zn food (LM treatment) or high-Zn food (LH treatment), and the amounts eaten from the two available dishes were recorded for each individual locust. Locusts in both treatments showed a significant preference for the low-Zn food (one-sample t -test with $H_0 = 0$: LM treatment, d.f. = 11, $t = 3.49$, $P < 0.01$; LH treatment, d.f. = 10, $t = 14.04$, $P < 0.01$), but this preference was much stronger for locusts on the LH treatment (ANCOVA: $F_{1,20} = 40.88$, $P < 0.01$; Fig. 4). Feeding preference was also examined over time, but no significant time–treatment interaction was found (RMA: $F_{2,40} = 1.66$, $P = 0.205$). Although locusts from the LH treatment showed a stronger preference for the low-Zn food, calculations showed that they had a significantly higher Zn intake compared with locusts on the LM treatment (ANCOVA: $F_{1,20} = 12.63$, $P = 0.002$; Fig. 4). This difference in Zn intake had little impact on performance, as survival on both treatments was near 100%, and all locusts that survived moulted. Additionally, the average development time for locusts on the two treatments did not differ significantly (failure time: d.f. = 1, $\chi^2 = 3.14$, $P = 0.076$; LM treatment = 8.25 ± 0.30 days, LH treatment = 9.09 ± 0.09 days). Finally, when mass gain on the two treatments was compared, no differences were found (ANCOVA: $F_{1,20} = 0.10$, $P = 0.760$).

Time course of deterrent response (experiment 5)

During the behavioural observations all locusts came into contact with the test food. On the control treatment

nine out of 12 locusts were observed having a meal (defined as feeding for longer than 18 s; Behmer & Elias 1999), compared with six out of 12 on the high-Zn treatment, although a χ^2 analysis showed that this difference was not statistically significant (d.f. = 1, $\chi^2 = 1.55$, $P = 0.213$). The length of the first meal was therefore compared for locusts observed eating the diets. Using a Mann–Whitney U -test we found that the meal lengths on diets with high Zn (5.5 mg g^{-1} dry mass) did not differ compared with diets lacking Zn (U -test: $Z = -1.53$, $P = 0.123$). On both treatments the median feed time of the first meal was greater than 500 s. These results suggest that naïve locusts were not directly deterred by Zn.

Test for aversion learning towards zinc (experiment 6)

Although the short-term experiments indicated that locusts were not immediately deterred by Zn, over longer feeding periods locusts showed a tendency to avoid foods with elevated Zn content. This aversion behaviour could be attributed to a toxic effect of Zn *per se*, or to aversion learning in which the consequences of ingesting Zn are coupled with previously unassociated sensory cues, such as a flavour. We tested for flavour-mediated aversion learning by subjecting locusts to a 48 h pretreatment training period followed by a 48 h testing period to identify what, if anything, was learned during the pretreatment period. Our initial experiment showed that fifth-stadium locusts from pretreatment 1 (one dish of control food and one dish of coumarin + Zn food), when presented with a choice between a control and coumarin-flavoured food, or between a control and Zn food, showed a significant preference for the control food in both cases (one-tailed t -test: d.f. = 9, $t = 4.478$, $P < 0.01$; d.f. = 9, $t = 4.130$, $P < 0.01$, respectively; Fig. 5a). There was, however, no significant difference in the strength of the preference (calculated as the difference between the amount of control and test food eaten) between the two food-treatment combinations (ANOVA: $F_{1,18} = 1.52$, $P = 0.233$). Locusts from pretreatment 2 (two dishes of food with coumarin + Zn) also showed a strong preference for the control food when presented with a choice between control food and either coumarin-flavoured or Zn food (one-tailed t -test: d.f. = 9, $t = 4.163$, $P < 0.01$; d.f. = 9, $t = 2.24$, $P < 0.05$, respectively; Fig. 5b). Here, however, the preference for the control food was significantly stronger when it was paired with the flavoured food (ANOVA: $F_{1,18} = 4.81$, $P < 0.05$). Coumarin itself was shown to be neither a phagostimulant nor a deterrent, as shown by the results in Fig. 5c; locusts from pretreatment 3 (two dishes of coumarin-flavoured food, no Zn) showed no preference for either the control or coumarin-flavoured food (two-tailed t -test: d.f. = 9, $t = 1.10$, $P = 0.299$). On the other hand, locusts from pretreatment 4 (two dishes of Zn food) showed a preference for the control food over the food containing Zn (one-tailed t -test: d.f. = 9, $t = 3.46$, $P < 0.01$; Fig. 5c).

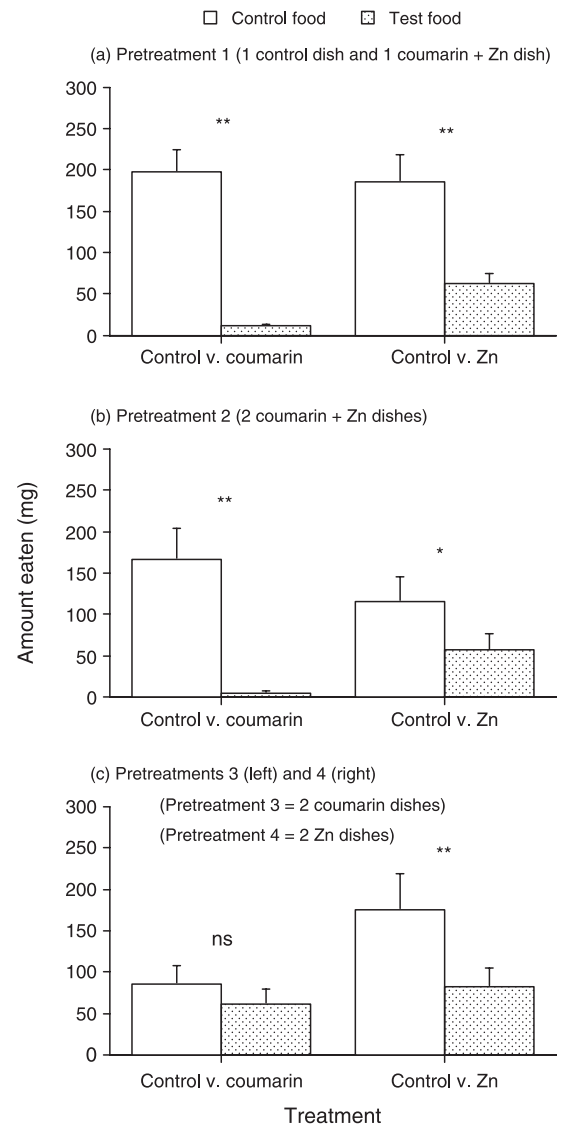


Fig. 5. Food consumption by fifth-stadium locusts in choice experiments designed to test for aversion learning towards Zn. In each case locusts were fed wheat on the first day following the moult, then given two dishes of synthetic food for the next 48 h (days 2 and 3). The nature of the food given to the locusts during these 48 h pretreatment periods is indicated above each graph. At the end of the pretreatment, foods were removed and replaced with one dish of control food (no coumarin or Zn) and a second dish containing either coumarin or Zn (indicated on the x-axis). These foods were left in the arenas for 48 h (days 4 and 5) and the amount eaten from each dish was measured (values are means \pm SE). Asterisks above the bars indicate a significant difference in amounts eaten from the two food dishes provided during the final 48 h period (*, $P < 0.05$; **, $P < 0.01$; ns, not significant).

Discussion

Our results are consistent with previous studies that have demonstrated both aversion responses by invertebrate herbivores towards plants that hyperaccumulate metals (Pollard & Baker 1997; Jhee *et al.* 1999), and the negative consequences of continual feeding on such plants for growth and survival of the herbivore (Boyd & Martens 1994; Martens & Boyd 1994; Boyd &

Moar 1999). The present experiments, though, have revealed the mechanisms by which Zn influences the feeding behaviour, growth and development of a generalist herbivore. Our results suggest that herbivores are not deterred by Zn on first contact, but instead develop an aversion to metal-containing foods post-ingestively by a process involving associative learning. With respect to measures of herbivore performance, development time increases and mass gain decreases as the metal concentration in the food increases. We also found that the conversion of ingested food and assimilated food into body mass decreases as food Zn concentration increases, but that Zn intake does not affect digestibility.

When locusts were given access to *T. caerulescens* plants containing low, intermediate or high foliar Zn concentrations, they consistently showed a preference for plants with low Zn content. Over the 7-day duration of the experiment, however, locusts still consumed relatively large amounts (on average >1 mg fresh biomass) of vegetative tissue from plants containing intermediate and high Zn concentrations. Such a result is consistent with the notion that locusts may not taste Zn directly, which is supported by two additional findings. First, when naïve locusts were presented with *T. caerulescens* containing different foliar Zn concentrations, there was no difference in the duration of their initial meal (Fig. 1a). Second, when locusts were presented with synthetic foods that lacked or contained Zn, there was no difference in the number of meals initiated on these two foods, or in the length of the first meal for locusts that had initiated feeding. Locusts, like most other plant-feeding insects, have well developed chemoreceptors on their mouthparts that allow them to respond to a diverse range of plant compounds, including primary metabolites (e.g. sugars and amino acids) and secondary compounds (e.g. phenolics, alkaloids, terpenes and – in the case of plants in the Brassicaceae – glucosinolates; Fahey, Zalcmann & Talaly 2001; Renwick 2002; Wittstock & Gershenson 2002). If locusts could taste Zn directly, and were deterred by it, their time feeding during the first meal on plants and synthetic foods with higher Zn concentrations should have been significantly reduced. Further studies, using electrophysiological techniques, will be needed to determine conclusively whether locusts are unable to taste Zn.

If unable to taste Zn, the preference shown by locusts for low-Zn plants could be explained most readily by a post-ingestive feedback mechanism. Such mechanisms are well established in grasshoppers, and can operate either (1) as a direct feedback on mouthpart taste receptors, mediated through the haemolymph (blood); or (2) via a learned association with some feature of the food. Direct nutrient feedbacks are critical to nutrient regulation in *Locusta migratoria* (Abisgold & Simpson 1988; Simpson, Chyb & Simpson 1990; Simpson & Raubenheimer 1993), but for such mechanisms to operate there must be gustatory responsiveness

to the compounds in question. In the case of Zn this seems unlikely, as plant material generally contains sufficient Zn to satisfy the dietary requirement of herbivorous insects (Chapman 1998). In the current study, post-ingestive feedbacks via associative learning appear to be the more likely aversion mechanism, for which there is a precedent in the closely related species *Schistocerca americana*. For example, Lee & Bernays (1990) found that last-stadium *S. americana* nymphs learnt to associate the gustatory cues of an initially acceptable novel food with detrimental effects caused by injection of plant secondary compounds into the haemolymph after feeding, leading to reduced acceptability of what was previously a highly acceptable food. Likewise, Behmer, Elias & Bernays (1999) found that *S. americana* nymphs regulated the intake of unusable dietary sterols by means of a post-ingestive feedback system and associative learning. Using synthetic foods containing various combinations of sterols and flavours, the same study showed that grasshoppers modified their feeding behaviour by learning to associate the taste of their foods with the presence of unusable sterols.

What would locusts in the current study have learned about their foods? Our experiments using synthetic food with Zn and added flavour clearly show that locusts learn to associate specific tastes with the presence of Zn. For example, when locusts were exposed to Zn-containing synthetic foods flavoured with coumarin, and then 48 h later were presented with a food containing coumarin but no Zn, they ate very little of this food even though it contained no Zn (Fig. 5a,b). A pretreatment period of 48 h was evidently sufficient for this association to be learned, yet not so long that the locusts suffered from acute Zn poisoning. This was shown by the feeding behaviour of locusts in the subsequent test period, which was similar irrespective of whether or not they had access to Zn-free food in the pretreatment period (Fig. 5a,b, respectively). Locusts might also learn to associate Zn-containing foods with some type of positional cue when the location of a particular food dish is maintained throughout an entire experiment (Dukas & Bernays 2000). However, the expression of positionally based learning may be related to the toxicity of a food, as locusts in our two treatments ate much more of the intermediate-Zn food than the high-Zn food (Fig. 4). To verify that positionally based learning in response to Zn can occur, it would be necessary to include a treatment in which the positions of foods in the arenas were regularly alternated.

In the experiment with plants cultivated on different Zn concentrations, locust preference for low-Zn plants could be attributed to aversion learning based both on taste and position. Glucosinolates give plants in the Brassicaceae a distinctive flavour (Renwick 2002; Wittstock & Gershenson 2002), and experiments by Tolrà *et al.* (2001) show that increases in Zn hyperaccumulation can decrease shoot concentrations of sinalbin, the most abundant glucosinolate in *T. caerulescens*. A change in glucosinolate content, or some other compound,

may have altered how locusts perceived their different test plants, and would have provided an opportunity to develop learned aversions based on taste. At the positional level, we kept the location of a particular plant in the arena constant during the experiment (7 days), sufficient time for positionally based learning to develop. It is notable, however, that locusts showed little discrimination between intermediate- and high-Zn plants. This suggests that foliar Zn concentration in the intermediate plants, which was threefold higher than in low-Zn plants, was already sufficient to exert the maximum feeding-deterrent effect.

The negative effects of Zn on locust growth and development increased as concentration in the food increased, and this result was similar whether the locusts were fed on plants (Fig. 1) or synthetic foods (Fig. 2). Few studies have examined precisely how metals influence insect physiology, but Martoja, Bouquegneau & Verthe (1983) found that the injection of cadmium and mercury into the haemolymph of locusts caused biochemical changes to the fat body, which led to reduced energy reserves. Cadmium contamination led to decreased storage of glycogen, with a resultant increase in the synthesis of phosphorylase a (Samarawickrama 1979), while lipid storage decreased in mercury-contaminated insects. In our experiments Zn was ingested by the locusts, rather than injected, but because we did not void the guts of contaminating plant tissues we could not accurately measure Zn uptake into insect body tissues. Hanson *et al.* (2003) found that snails (*Mesodon ferrissi*) fed on selenium-containing plants had higher Se concentrations in their bodies and shells compared with snails fed Se-free plants. However, the Se concentrations reported in the soft tissues may have been influenced by plant material in the alimentary tract, as snails can retain material in their guts for more than 2 weeks.

Two noteworthy physiological effects were observed as the Zn concentration of the locust food increased. First, we saw a change in food-consumption patterns at higher Zn concentrations (Fig. 2). In general, the rate of feeding was similar during the first 3 days for all treatments except the high-Zn one, for which feeding rates were low. Between days 3 and 5, however, feeding rates on all three Zn treatments decreased relative to the control. By day 5 all locusts on all three Zn treatments had developed aversions to the food, but as this was a no-choice experiment they had no option but to eat Zn-containing food to avoid starvation. Locust feeding may have slowed because of toxicological effects associated with ingesting Zn in excess of normal physiological levels. Interestingly, we observed unusual twitching behaviours in some of the locusts reared on individual *T. caeruleus* plants with intermediate and high foliar Zn concentrations. In vertebrates, Zn²⁺ ions can block chloride channels and might thereby perturb electrical membrane potential (Hille 2001), and a similar effect may occur in locusts. No differences in amounts eaten during the final time period (up

to the end of the fourth stadium) were noted between the control, low- and intermediate-Zn treatments, but food consumption in the high-Zn treatment was elevated (Fig. 2b). This reflected the lengthened developmental period for locusts on the high-Zn treatment, which was almost twice that of the controls (Fig. 2a), suggesting that the locusts needed to acquire a minimal amount of nutrients to grow and moult to the next developmental stage.

The second physiological effect detected was a direct relationship between Zn content of the food and the ability of locusts to utilize both ingested and digested food. Plots of the efficiency of conversion of either ingested (ECI) or digested (ECD) food showed that higher Zn concentrations in the food were associated with lower mass gain per unit of food ingested or digested (Fig. 3). In contrast, we found no evidence that Zn concentration had any effect on the approximate digestibility of the test food. Measurements of the conversion of ingested food into body nitrogen or lipid would be needed to provide information related to body growth, metabolism and storage. Investigation of the mechanisms by which Zn reduces the efficiency of food conversion into body mass would be a valuable area for future research.

Our findings support the hypothesis (reviewed by Boyd 1998) that metal hyperaccumulation in plants can provide an effective defence against herbivores, particularly against polyphagous insects. Our results show for the first time that the deterrent effect of high Zn concentrations begins to develop only after an initial feeding experience, which might explain why metal hyperaccumulation may be an ineffective defence against large vertebrate herbivores (Martens & Boyd 2002). Across a reasonably large, homogeneous stand of plants, however, metal hyperaccumulation could work against vertebrate herbivores via a group protection strategy. Even among invertebrates, there may be differences in the responses of various species to hyperaccumulator plants, as shown by work on gastropods. For instance, Pollard & Baker (1997) showed that the slug *Deroceras carvanae* avoids plants containing high foliar Zn concentrations; and Boyd *et al.* (2002) found that the snail *Helix aspersa* tended to avoid *Senecio coronatus* leaves that hyperaccumulate nickel. Huitson & Macnair (2003) studied the feeding pattern of *H. aspersa* on *F₂* plants derived from a cross between the Zn-hyperaccumulator *Arabidopsis halleri* and the non-accumulator *Arabidopsis lyrata* ssp. *petraea*. Although a negative correlation between herbivory and foliar Zn concentration was observed, no significant relationship remained when the effect of experimental treatment (plant growth media containing different Zn concentrations) was statistically eliminated. Strikingly, Hanson *et al.* (2003) found that the snail *Mesodon ferrissi* showed a strong preference for *Brassica juncea* tissues with high Se content. Further investigations would be needed to determine whether this reflects a special dietary requirement for Se by *M. ferrissi*, or

whether Se hyperaccumulation reduces the amounts of other chemicals in the plant that normally protect against herbivores (similar to the lower glucosinolate concentrations observed with increased Zn accumulation in *T. caerulescens* by Tolrà *et al.* 2001). At any rate, these studies suggest that broad generalizations about the effectiveness of metal hyperaccumulation as a feeding deterrent may not be possible, and emphasize the need for detailed investigation of specific plant – herbivore interactions.

A final conclusion to be drawn from the experiments using nutritionally balanced synthetic food is that differences in Zn concentration alone are sufficient to alter the feeding behaviour of *S. gregaria*. This result is important because it demonstrates that the metal *per se* can deter feeding. In experiments with whole plants, it is difficult to exclude the possibility that herbivore feeding behaviour is influenced by some other factor that covaries with leaf metal concentration (Boyd & Moar 1999; Macnair 2003), such as an alteration in chemical composition or plant texture. Moreover, the present experiments demonstrate that Zn has a feeding-deterrent effect in the concentration range 0.5–5 mg g⁻¹ (0.05–0.5% on a dry mass basis), which is significantly below the value of 1.0% (w/w) regarded as the threshold for defining Zn hyperaccumulation in shoots of plants (Baker & Brooks 1989; Reeves & Baker 2000). This is notable when considering the evolutionary origins of the hyperaccumulation trait (Boyd & Moar 1999; Huitson & Macnair 2003; Macnair 2003), because our results suggest that comparatively small differences in tissue metal concentration might be sufficient to confer an adaptive advantage on the plant by influencing the feeding behaviour of polyphagous insect herbivores.

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