1. Introduction

Approximately 30% of all proteins are considered to require a metal cofactor, usually a transition metal such as Cu, Fe, Mn or Zn [1]. Moreover, metal ions and proteins are also functionally interdependent in other ways, including metal-mediated control of gene expression [2], direct [3] and indirect [4] metal ion involvement in intracellular signalling, and the roles of certain proteins as metallochaperones [5] and metallotransporters [6]. The requisite selectivity of these molecular events crucially depends on discriminatory metal sensors [7]. It is almost inevitable that imbalances in the homeostasis of these essential transition metals can lead to cytotoxicity and disease processes in both invertebrates and vertebrates because surpluses of redox-active species often induce reactive oxyradical generation [8]. For example, elevated levels of Fe are associated with neurodegenerative conditions such as Alzheimer’s disease [9], while the progressive liver degeneration in the genetic disorder Wilson’s disease is characterized by gross Cu deposition within hepatocytes [10]. Thus, cells and organisms are both...
beneficiaries and hostages of the coordination chemistries and redox properties of metabolically essential transition metals.

In general, essential and non-essential metals are heterogeneously distributed among biological tissues and are often compartmentalized within individual cells as a function of their identities, life-stage or health [11,12]. Significant insights into the physiology and pathophysiology of metals can potentially, therefore, be provided by bio-imaging techniques [13–15]. While technically challenging, combining the mapping of metals within subcellular compartments and simultaneously determining their chemical states (i.e. ‘speciation’) is a necessary prelude to better understanding metal homeostasis [2]. Synchrotron-based X-ray absorption spectroscopy (XAS) bio-imaging, in different modes, can map metal distributions in a spatially resolved manner, as well as providing information about oxidation states and covalence numbers. In addition, this family of intense brightness techniques can furnish structural information about the identities of neighbouring atoms, as well as information about the bond lengths separating neighbouring atoms from the metal atom cores being probed spectrascopically [16], unique properties beyond the capabilities of X-ray analyses in electron and proton probe instruments [13].

Molecular-genetic evidence indicates that Cu and Fe transport and homeostatic pathways are highly conserved from yeast to mammals [17,18]. A corollary of this statement is that the interdependent features of the Cu and Fe transport networks in mammals are illuminated by observations in lower organisms. This principle has motivated major research efforts on Cu and Fe metabolism in yeast as a model system [8,19], but has not hitherto engendered equivalent detailed studies on the diverse, often highly discriminating, intracellular metal-sequestering organelles of invertebrates. We propose to address this shortcoming by describing spatially resolved Cu and Fe redox states as well as ligand-binding speciation in the midgut (hepatopancreas) of terrestrial isopods, whose constituent cells offer an impressive example of transition metal specificity, partitioning and homeostasis.

Terrestrial isopods (suborder Oniscidea; commonly and variously referred to as ‘woodlice’, ‘sowbugs’ and ‘slaters’) are the most successful crustacean land colonizers [20]. They have long been recognized as generalist detritivores [21], but recent observations suggest that they warrant the status of keystone fungal grazers in temperate woodland habitats [22]. Recent observations suggest that they warrant the status of keystone fungal grazers in temperate woodland habitats [22].

Numerous studies have shown that the woodlouse hepatopancreas comprises two distinct cell types in roughly equal numbers and possibly forming functionally integrated units: (i) small conical ‘S’ cells that are mainly absorptive, and basally contain numerous discrete Cu-storing organelles, the cuprosomes, with an S-donating matrix; and (ii) large binucleate ‘B’ cells projecting into the organ’s lumen that are involved in absorption and secretion, and contain glycogen, prominent lipid droplets and multivesicular organelles with floccular Fe deposits sequestered [28–31] within a phosphate-rich matrix [25,28–31]. The half-life of S cells and their cuprosomes is relatively long, with some authors (e.g. [32]), but not all [33], claiming that Cu loss is negligible even in woodlice consuming a Cu-impoverished diet. The nature of the Cu-binding ligand chemistry of cuprosomes has not hitherto been described; Donker et al. [34] concluded that Porcellio scaber hepatopancreas may not contain thiol-rich metallothionein (MT), although Žnidarič [35] did identify MT-like protein in the hindgut of the same woodlouse species. It is reasonable to hypothesize that cuprosomes not only immobilize Cu to protect the storage cell from the potential toxicity of this redox-active metal, but also to release Cu to serve haemocyanin synthesis and phenoloxidase-related nutritional and immune function requirements. The cytology and metalome of B cells are even less well understood than those of S cells. Hames & Hopkin [36] reported that B cells of the predominantly nocturnal woodlice P. scaber and Oniscus asellus are morphologically plastic, undergoing a striking diurnal cycle during which the contents of the apical cytoplasm, including the Fe inclusions and lipid, are extruded at the end of the dark phase and well into the light phase in a manner reminiscent of apocrine secretion; this is followed by gradual restitution beginning towards the end of the light phase and completed during the dark phase when the cytoplasm becomes fully recharged. Thus, according to Hames & Hopkin [36], there appears to be a continuous daily cycle of Cu acquisition and excretion from B cells. By contrast, Lesšer et al. [29], in a less temporally resolved study, also observed B-cell lipid droplet extrusion in P. scaber that did not appear to follow a distinctive daily pattern. If daily B-cell rhythmicity as described by Hames & Hopkin [36] does occur, it begs fundamental questions regarding either the metabolic wastefulness or metabolic functions of wholesale release of lipid and mineralized Fe into the midgut lumen. On the other hand if, as according to Lesšer et al. [29], it does not occur, we are still left with unanswered questions relating to the chemical states and possible metabolic interactivity of Cu and Fe within the contiguous S and B cells.

The broad aim of this study was the in situ characterization of the distinct Cu-phile and Fe-phile organelles in two specialized epithelial cell types in order to better understand how transition metal storage and metabolism in woodlice contribute to their success as land colonizers, and also to explore the possibility that these organelles might provide tractable model systems for probing vital aspects of transition metal speciation and interactions at the subcellular level. Of all the available bio-imaging techniques, high-brightness and highly coherent synchrotron-based X-ray fluorescence (XRF) microscopy is uniquely capable of providing the necessary analytical sensitivity, spatial resolution and ability to determine metal oxidation states [15,16,37] to pursue these goals. Our study had a core specific aim: to determine in situ the oxidation states and ligand-binding speciation of Cu and Fe in the S and B cells of the hepatopancreas in laboratory-
acclimated woodlouse (O. asellus) at two extreme nodes of the presumptive daily B-cell extrusion/restitution cycle, using preparative procedures that preserve simultaneously the integrity of cellular morphology and chemistry. We undertook an adjunct experiment using light microscope-based morphometry to determine the presence of a daily wave of apocrine secretory activity in the B cells of our cultured woodlouse.

2. Material and methods

2.1. Whole hepatopancreas elemental analyses

Pooled hepatopancreas tubules (each replicate dissected from five individual Oniscus asellus) were digested in 2 ml boiling 16N HNO₃ on a sand bath. Digests were made up to 10 ml with ultrapure water, and analysed in a YJ Horiba Ultima-2 inductively coupled plasma optical emission spectrometer (ICP-OES). Five replicates of hepatopancreas were collected at 02.00 and 12.00 h, these intervals corresponding with the times at which the tubules for synchrotron-based imaging and analysis were taken. Analyses of a standard tissue (marine mussel, GBW 08571; State Bureau of Technical Supervision, China) indicated that the combined tissue-processing and analysis were taken. Analyses of a standard tissue (marine mussel, GBW 08571; State Bureau of Technical Supervision, China) indicated that the combined tissue-processing and analytical protocols yielded values within 8% of expectations.

2.2. Hepatopancreas morphometry

For the morphometry experiment, adult inter-moults woodlice (O. asellus; Crustacea, Isopoda, Oniscidae) were collected from an unpolluted reference site at Coopers Field, Bute Park, Cardiff, UK (NGR 317819 176785, 51 29'20.4"N, 3'11'20.4" W). Eight individuals were placed into each of 12 plastic pots (8 cm diameter, 5 cm high) with perforated lids. Dry, heat-sterilized soil from the sampling site had already been inserted into the pots to a depth of 2 cm and then moistened thoroughly with deionized water. Leaf litter (Acer pseudoplanatus) from Coopers Field was added to the pots as a food source. Soil moisture and food were checked at weekly intervals during the acclimation period, and replenished as required. The woodlice were maintained in their pots for four weeks in a Binder GmbH incubator (Tuttlingen, Germany) at 20 °C, 75% humidity, and a 12 L: 12 D regime (light on at 06.00, light off at 18.00). Hepatopancreas samples for microfocus analyses were dissected and immediately processed from woodlice collected at 12.00 and 02.00 h (i.e. 6 h into the light and dark phases, respectively). These temporal ‘nodes’ were initially chosen because they corresponded approximately to the mid-extrusion and mid-restitution phases of the diurnal apocrine cycle within the hepatopancreas as described by Hames & Hopkin [36]. The preparative procedure used was compared in a previous microfocus study on earthworms [38] with cryo-sectioning of unfixed fresh tissues, and was deemed to maintain faithfully both the morphological and compositional fidelity of tissues and cells to degrees permitting meaningful microfocus imaging, mapping and analysis. It involved gentle fixation for approximately 3 days in 70% alcohol of one or more tubules from individual woodlice, followed by glycol methacrylate embedding and transverse sectioning in the range of 2–10 μm. Sections were initially chosen because they corresponded approximately to the mid-extrusion and mid-restitution phases of the diurnal apocrine cycle within the hepatopancreas as described by Hames & Hopkin [36]. The preparative procedure used was compared in a previous microfocus study on earthworms [38] with cryo-sectioning of unfixed fresh tissues, and was deemed to maintain faithfully both the morphological and compositional fidelity of tissues and cells to degrees permitting meaningful microfocus imaging, mapping and analysis. It involved gentle fixation for approximately 3 days in 70% alcohol of one or more tubules from individual woodlice, followed by glycol methacrylate embedding and transverse sectioning in the range of 2–10 μm. Sections were mounted on 25 × 50 mm spectroscopic-quality Spectrosil 2000® or fused quartz Vitreosil 077® glass slides (UQG Optics Ltd., Milton, Cambridge). Slides were inserted into the standard Diamond I18 beamline sample holder, orientated and imaged under brightfield illumination, then subjected to metal mapping and μ-focus spectroscopy, with different acquisition times and levels of spatial resolution, almost exclusively at ambient temperature.

XAS data were collected on beamline I18 at Diamond Light Source using a Si(111) double crystal monochromator, and the Kirkpatrick-Baez focusing mirrors, which provide a 3 μm spot size, were also used to remove harmonic contamination [39]. Calibration spectra for Cu, Fe and Zn foils were recorded in transmission mode. Data for the samples were collected in fluorescence mode using an Ortec (Oakridge, USA) 9-element Ge detector. Elemental distribution maps for Cu, Fe and Zn were acquired and processed essentially as described previously for Pb and Zn in sectioned earthworm tissues [40]; these were used to determine the areas to collect the XAS spectra. The XAS data were reduced in the program Athena [41]. X-ray Absorption Near Edge Structure (XANES) data were modelled as linear combinations of the XANES spectra of selected standards, collected previously and energy corrected using the calibration spectra [42]. The Extended X-ray Absorption Fine Structure (EXAFS) data were analysed using exact.
curved wave theory [43] in DL-EXCURV [44]. Phaseshifts were derived in the program from \textit{ab initio} calculations using Hedin–Lundqvist potentials and von Barth ground states [45]. The data were fitted for each sample by defining a theoretical model and comparing the calculated EXAFS spectrum with the experimental data. Shells of backscatterers were added around the absorber atom and by refining an energy correction $E_F$ (the Fermi energy), the absorber–scatterer distance and the Debye–Waller factor for each shell, a least-squares residual (the $R$-factor [46]) was minimized. For fits with two shells, a reduced $\chi^2$-statistical test was used to check that inclusion of the additional fitting parameters was justified.

The stability under beam irradiation of the Cu, Fe and Zn signals was evaluated by collecting a series of consecutive XANES spectra under standard operational conditions from selected locations in S and B cells in the resin-impregnated tissue sections.

Some $\mu$XRF maps for P K-edge (2014 eV) and S K-edge (2308 eV) were acquired using a four element Si drift detector with a beryllium window (Hitachi Inc.) positioned close to the specimen. The relatively strong Ar K-edge (3.203 eV) signal from ambient air interferes with the P and S emissions; it was reduced but not eliminated by enclosing the specimen and detector inside a bespoke bag under flowing (300–400 ml min$^{-1}$) He to give a largely He environment during analysis. No $\mu$XANES scans were collected at either the P or S K-edge. $\mu$XRF maps for these two anionic elements were used qualitatively to determine their co-distributions with the transition metals of interest.

3. Results

3.1. Elemental composition of whole hepatopancreas

There were no significant differences in the Cu, Fe, Zn and P concentrations in the hepatopancreas tubules of woodlice sampled at 02.00 and 12.00 h (table 1). Cu was the dominant transition metal with a concentration approximately $\times 6.5$ higher than Fe, and approximately $\times 2$ higher than Zn in these physiologically ‘normal’ samples. The concentrations of each of the Group 2 elements analysed (Mg, Ca, Sr, Ba) tended to be higher during the day (12.00 h) compared with the night (02.00 h); interestingly, metabolically essential Mg and Ca are recognized as predominantly extracellular in distribution. The concentration of K, a predominantly intracellular electrolyte, did not differ at the two time points.

3.2. Hepatopancreas morphometry

Epithelial thickness in the woodlouse hepatopancreas essentially reflects the height of the B cells protruding into the tubule lumen (figure 1a,b). Morphometric analysis at 2-hourly intervals provided evidence of a diurnal pattern of extrusion/restitution change in epithelial thickness (Kruskal–Wallis, $p < 0.05$) in woodlice acclimated to the experimental light regime (figure 1c–e). From the middle of the dark period (04.00 h), the mean epithelial thickness increased from its nadir of 33.2–68.0 $\mu$m at the end of the dark period (08.00 h). The B cells subsequently appeared to be more-or-less fully charged from around the night/day transition, rising to a peak at midday (12.00 h), from which point the

<table>
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<tr>
<th>Treatment</th>
<th>Cu (mg/g dry weight)</th>
<th>Fe (mg/g dry weight)</th>
<th>Zn (mg/g dry weight)</th>
<th>P (mg/g dry weight)</th>
<th>Na (mg/g dry weight)</th>
<th>Mg (mg/g dry weight)</th>
<th>Ca (mg/g dry weight)</th>
<th>Sr (mg/g dry weight)</th>
<th>Ba (mg/g dry weight)</th>
</tr>
</thead>
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<tr>
<td>02.00 h</td>
<td>4.94 (± 0.67)</td>
<td>7.65 (± 0.86)</td>
<td>2.59 (± 0.56)</td>
<td>19.02 (± 0.52)</td>
<td>12.10 (± 0.48)</td>
<td>14.07 (± 0.51)</td>
<td>26.44 (± 1.76)</td>
<td>46.03 (± 0.93)</td>
<td>50.02 (± 0.93)</td>
</tr>
<tr>
<td>12.00 h</td>
<td>4.82 (± 0.40)</td>
<td>4.92 (± 0.31)</td>
<td>3.11 (± 0.18)</td>
<td>26.79 (± 0.37)</td>
<td>32.81 (± 0.29)</td>
<td>15.76 (± 0.39)</td>
<td>27.31 (± 3.33)</td>
<td>67.06 (± 3.33)</td>
<td>57.06 (± 3.33)</td>
</tr>
</tbody>
</table>
thickness parameter fluctuated (reflecting considerable B-cell asynchrony, and inter-individual variability) but, nevertheless, tended to decline until the cytoplasmic restitution was initiated at night. Direct measurements of B-cell height confirmed this cyclical diurnal pattern (data not shown). A reversed light regime experiment (i.e. switching O. asellus from light: dark to dark: light over a four-week acclimation period) provided further evidence of rhythmicity; a distinct, albeit incomplete, shift in the pattern of cell morphology was observed with the extruded cells firmly confined to the light period and restitution peaking in the dark period (electronic supplementary material, figure S1).

3.3. μXAS in situ measurements: Cu, Fe and Zn K-edges in thin sections

The μXRF maps more so than the corresponding unstained sections demonstrate that, in a given transverse optical plane, there is a fundamental symmetry in the arrangement of the two epithelial cells in the hepatopancreas, with each B cell flanked by two S cells (figure 2; electronic supplementary material, figures S2 and S3). μXRF maps indicate that Cu is concentrated as focal intracellular deposits in the basal cytoplasm of S cells in hepatopancreas tubules sampled at both 02.00 and 12.00 h (figure 2b,c,f; electronic supplementary material, figures S2b, S2c and S3). By contrast, Fe displayed a dispersed distribution pattern within B cells at both time intervals, but with an apparent bias towards the apical cytoplasm near the interval (02.00 h) when extrusion approaches completion and switches to restitution (figure 2b,c,f; electronic supplementary material, figures S2b, S2c and S3). Some images and μXRF maps (especially figure 2b; electronic supplementary material, figures S3a, S3b) provide evidence for the formation of large apical blebs in a number of B cells, with traces of diffuse material of apparently similar composition free in the lumen (figure 2b; electronic supplementary material, figure S3b). Cu was co-distributed with S, while Fe was co-distributed with P (cf. figure 2b,c). Zn was almost exclusively associated with Cu within the S cells of O. asellus.
Figure 2. \(\mu\)XRF maps of element distributions in unstained methacrylate-embedded thin mid-tubule sections of woodlouse hepatopancreas. (a) Light micrograph of a transverse section from a woodlouse sampled at 02.00 h (i.e. the beginning of B-cell restitution during darkness). Note that the morphology of the sections used for \(\mu\)XRF is relatively unclear due to a lack of differential contrast in unstained, methacrylate-embedded sections. (b) Superimposed Cu, Fe and Zn \(\mu\)XRF maps of the section depicted in (a); note the co-distribution of Cu and Zn in S cells (arrowheads), and the distribution of diffuse Fe mainly in B-cell apical cytoplasm that might be undergoing blebbing (arrows), and some Fe signal within the tubule lumen (broken arrows). (c) Superimposed Cu, P and S \(\mu\)XRF maps of the section depicted in (a); note the co-distribution (yellow) of Cu and S in S cells (arrowheads), and the mainly apical distribution of P in B cells (arrows). (d) Light micrograph of a mid-tubule transverse section from a woodlouse at 12.00 h (i.e. at the beginning of B-cell extrusion during the day). (e) Superimposed Cu, Fe and Zn \(\mu\)XRF maps of the section depicted in (d); note that the transition metal distribution patterns are similar to those seen in (b). (f) Expanded view \(\mu\)XRF maps for the region delineated by a broken-lined rectangle in (e); note that Fe is distributed above and below the nuclear plane (arrow) of the prominent B cell, albeit mainly in the apical cytoplasm. (g) and (h) are schematic diagrams derived from Hames & Hopkin [36], illustrating the gross difference between B- and S-cell morphologies (g) and showing a ‘clock’ summarizing the diurnal cycle of apocrine secretion in B cells in relation to the light : dark regime with our two XAS sampling points superimposed upon it (h).

**asellus** hepatopancreas (figure 2b,e,f; electronic supplementary material, figures S2b, S2c and S3). Although the spatial resolution of the images obtained from the synchrotron is limited, some \(\mu\)XRF maps (e.g. electronic supplementary material, figure S2c) give the impression that the Cu-rich cytoplasm of S cells extends under the basal regions of the neighbouring B cells, indicating a close morphological intimacy between the two cell types.

The Cu K-edge XANES spectra exhibit three main features: a peak (or shoulder) on the absorption edge at 8982.5 eV, the edge crest at 8995 eV and a peak at 9011 eV (figure 3a). For the XANES spectra of the model compounds, only Cu$_2$O shows the first peak (figure 3b), and its height in the spectra derived from methacrylate-embedded sections of woodlouse hepatopancreas correlates with the contribution from Cu$_2$O in the XANES fitting (table 2). Moreover, the Cu edge values (8980 and 8981 eV) determined for spectra collected from S cells correspond well with literature values obtained for the maximum of the first derivative of XANES from a number of Cu(I) compounds (ranging from 8980.9 to 8983.1 eV) compared with Cu(II) compounds (8984.4 to 8988.0 eV) (electronic supplementary material, table S1). In the majority of cases, B-cell spectra displayed a higher contribution from the characteristic Cu(I) peak than did the spectra derived from S cells, perhaps suggesting that the B cells contain mixed Cu species albeit with a dominant S-bound phase. Calculated Cu K-edge values from B cells (8980.9 and 8981.0 eV) confirm the presence of reduced Cu(I) species in this cell type (electronic supplementary material, table S1). Both the XANES fitting (table 2) and the EXAFS fitting for four relatively noise-free spectra (figure 4a,b and table 3) showed that Cu, probably in its reduced Cu$^+$ state, is mainly coordinated to S-donating ligands, with no indication of any outer shells. EXAFS indicated that there was no significant difference between the fit for an S cell at the time (02.00 h) roughly corresponding to the extrusion/restitution cross-over and the fits for B cells at the same time interval and near the restitution peak (12.00 h) (table 3). The data were not good enough to justify fitting the inner coordination sphere with a mixture of oxygen and sulfur scatterers.

The Fe K-edge XANES spectra were all very similar, with a pre-edge feature (1s-3d) at 7117 eV, the edge crest at 7131 eV and a peak at 7137 eV (figure 5a). The Fe XANES spectra from model compounds (figure 5b) most strongly corresponding to those from hepatopancreas thin sections...
were acquired from octahedrally oxygen-bound Fe$^{3+}$ models, Fe$_2$O$_3$ and Fe(NO$_3$)$_3$ (cf. figure 5a,b). The XANES fitting (table 2) showed predominantly oxygen coordination for Fe, which was confirmed by EXAFS fitting (table 3 and figure 6a,b). The Fe–O bond length of approximately 2 Å is consistent with mainly six-coordinate Fe$^{3+}$. An outer shell

Table 2. K-edge (Cu, Fe) XANES fitting of spectra derived from the two cell types in O. asellus hepatopancreas sampled at 02.00 (un-shaded rows) and 12.00 h (shaded rows).

<table>
<thead>
<tr>
<th>metal</th>
<th>cells</th>
<th>clock time (hours)</th>
<th>metal in the form of (%)</th>
<th>metal in the form of (%)</th>
<th>metal in the form of (%)</th>
<th>metal in the form of (%)</th>
<th>R factor (%)</th>
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<tbody>
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<td>CuS</td>
<td>CuO</td>
<td></td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>S</td>
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<td>8</td>
<td>87</td>
<td>5</td>
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<td>—</td>
<td>95</td>
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<td>12</td>
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<tr>
<td>Fe</td>
<td>B</td>
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<td>Fe$_2$O$_3$</td>
<td>Fe(NO$_3$)$_3$</td>
<td>FeS</td>
<td>FeO</td>
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<td></td>
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of Fe scatterers at approximately 3.05 Å could be fitted for the three EXAFS spectra derived from B cells (one at 02.00 h and two at 12.00 h), possibly implying the presence of an iron oxyhydroxide phase. The short usable data range of the single S-cell spectrum (12.00 h) prevented any outer shell fitting for this sample. There were no significant observable differences in Fe ligand-speciation binding between S and B cells, either in the XANES (table 2) or the EXAFS (table 3) datasets.

XANES and EXAFS spectra (data not shown) indicated that Zn is predominantly bound by O-donating ligands, with a relatively minor S-bound phase. This implies that while Zn is distinctly co-distributed with Cu within S cells (figure 2b,e,f; electronic supplementary material, figures S2b, S2c and S3), the cell may sequester redox-inactive Zn in two major pools. The electronic supplementary material, figure S2c provides spatial evidence that there is a shared Cu + Zn compartment, as well as separate Cu-only and Zn-only compartments in individual S cells. (The issue of Zn speciation under physiological and hyper-accumulation conditions is the subject of a separate detailed XAS study [47].)

Sequential analyses of a given spot on randomly selected hepatopancreas sections yielded some indication that the Cu XANES signal derived from B cells was initially stable under irradiation, but then showed some progressive erosion of the shoulder on the absorption edge at 8982.5 eV from the third spectrum onwards (figure 7a–c). Flattening after three or more acquisition scans of the Cu XANES signal derived...
from S cells was more subtle, possibly because the edge feature at 8982.5 eV was less pronounced (electronic supplementary material, figure S4a–c). Fe XANES spectra from both cell types were stable under repeated irradiation (figure 8a,b). Zn XANES spectra derived from S cells revealed some evidence of radiation-induced change in later scans (electronic supplementary material, figures S5a and S5b). Overall, these qualitative observations provide confidence in the biological veracity of the acquired qualitative and quantitative XAS data.

4. Discussion

Hames & Hopkin [36] qualitatively examined the fine structure of the S and B cells of two terrestrial isopod species (O. asellus and P. scaber) at hourly intervals over a 24 h period, and concluded that while the morphology of the S cells remained unchanged, B-cell morphology displayed the hallmarks of a diurnal pattern of apocrine secretion. By contrast, Leser et al. [29] morphometrically examined the hepatopancreatic epithelium of P. scaber at four time points during a 24 h
period and observed that neither epithelium thickness nor lipid droplet abundance differed significantly, although lipid droplets were concentrated conspicuously in B-cell apical cytoplasm 2 h into the light phase. Our findings on O. asellus, using the morphometric method described by Leser et al. [29], were in general agreement with those of Hames &

Figure 7. Radiation sensitivity of Cu speciation assessed by collecting a sequential series of five XANES spectra from given regions in three different B cells (a–c). The thin methacrylate-embedded sections were obtained from a woodlouse sampled at 02.00 h. In each panel, the large down-pointing vertical arrow indicates that the top spectrum was collected first and the lowest spectrum last. Note that the shoulder on the absorption edge at 8982.5 eV (broken arrows), while remaining visible, is progressively eroded from the third acquired spectrum onwards.

Figure 8. Radiation sensitivity of Fe speciation assessed by collecting a sequential series of five XANES spectra from given regions in a B cell (a) and in an S cell (b). The thin methacrylate-embedded sections were obtained from a woodlouse sampled at 02.00 h. In both panels, the large down-pointing vertical arrow indicates that the top spectrum was collected first and the lowest spectrum last. Note that successive spectra remain very similar.
Hopkin [36]. Indeed, we also found that a cyclical apocrine pattern was evident, albeit less pronounced, in woodlice exposed to a reversed lighting regime for four weeks (electronic supplementary material, figure S1). It is difficult to reconcile the disparities relating to the presence or the absence of a B-cell apocrine cycle linked to a diurnal trophic pattern, but it is germane that according to Lesér et al. [29] the properties of the hepatopancreas epithelium are altered progressively after transfer from favourable field conditions to laboratory culture where, for example, the diet is typically less heterogeneous. Other potential confounding factors are weak synchronicity in the B-cell population at certain time points, as well as a relatively high degree of inter-individual variation apparent in our morphometry dataset that probably reflects asynchronous feeding patterns. In any debate about the existence and pattern of apocrine secretion in isopod B cells, it is not inconsequential that these cells are binucleate. ‘Normal’ binucleate cells that are involved in forms of apocrine secretion are fairly common in insect taxa [48] where, according to Anhe & Azeredo-Oliveira [49], the additional genetic material is essential to drive cell restitution after the extensive secretory phase. Also pertinent is that a daily cycle of changes in haemolymph osmolality, ammonia and glucose levels linked to nocturnal feeding has been recorded in a semi-terrestrial isopod [50]. Moreover, Nakamura & Wright [51] recently described a diurnal cycle of glutamine storage and ammonia excretion in fully terrestrial isopods, probably associated with cyclical absorption and subsequent catabolism of dietary proteins.

In this study, whole hepatopancreas analyses, and in situ Cu and Fe K-edge μXAS analyses, did not reveal any striking temporal differences in metal content or ligand-binding speciation at the two selected time points (i.e. 6 h into the light and dark phases, respectively). Given the variations in cell morphology alluded to above, and the replication constraints imposed by the intrinsically low throughput of XAS micro-beam analysis, this may not be surprising.

Wieser [27] remarked that the woodlouse S cell is so densely packed with Cu-rich vesicles that it is difficult to envisage these small cells having the capacity to do anything other than sequester and store Cu. If we assume that the bulk of hepatopancreatic Cu resides in S cells, and that the estimated average volume fraction of these cells including the nucleus during the daily cycle is 20%, then extrapolating from tubule absorption and subsequent catabolism of dietary proteins.

To the extent that the hepatopancreas is a distinctly oxic environment [61], the Fe3+ oxidation state was not unexpected. The notion that Fe is regularly released along with lipid droplets from B cells into the alimentary lumen appears well-founded [36], whether a well-defined diurnal cycle of apocrine secretion occurs or not. Leaf litter rich in recalcitrant lignocelluloses is ingested by terrestrial woodlice and is ultimately digested by enzymes derived from litter-colonizing microflora and by endosymbionts [56] as well as from truly endogenous sources. Elegant work on wood-decaying fungi [62,63] shows that polysaccharide depolymerization by oxyl radicals generated via Fenton-type reactions, involving Fe2+ and Fe3+ as well as Cu2+, is an important prelude facilitating access of enzymes to the dietary substrates. The micro-oxic/anoxic conditions prevailing in the radial centre of the posterior hindgut of woodlice [61] provide the reducing environment conducive to these reactions. However, the digestibility of cellulose is suppressed by Fe3+-mediated oxidation and cellules can be inhibited by Fe3+ [64,65] but, again, reducing conditions in proximity to alimentary sites of lignocellulose digestion to monomeric products would promote enzyme activities. Polyphenols (tannins) ingested in large quantities by woodlice can not only potentially inhibit digestive enzyme activity by precipitation [66], but can also scavenge oxyl radicals and block Fenton reactions by binding Fe2+ and Cu2+ [67,68]. It is known that the gut fluids of marine [69] and terrestrial isopods are rich in surfactants [70]. Surfactants are diverse in composition and sources, but they are often lipid derivatives [71]. Apart from facilitating the transport of hydrophobic digested lipids towards absorptive gut surfaces [72], they are known to counteract protein binding by polyphenols and, thus, can liberate the activities of phenoloxidases, catalases and other gut enzymes [70]. It is an intriguing possibility that lipid droplets regularly secreted by hepatopancreas B cells in harmony with the trophic cycle provide a continuous to gut flora [56], it is plausible that the hepatopancreas epithelia are another potential source of the Cu-containing enzyme. At the present time, the definitive molecular nature of the dominant Cu storage fractions in woodlouse hepatopancreas is not known. While not excluding the possibility that thiol-rich MTs may be involved in Cu storage, Donker et al. [34] favoured a role for haemocyanin and/or its metabolites. Since crystallographic study [57] shows that arthropod prophenoloxidase has the ‘canonical’ three di-nuclear Cu centre, with each Cu ion coordinated to non-thiol histidine, our μXANES and μEXAFS observations tend to suggest the involvement of Cu sequestering roles for MT-like peptides [58] in isopod S cells. It is pertinent that Engel & Brouwer [59] presented evidence that Cu-MT in a marine crustacean can transfer Cu to the active site of apo-haemocyanin. Hopkin [60] stated unequivocally that wholesale voiding of cuprosomes from S cells into the lumen does not occur. The previously unsuspected detection of Cu in the apocrine B cells might lend credence to the hypothesis that the hepatopancreas exports Cu-containing molecules into the hepatopancreas lumen via this cell type, although our microfocus findings did not implicate a type 3 Cu protein.

Iron is the transition metal ion with the greatest variety of binding sites in proteins [58]. However, hardly any attention has previously been devoted to the coordination chemistry or functional aspects of Fe in woodlouse hepatopancreas. We detected Fe in both epithelial cell types, where its redox state and coordination chemistry was very different to that of Cu; Fe was always found as Fe3+ with predominantly O-coordination. To the extent that the hepatopancreas is a distinctly oxic environment [61], the Fe3+ oxidation state was not unexpected. The notion that Fe is regularly released along with lipid droplets from B cells into the alimentary lumen appears well-founded [36], whether a well-defined diurnal cycle of apocrine secretion occurs or not. Leaf litter rich in recalcitrant lignocelluloses is ingested by terrestrial woodlice and is ultimately digested by enzymes derived from litter-colonizing microflora and by endosymbionts [56] as well as from truly endogenous sources. Elegant work on wood-decaying fungi [62,63] shows that polysaccharide depolymerization by oxyl radicals generated via Fenton-type reactions, involving Fe2+ and Fe3+ as well as Cu2+, is an important prelude facilitating access of enzymes to the dietary substrates. The micro-oxic/anoxic conditions prevailing in the radial centre of the posterior hindgut of woodlice [61] provide the reducing environment conducive to these reactions. However, the digestibility of cellulose is suppressed by Fe3+-mediated oxidation and cellulases can be inhibited by Fe3+ [64,65] but, again, reducing conditions in proximity to alimentary sites of lignocellulose digestion to monomeric products would promote enzyme activities. Polyphenols (tannins) ingested in large quantities by woodlice can not only potentially inhibit digestive enzyme activity by precipitation [66], but can also scavenge oxyl radicals and block Fenton reactions by binding Fe2+ and Cu2+ [67,68]. It is known that the gut fluids of marine [69] and terrestrial isopods are rich in surfactants [70]. Surfactants are diverse in composition and sources, but they are often lipid derivatives [71]. Apart from facilitating the transport of hydrophobic digested lipids towards absorptive gut surfaces [72], they are known to counteract protein binding by polyphenols and, thus, can liberate the activities of phenoloxidases, catalases and other gut enzymes [70]. It is an intriguing possibility that lipid droplets regularly secreted by hepatopancreas B cells in harmony with the trophic cycle provide a continuous to gut flora [56], it is plausible that the hepatopancreas epithelia are another potential source of the Cu-containing enzyme. At the present time, the definitive molecular nature of the dominant Cu storage fractions in woodlouse hepatopancreas is not known. While not excluding the possibility that thiol-rich MTs may be involved in Cu storage, Donker et al. [34] favoured a role for haemocyanin and/or its metabolites. Since crystallographic study [57] shows that arthropod prophenoloxidase has the ‘canonical’ three di-nuclear Cu centre, with each Cu ion coordinated to non-thiol histidine, our μXANES and μEXAFS observations tend to suggest the involvement of Cu sequestering roles for MT-like peptides [58] in isopod S cells. It is pertinent that Engel & Brouwer [59] presented evidence that Cu-MT in a marine crustacean can transfer Cu to the active site of apo-haemocyanin. Hopkin [60] stated unequivocally that wholesale voiding of cuprosomes from S cells into the lumen does not occur. The previously unsuspected detection of Cu in the apocrine B cells might lend credence to the hypothesis that the hepatopancreas exports Cu-containing molecules into the hepatopancreas lumen via this cell type, although our microfocus findings did not implicate a type 3 Cu protein.
source of surfactant substrate and contribute, along with metallo-compound secretions, towards the efficient digestion of recalciitrant dietary macromolecules.

In conclusion, the findings of this study contribute to the general notion that alimentary function in woodlice such as O. asellus is a multifactorial network of interactions involving secretions from structurally and functionally differentiated regions of the invertebrate’s alimentary canal, the activities of regionally specialized members of its microbiome, and ingested biotic and abiotic materials. More specifically, the findings call for a re-appraisal of the relationships between the hepatopancreatic B and S cells. These very specialized epithelial cells have to date been examined by a variety of microscopic and relatively insensitive microprobe analytical methods as if they are free-standing entities. High-resolution μXRF mapping with a high-brightness synchrotron beam suggests otherwise: a sounder hypothesis views them as morphologically integrated functional units. This view is supported by the intimacy of the highly infolded basal membranes of neighbouring cells [36] and the hitherto unsuspected ‘sharing’ of common Cu and Fe ligand-binding species within both cell types. The new paradigm envisages the partners forming these functional units having particular roles, with the S cells serving as dominant metal acquisition/storage hubs, while the B cells are primarily engaged in distribution logistics involving metals, metallo-compounds including pro-enzymes, and lipids. The paradigm should engender the formulation of novel hypotheses that can be tested with the full spectrum of contemporary bio-imaging and ‘omics’ tools.

Data accessibility. Raw spectral data associated with figures 2–8 and electronic supplementary material, figures S2–S5 are available from Dryad Digital Repository using the following link: http://dx.doi.org/10.5061/dryad.8734c.

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