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1	The crustacean cuticle does not record chronological age:
2	New evidence from the gastric mill ossicles
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4 5	Carola Becker ^{1,2,*} , Jaimie T. A. Dick ^{1,3} , Eoghan Mánus Cunningham ¹ , Clemens Schmitt ⁴ , Julia D. Sigwart ¹
6	¹ Queen's University Marine Laboratory, 12-13 The Strand, Portaferry BT22 1PF, Northern Ireland
7 8	² Humboldt Universität zu Berlin, Department of Biology, Comparative Zoology, Philippstr. 13, Haus 2, 10115 Berlin, Germany (present address)
9 10	³ Institute for Global Food Security, School of Biological Sciences, Queen's University Belfast, MBC, 97 Lisburn Road, Belfast BT9 7BL, Northern Ireland
11 12	⁴ Max Planck Institute of Colloids and Interfaces, Potsdam-Golm Science Park, Am Mühelnberg 1 OT, Golm, Germany
13	
14	* Corresponding author: Carola Becker, carolabecker.germany@gmail.com
15	Co-authors email addresses: j.dick@qub.ac.uk, ecunningham18@qub.ac.uk,
16	clemens.schmitt@mpikg.mpg.de, j.sigwart@qub.ac.uk
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26 Abstract

A proposed method to determine chronological age of crustaceans uses putative annual bands in 27 28 the gastric mill ossicles of the foregut. The interpretation of cuticle bands as growth rings is based on the idea that ossicles are retained through the moult and could accumulate a continuous record 29 of age. However, recent studies presented conflicting findings on the dynamics of gastric mill 30 ossicles during ecydysis. We herein study cuticle bands in ossicles in four species of commercially 31 32 important decapod crustaceans (Homarus gammarus, Nephrops norvegicus, Cancer pagurus and 33 Necora puber) in different phases of the moult cycle using dissections, light microscopy, micro-34 computed tomography and cryo-scanning electron microscopy. Our results demonstrate that the 35 gastric mill is moulted and ossicles are not retained but replaced during ecdysis. It is therefore not plausible to conclude that ossicles register a lifetime growth record as annual bands and thereby 36 37 provide age information. Other mechanisms for the formation of cuticle bands and their correlation to size-based age estimates need to be considered and the effect of moulting on other 38 cuticle structures where 'annual growth bands' have been reported should be investigated 39 40 urgently. Based on our results, there is no evidence for a causative link between cuticle bands and 41 annual moult increments, meaning it is unreliable for determining crustacean age.

42 **Keywords:** lobster; crab; age determination; sclerochronology; ecdysis; moulting.

43

44 **1. Introduction**

45 A record of the age of individual organisms can manifest as annual rings or bands in hard skeletal 46 structures if growth is subject to cyclic variation in environmental conditions, such as seasons. Dendrochronology, for example, uses tree rings to determine age and improve the understanding 47 48 of past and future environmental impacts on tree growth (Creber, 1977). Similarly, ageregistering hard structures are used in a variety of vertebrate and invertebrate groups. In fish, 49 50 otoliths or scales can record annual growth rings (Campana, 2001); in bivalves, shells possess 51 annual bands (Abele et al., 2009); and the statoliths of squid can show rings which have been 52 interpreted as daily growth increments (Rodhouse and Hatfield, 1990). In crustaceans, it has been assumed that growth bands do not exist due to the loss of hard structures with the moult (Farmer, 53 54 1973; Hartnoll, 2001; Vogt, 2012).

55 **1.1 Previous methods of crustacean age determination**

As direct anatomical ageing methods have not been available for crustaceans, determination of
 chronological age (as opposed to simply body size or sexual maturity) has mainly relied on several

indirect methods. The conventionally used method is to calculate growth models, which are most 58 59 commonly based on size modal analysis or on the observation of intermoult duration and increase 60 in size at moulting, from specimens in captivity or tagging in wild populations (Vogt, 2012; Wahle 61 & Fogarty, 2006). This method requires well constrained seasonality of reproductive cycles or 62 recruitment events, and is only applicable if growth increments and intermoult periods are not too variable among individuals. Separately, a biochemical approach, the lipofuscin method, uses 63 64 the accumulation of this so-called "age pigment" as a by-product of mitotic activity in ageing cells (Matthews et al., 2015). Lipofuscin is, however, an indicator of physiological rather than 65 chronological age, since its deposition rate is influenced by metabolic and environmental 66 conditions (O'Donovan and Tully, 1996). The lipofuscin approach therefore requires calibration 67 for each species and different environmental conditions by using specimens of known age 68 69 (Maxwell et al., 2007). Direct observations of crustacean ageing and longevity in the field are only 70 possible through mark-release-recapture approaches, which can be very cost-intensive and time 71 consuming (see Vogt, 2012 for a comprehensive review on ageing crustaceans).

72 **1.2 Crustacean age determination through cuticle growth bands – fact or fiction?**

An apparently promising new anatomical approach to ageing crustaceans was first published in
2011 (Leland et al., 2011) and has been increasingly applied since then (e.g. Sheridan et al., 2015;
Kilada et al., 2015; Krafft et al., 2016). This method used strongly calcified cuticular structures and
interpreted bands observed in cross-sections of the endocuticle as annual growth increments.

The numbers of observed endocuticular bands increase with body size, and seem to correspond
to size-based age estimates (Kilada et al., 2012; Leland and Bucher, 2017). The calcified basis of
the eyestalk is the structure predominantly used in krill (Krafft et al., 2016; Kilada et al., 2017a)
and shrimp (Kilada et al. 2012; Kilada and Acuña, 2015; Kilada et al., 2015; Kilada et al., 2017b).
In most of the larger decapod crustaceans, calcified endoskeletal structures of the foregut, the
gastric mill ossicles, are used (Table 1).

An important premise of this method is an assumption that the structures used for ageing are 83 84 retained through the moult. Evidence for such retention was reported in the original publications, 85 as: (1) the absence of certain gastric mill ossicles in exuviae; and (2) the perpetuation of a calciumbinding fluorescent live marker in the endocuticle of ossicles through several moults (calcein 86 87 detection) (Leland et al., 2011; Kilada et al., 2012). However, recent studies demonstrated gastric mill ossicles are not retained through moulting in the Norway lobster, *Nephrops norvegicus*, the 88 89 white-clawed crayfish, Austropotamobius pallipes (Sheridan et al., 2016) and the shore crab 90 Carcinus maeans (Sheridan & O'Connor, 2018) and instead the ossicles were shed into the stomach 91 contents. Although the ossicles were apparently lost at moulting, a calcein fluorescent stain applied before the moult was still detected in ossicles of post-moult specimens (Sheridan et al.,
2016). A study on the dynamics of certain gastric mill ossicles during the moult cycle of the blue
crab, *Callinectes sapidus*, demonstrated that these structures are partially resorbed and shed at
ecdysis (Vatcher et al., 2015).

96 All of these studies demonstrate the current scientific controversy around the interpretion of 97 crustacean cuticle bands as annual rings. Nevertheless, gastric mill ossicles are increasingly being 98 used to determine crustacean age by counting bands in the endocuticle (Kilada et al., 2017b). 99 There are thus significant questions remaining about the actual mechanism that shapes the 100 observed bands. Yet this novel method has been mentioned alongside the traditional approaches 101 of ageing crustaceans in a recent review (Kilada and Driscoll, 2017).

102 (insert table 1)

103 **1.3 The crustacean cuticle and the moult**

Prior research on crustacean moulting has mostly focussed on the cuticle of the carapace and 104 105 limbs (Welinder, 1974; 1975a, b). However, the cuticular exoskeleton is a continuous sheet that 106 not only forms the outer shell but also lines several internal organs, including the structures in the 107 foregut, and develops skeletal elements inside the body (Davie et al., 2008). The crustacean cuticle 108 is always organised in distinct layers, which are characterised by their location, chemical 109 composition (especially mineralisation), structural and mechanical properties (Fabritius et al., 2016). An underlying hypodermis secretes the cuticle, which is divided into a procuticle and an 110 epicuticle. The outermost epicuticle is a generally thin and waxy layer which can also form scales, 111 setae and teeth (as in the zygocardiac and urocardiac osscicle, see Fig. 3c). The procuticle 112 113 constitutes the main component of the exoskeleton and is divided into a proximal endocuticle and 114 a distal exocuticle. The membranous layer is per definition the unmineralised, most basal part of 115 the endocuticle and directly overlies the hypodermis (Fabritius et al., 2016). In the procuticle, the 116 protein-chitin fibers or fibrils are arranged in horizontal planes which are stacked helicoidal and 117 form a twisted plywood structure (Davie et al., 2015; Fabritius et al., 2016; see also Fig. 2b). Endo-118 and exocuticle therefore appear multi-lamellate. These lamellae do not correspond to the light 119 and dark cuticle bands, which have been interpreted as annual bands. Each of these bands comprises several lamellae and therefore represent a higher structural hierarchy (see Figs. 1, 8f). 120

121 **1.4 Aims**

Since its first publication, the new approach to determine crustacean age through 'growth bands'
in the cuticle has become widely used and raised hope that studies on population structure,
longevity, and size or reproductive maturity at age could have dramatically improved resolution

125 (Carmichael, 2013). All those parameters form the basis for stock assessment and the sustainable 126 management of fisheries species. It is, however, absolutely critical that evidence-based fisheries 127 management rests upon accurate interpretation of data. In the present study, we applied different 128 morphological methods to visualise cuticle bands in gastric mill ossicles and study their fate during the moult cycle in four commercially important species of decapods, Homarus gammarus 129 130 (European Lobster), Nephrops norvegicus (Norway Lobster), Cancer pagurus (Brown Crab) and 131 *Necora puber* (Velvet Crab). We first used the previously published approach (e.g. Kilada et al., 2012) and prepared sections of ossicles in intermoult specimens at thicknesses between 180 and 132 250 μm. Additionally, synchrotron micro-computed tomography and cryo-scanning electron 133 134 microscopy was used to study the nature of cuticle bands in intermoult specimens. We then tested 135 the transmoult retention of gastric mill ossicles used for ageing, through moulting experiments and morphological comparisons of gastric mill ossicles in premoult, intermoult and recently 136 moulted specimens using stereo microscopy, light microscopy and micro-computed tomography. 137

138

139 2. Methods

140 **2.1 Specimen sampling**

141 Intermoult specimens of *N. norvegicus*, *C. pagurus* and *N. puber* were collected between March 2016 and April 2017 by deploying creels in Strangford Lough, N. Ireland, in both the south basin 142 (54°23'.45N 05°37'.45W) and centrally in the lough (Tip Reef, 54°27'.55N 05°34'.80W). 143 Additionally, freshly moulted specimens of *H. gammarus* and *C. pagurus* were obtained directly 144 145 from commercial fishermen (B & M Chambers LTD, Annalong, County Down, N. Ireland) who deployed creels in the Irish Sea off the coast of Annalong (coordinates for *H. gammarus*: 146 147 54°10'.590N 05°45'.300 W, C. pagurus: 54°08'.370N 05°44'.900). All live animals were transported to the Queen's University Marine Laboratory, Portaferry, and housed and maintained 148 on flow-through seawater until sacrificed. Live intermoult specimens were individually labelled 149 150 and measured (carapace length, CL and carapace width, CW). Specimens were maintained in large 151 outdoor tanks with a sea water flow through system and air supply at Queen's Marine Laboratory 152 in Portaferry, UK (QML). Each species was kept in groups of five to 20 specimens per tank. Animals 153 were fed ad libitum using locally sourced fish and mussels. Pipe sections of different sizes were 154 provided as shelter for *H. gammarus* and *N. norvegicus*. Outdoor tanks were covered for shade and 155 to exclude potential predators.

Specimens were monitored frequently to recover freshly moulted specimens together with theirshed exuvia. All specimens used for the present study are listed in suppl. Table 1. Freshly moulted

specimens and their exuviae were frozen immediately after recovery for subsequent dissections
and analysis. Intermoult specimens used for comparisons of gastric mill ossicles were taken from
holding aquaria, cold-anaesthetised for 20 minutes, and subsequently sacrificed for dissections.

161 **2.2 Dissections and sample preparation**

162 In total, two specimens of *H. gammarus*, seven of *N. norvegicus*, three of *Necora puber*, and six 163 specimens of *C. pagurus* were dissected for comparisons of gastric mill ossicles in different moult stages (see suppl. Table 1). The foregut was dissected out of fresh or defrosted samples. Gastric 164 mills were immersed in a solution of 10% potassium hydroxide in distilled water and boiled for 165 10-25 minutes to remove soft tissues to improve observations. Gastric mills were then opened 166 through a longitudinal incision on the ventral face to expose the zygocardiac, urocardiac, 167 mesocardiac and pterocardiac ossicles. The stomach content of freshly moulted specimens was 168 169 searched for ossicles. The gastric mills in exuviae were soft and collapsed and therefore immersed 170 in distilled water to facilitate dissections and observation. Samples were studied and photographed using an Olympus SZX16 dissection microscope equipped with an Olympus E600 171 172 camera. Images were processed in Adobe Photoshop version CS5.1 and assembled to figure plates.

173 **2.3 Microscopic visualisation**

174 Gastric mills were preserved in a mix of glycerol, distilled water and a 96% ethanol solution (30:10:60) for two weeks and then immersed in a potassium hydroxide (KOH) solution and boiled 175 for 10-25 minutes to remove soft tissues before gastric mills were dissected and ossicles 176 177 extracted. Ossicles were subsequently air-dryed, embedded in epoxy resin (Logitech Epo-Flo type 178 301) and hardened overnight at 40°C. Sample blocks were then cut with a Logitech GTS10 179 diamond saw and processed in a diamond lab. Blocks were polished using silicate carbide grinding 180 paper at grade 320 and loose silicon carbide powder at grade 600 on a glass plate. The processed 181 surfaces of sample blocks were glued on glass slides. A Jones-Shipman 540 surface grinder was used to produce a section thickness of 180-250 µm. Sections were subsequently polished using a 182 183 WG2 Logitech polishing unit and studied and photographed using an Olympus BX41 compound 184 microscope equipped with an Olympus E600 camera. Images were processed and assembled to 185 figure plates in Adobe Photoshop (ver. CS5.1).

One specimen of *N. norvegicus* close to moulting was used for paraffin histology at Queen's
University Marine Laboratory in Portaferry (Northern Ireland). The gastric mill was dissected and
preserved in "Susa Heidenhain" (MORPHISTO® Evolutionsforschung und Anwendung GmbH,
Frankfurt am Main, Germany) for two weeks. The sample was washed in a descending series of
ethanol and treated with Ethylenediaminetetraacetic acid (EDTA) for 72 hours for decalcification.

191 The thorax of this sample was then dehydrated through an ascending series of ethanol solutions 192 and infiltrated (Shandon Hypercenter XP, Thermo Fisher Scientific, Waltham, Massachusetts, 193 USA) and embedded for transverse sections through the gastric mill in a paraffin block. Sections 194 were prepared at 6 µm using a Leica RM2255 rotary microtome (Leica Microsystems GmbH, Wetzlar, Germany). The histological sections were stained using a trichromatic Masson-Goldner 195 196 "light green" stain (MORPHISTO®, Germany). Covered slides were studied and photographed 197 using an Olympus BX41 compound microscope equipped with an Olympus E600 camera. Images were processed and assembled to figure plates in Adobe Photoshop (ver. CS5.1). 198

199 The thorax of a specimen of *N. norvegicus* (see Table 2) was prepared for tomography by 200 dehydration through an ascending ethanol series and chemically dried using 201 hexamethyldisilazane (HMDS). The micro-computed tomography scan was conducted at the 202 Museum für Naturkunde Berlin (Germany) using a Phoenix nanotom X-ray|s tube at 100 kV and 203 150 μA, generating 2000 projections. The effective voxel size was 20 μm, the detector timing 750 204 ms. The cone beam reconstruction was performed using datos|x-reconstruction software (GE Sensing & Inspection Technologies GMBH phoenix|x-ray) and a stack of virtual sections was 205 produced and exported with software VGStudio Max (Volume Graphics, Heidelberg). Image stacks 206 were processed for volume renderings and surface reconstructions with software Amira version 207 208 5.3.3 for Mac (FEI Visualization Sciences Group, Bordeaux).

Samples were scanned by synchrotron hard x-ray microtomography at beamline 8.3.2 at the Advanced Light Source at Lawrence Berkeley National Laboratory. Monochromatic X-rays were used, at either 23 keV, with an Optique Peter lens system with an olympus 10x lens and a a LuAG:Ce 20 micron thick scintillator. A PCO.edge sCMOS camera was used, yielding an effective pixel size of 0.645 microns. Samples were rotated through 180 degrees while 2049 images were collected. Reconstruction was performed with Tomopy and Xi-cam (alpha release, 2016, http://www.camera.lbl.gov/xi-cam-interface).

216 Scanning electron micrographs were obtained in cryo conditions with a Cryo SEM (Jeol JSM7500F) 217 equipped with a cryo preparation system (Gatan Inc., ALTO-2500, Abingdon). Samples were plunge frozen in liquid nitrogen slurry, transferred into the cryo-stage of the preparation chamber 218 (-130°C), and cracked open with a scalpel blade. Sublimation was performed heating the sample 219 preparation chamber from -130°C to -95°C (which took approximately 5 minutes) to remove 220 221 contamination by condensed ice crystals. To avoid charging artifacts, samples were palladium sputter coated (3 nm thickness) in the frozen condition. Imaging was performed using a gas cooled 222 223 SEM stage at -130°C and a accelerating voltage of 3kV.

224

225 **3. Results**

Our observations show that gastric mill ossicles contain cuticle bands (Figs. 1 and 2), but the
growth dynamics of the gastric mill in recently-moulted specimens demonstrate that the ossicles
were not retained through the moult in the lobster species studied, *H. gammarus* (Fig. 3) and *N. norvegicus* (Figs. 4, 5), nor in the crab species *N. puber* (Fig. 6) and *C. pagurus* (Fig. 7). Histological
sections of premoult *N. norvegicus* further confirm that the banded endocuticle is completely shed
(Fig. 8).

232 **3.1 Cuticle structure**

Our data on the fate and growth dynamics of gastric mill ossicles includes two major lines of 233 234 evidence. First, a combination of microscopic visualisation techniques allowed us to infer the 235 internal construction of previously described bands within the cuticle. Sections of gastric mill 236 ossicles showed cuticle bands (Fig. 1). However, the readability of sections cut at $\sim 200 \,\mu m$ varied. In images obtained from synchrotron micro-computed tomography at three-dimensional 237 238 resolutions of ~8 µm, internal bands of differential density were visible (Fig. 2a). In cryo-scanning 239 electron micrographs, bands were not visible at the same scale as seen via other techniques, only the internal plywood structure of the cuticle was apparent (Fig. 2b). This incidates that the 240 241 variation in material density within the cuticle is not readily visible from surface structure (SEM), 242 but it is only observable when viewed by x-ray or transmitted light.

243 3.2 Moulting experiments

244 The second major line of evidence comprises data from whole animals observed through the 245 moulting process in several species. The gastric mill in all the study species is located anterior to the heart, directly below the dorsal carapace (Figs. 3a, 5). In intermoult specimens, gastric mill 246 ossicles are calcified, which is visually apparent from their white to creamy colour and 247 opaqueness (Figs. 3b, c, 4b, 7b). In freshly moulted specimens, the gastric teeth are present but 248 249 the ossicles remain incomplete, they lack calcification and therefore appear translucent (Figs. 4c, 250 6d, 7c). This is also the case in the gastric mill seen in the moulted exuvia; the teeth are complete but the underlying ossicles are absent (Fig. 6b, c). The missing parts of ossicles were found loose 251 in the stomach of one freshly moulted specimen of H. gammarus and five specimens of N. 252 253 norvegicus among the disintegrated subunits of the gastroliths (Figs. 3d, 4d, e, 5). However, only in very soft, presumably just recently moulted specimens, the stomach was filled with gastrolith 254 255 material and different sets of ossicles. Due to the moult occurring during the night, the exact delay 256 between moulting and specimen recovery could not be determined.

In the freshly moulted *H. gammarus* studied, the paired zygocardiac and pterocardiac ossicles were found with the zygocardiac and pteriocardiac ossicles of each body half attached to each other (Figs. 3d). Furthermore, the transversely elongated mesocardiac ossicle was present plus an additional pair of ossicles, we could not further determine (Figs. 3d). The very soft *H. gammarus* specimen, which was recovered the morning after the overnight moult (assumably up to 12 hours postmoult) had ossicles and gastrolith material in the stomach content, while the intermoult specimen had food remains in the stomach while ossicles and gastrolith material were absent.

264 Gastrolith material (Fig. 4d, 5) and zygocardiac, pterocardiac and mesocardiac ossicles (Figs. 4e, 265 5) were present in the stomach of five specimens of *N. norvegicus* recovered the day after an 266 overnight moult (maximum up to 18 hours postmoult). In specimens that were recovered later 267 than 24 h post-moulting, and in which the hardening of the exoskeleton had progressed, ossicles and remains of gastroliths were absent. At approximately 36-48 hours after the moult, specimens 268 of *N. norvegicus* generally resumed feeding, and in five specimens sacrificed at this stage the 269 270 stomach was filled with crushed food remains rather than gastrolith material (not listed in suppl. 271 Table 1).

Necora puber did not moult on a regular basis in our experimental tanks. Out of the two specimens
we recovered, one was very soft and the zygocardiac and pterocardiac ossicles were recovered
from the stomach content (Fig. 6d). The other specimen was slightly harder and food remains
were found in the stomach, indicating that the specimen had already resumed feeding.

The two specimens of *C. pagurus*, which had moulted in our tank were discovered when they were 276 277 only slightly soft. The three specimens received from fishermen were however extremely soft, but 278 the exact time elapsed since moulting was unknown as the moult occurred in a deployed creel. In 279 contrast to the other species, in *C. pagurus*, the stomach of the three freshly moulted specimens 280 was found to be empty. Even in extremely soft, jelly-like specimens of freshly moulted C. pagurus, 281 ossicles were not present in the gastric mill, nor were any food remains apparent. However, in 282 recently moulted specimens of *C. pagurus* (as in all studied species), the gastric mill ossicles were 283 completely decalcified and translucent (compare Fig. 7b and c). The same uncalcified gastric ossicle precursors were observed in *H. gammerus*, *N. norvegicus* and *N. puber* (Figs. 4c, 6d), and 284 285 any observed variation in colour or texture is likely to be related to the unquantified time elapse 286 between actual moult and preservation of specimens.

287

288 3.3 Light microscopic observations

289 Histological sections of a premoult specimen of *N. norvegicus* allowed the observation and study 290 of the old cuticle that is going to be shed at the imminent moult and the new cuticle which is 291 forming underneath simultaneously (Fig. 8). The zygocardiac ossicles were easily identified in 292 transverse sections of the gastric mill by their paired structure and the gastric teeth which are 293 characterised by a thick red-staining epicuticle that impregnates the tooth (Fig. 8a-d). Sections of 294 the zygocardiac ossicle in the region of the tooth show the thick old cuticle (Fig. 8a, b) already 295 delaminated from the gastric tooth. All cuticle layers (endo-, exo-, and epicuticle) are present in 296 the old cuticle and will be shed at the moult. Underneath the old cuticle, a new cuticle has started to form by secretions of the hypodermis of the gastric tooth (Fig. 8a, c, d). The new cuticle is still 297 298 incomplete and is composed only of the thick red-staining epicuticle and a thin underlying 299 exocuticle, which is still incomplete (Fig. 8d). An endocuticle has not yet formed (Fig. 8d). The basal region of the zygocardiac ossicle, which contains the cuticle bands interpreted as annual 300 301 bands, is structurally different from the gastric tooth by having a very thin epicuticle (Fig. 8e, f); 302 however, the signs of an imminent moult are the same as in the tooth: all layers of the old cuticle, 303 also the endocuticle which contains light and dark bands, are delaminated from the underlying 304 hypodermis which is secreting the new cuticle (Fig. 8f).

305

306 4. Discussion

307 4.1 Overview

The study presented herein confirms that the cuticle of gastric mill ossicles appears banded in some regions. Those bands could be visualised by light microscopy and x-ray tomography, but were not apparent on the microarchitectural level studied by cryo-SEM. Our results demonstrate that the dorsal ossicle complex in the gastric mill (including the parts that contain cuticle bands) is not retained, but shed and replaced through the moult, and therefore cannot accumulate a growth record in the form of annual bands.

There would be great value in a tool to directly determine the chronological age of crustaceans; however, previous work interpreting bands in the endocuticle of gastric mill ossicles as an indicator of age (Leland et al., 2015; Sheridan et al., 2015; Krafft et al., 2016) cannot be supported by our findings. The approach to age crustaceans by counting cuticle bands is refuted by the experimental evidence presented here, and also by the anatomical understanding of moulting. Ecdysis is an incredibly complex process in which the hard exoskeleton is shed and entirely reformed, to allow crustaceans to grow despite their rigid cast. In order to understand any potential utility of certain skeletal elements as potential indices of age, it is useful to consider a thought experiment about the fate of the skeleton during ecdysis. We have shown that the gastric mill ossicles are lost and not retained as a skeletal structure through the moulting process. Yet the gastric mill is a highly complex structure so it is worth some further consideration on where the ossicles fit within the gastric complex, and how are they identified in the skeleton and postmoult exuvia.

327 **4.2 The cuticle during the moult cycle**

The moult cycle of crustaceans can be divided into different stages (Roer and Dillaman, 1984). The 328 intermoult is the hard-shelled equilibrium condition in which a crustacean spends most of its 329 330 lifetime. The early premoult is characterised by a delamination of the old cuticle from the underlying hypodermis, to prepare for the formation of a new cuticle. Mineral resorption and the 331 332 formation of the new epi- and endocuticle take place in the late premoult, thus the next skeleton 333 is a separate structure, in place underneath the old skeleton before moulting, though it remains soft. The old cuticle is shed, revealing a soft-shelled crustacean. The deposition of the new 334 endocuticle and the mineralisation of the procuticle occurs gradually after moulting; it can take 335 several days to complete this process before a specimen has fully hardened (Roer and Dillaman, 336 1984). 337

338 The gastric mill is part of this skeletonised foregut, and includes structures that are supported by numerous paired and unpaired calcified skeletal elements, the ossicles, on which the stomach 339 musculature attaches. Several ossicles carry teeth and form a chewing apparatus, the gastric mill 340 341 (Maynard and Dando, 1974; Figs. 3-7). The epicuticle of the teeth is impregnated with silica, which 342 gives them an amber-coloured appearance (Vatcher et al., 2015; Figs. 3c, 5b, c, 7b). The largest 343 and most dominant structures of the dorsal ossicle complex within the gastric mill have been used 344 as putative ageing structures (see Table 1). Importantly, these structures are still connected 345 within the crustacean skeleton, all have the same ectodermal developmental origin, and all parts suffer the same fate and are completely replaced episodically during ecdysis. 346

347 The structures within the gastric mill are complex and the individual ossicles are often challenging 348 to identify. The paired zygocardiac ossicles are located laterally on each side of the gastric mill and form the lateral tooth plate. The central unpaired urocardiac ossicle carries the median tooth 349 350 plate and is anteriorly connected to the mesocardiac ossicle, which is flanked on both sides by the paired pterocardiac ossicles (Davie et al., 2008; Figs. 3c, 5b, c, 7b). Some of the previous ageing 351 352 work was not consistent with regard to which ossicles have been used and in which orientation they were sectioned (see Table 1). The structure of ossicles can vary among decapod crustaceans, 353 for example, the mesocardiac ossicle differs remarkably between the crab and lobster species we 354

herein studied. The mesocardiac ossicle of *H. gammarus* and *N. norvegicus* is wide, strongly 355 356 calcified and well separated from the adjoining urocardiac ossicle (see Fig. 3b). The same 357 structure is narrow and fused with the urocardiac ossicle in *N. puber* and *C. pagurus* (see Fig. 7b). 358 The pterocardiac ossicles were very different in terms of shape and size between the studied lobster and crab species as well (compare Figs. 3d and 4e to 6e). The structure of ossicles is so 359 360 diverse that it has frequently been used in phylogenetic reconstructions (Brösing, 2008; Brösing 361 et al., 2002; 2007; Brösing and Türkay, 2008, Reimann et al., 2011), while the gastric teeth often show adaptations to feeding and the specific food range of a species (Brösing, 2002; Castejón et 362 al., 2015a, b). The fate of gastric mill ossicles during ecdysis has previously only been studied by 363 364 Vatcher et al. (2015) and Sheridan et al. (2016). Vatcher et al. (2015) studied the mesocardiac and urocadiac ossicle in *C. sapidus* using light microscopy and elemental mapping, with special regard 365 to the mineralisation of those structures during the moult cycle. Sheridan et al. (2016) looked at 366 367 the fate of gastric mill ossicles in *N. norvegicus* from a macroscopic perspective. These studies also 368 found that gastric mill ossicles were lost during the moult.

369 **4.3 The dynamics of gastric mill ossicles during the moult**

That gastric mill ossicles are subject to ecdysis has been demonstrated unequivocally in the present study through 1) the observation of not fully calcified, incomplete ossicles in freshly moulted specimens of all four studied species (Figs. 4c, 6d, 7d), and 2) the finding of loose, shed ossicles within the stomach contents of recently moulted specimens of *H. gammarus*, *N. norvegicus* and *N. puber* (Figs. 3d, 4e, 3, 6e). If a skeletal element is shed during the moult, *ipso facto* it cannot be growing in a continuous way that would record chronological age.

The foregut of decapod crustaceans is a complex organ with a multitude of morphologically 376 377 varying ossicles (Brösing, 2002), such that identification and classification of individual 378 component structures can be very challenging. Different species examined herein showed 379 different sets of ossicles among the disintegrated gastroliths in the stomach. In the lobsters, H. gammarus and N. norvegicus, the transversely elongated mesocardiac ossicle was present 380 together with the paired zygocardiac and pterocardiac ossicles. In N. puber, only the zygo- and 381 pterocardiac ossicles were present while the mesocardiac ossicle was not found. The meoscardiac 382 383 ossicle in crabs is smaller and less calcified than the homologous structure in lobsters; this relative 384 fragility might explain why it was not recovered from the stomach in *N. puber*. In *H. gammerus*, an 385 additional distinctive pair of ossicles was found but we were not able to definitively identify it. We also note that other ossicles may have been overlooked in the present study, due to destruction 386 387 during the moult and because most of them are much smaller than the prominent teeth-bearing 388 ossicles mentioned above.

The only species in which we were not able to find any ossicles in the stomach content of freshly 389 390 moulted specimens was *C. pagurus*. However, the exact timing of the moult was not directly 391 established for these individuals. In contrast to our findings for the other study species, in which 392 the stomach was either filled with ossicles or food remains, the stomachs of C. pagurus were 393 completely empty. This may suggest that resorption and remobilisation of minerals may occur 394 differently in *C. pagurus* than in the other species, but importantly the ossicles were definitively 395 not retained in the skeleton. We also investigated the gastric mill in exuvia of the studied species, 396 revealing similarities with the gastric mills of freshly moulted specimens, in the lack of calcification and a resulting transparency of ossicles. The same was observed in an earlier study 397 398 by Brösing (2014), who investigated the foregut in exuviae of several decapod species (*C. pagurus*, 399 Maja crispata and Pseudosesarma moeschi) and found that ossicles of the gastric mill were 400 incomplete, degraded and decalcified. The gastric teeth, however, were present and complete in 401 the exuviae of those species. The observation of incomplete gastric mills in exuviae may explain 402 why earlier studies concluded that these internalized structures are not shed but retained through 403 moulting. Kilada et al. (2012) correctly described that the mesocardiac ossicle was missing in 404 exuviae of *H. americanus*, and the swamp crayfish, *Procambarus clarkii*. However, their conclusion 405 that the mesocardiac ossicle must be retained in these two species is neither supported by our 406 direct observations in the present species, nor by the nature of ecdysis. In particular, considering species as closely related as *H. americanus* and *H. gammarus*, it is hard to imagine that effects of 407 408 moulting on the gastric mill would be variable. We found it challenging to control the timing and 409 fate of moulting specimens; other reports of calcified gastric ossicles shortly after moulting (Kilada et al., 2012) could easily have been confounded either by the timing of the moult or by the 410 411 acknowledged challenges in describing the anatomy of this very complex structure. To date, there is no evidence to support the idea that any calcified ossicles are retained in the skeleton during 412 the moult. 413

414 4.4 Gastric mill ossicles do not preserve chronological age

Advocates of ageing crustaceans by counting annual bands have used different approaches to support the idea that gastric mill ossicles are sustained through the moult and could therefore retain record of growth and age. It may seem enigmatic that internal body parts, such as the gastric mill, would be shed. This may have contributed to the idea that ossicles are excluded from the process of moulting. However, our evidence clearly demonstrates that the ossicles are not fully developed in soft specimens immediately after the moult.

The idea of structures being retained through the moult was apparently supported by previous
evidence from live staining with the fluorescent marker calcein. The persistence of this marker
through the moult was interpreted as demonstrating that the endocuticle of ossicles was retained

through ecdysis (Kilada et al., 2012; Leland et al., 2015). It is important to consider those 424 425 experiments, and the anatomical explanation for their observations. Specimens of *H. americanus* 426 (Kilada et al., 2012) and the red claw crayfish *Cherax quadricarinatus* (Leland et al., 2015) were 427 dyed with the fluorescent marker calcein that binds to calcium. The live specimens were then 428 allowed to moult several times before they were sacrificed. In both studies, the calcein mark was 429 still present in the cuticle after the moult. This was interpreted as evidence for trans-moult 430 retention of the endocuticle (Kilada et al., 2012; Leland et al., 2015). In the study on *H. americanus*, 431 Kilada et al. (2012) presented their results in supplementary figures (suppl. figs. S10 and S11) and described their observation of a clear calcein mark in the most proximal part of the endocuticle of 432 the eyestalk and mesocardiac ossicle. However, this is not actually consistent with retention of the 433 434 structures. If calcified material stained with calcein had been retained through the moult cycle, 435 and the hypodermis of those structures had then secreted additional cuticle layers during growth 436 after the stain was applied, then the calcein mark should be found more distally in the endocuticle. 437 But in fact, the cuticle does not grow during the intermoult, it is only formed during ecdysis (Roer and Dillaman, 1984), and it must always grow outward. Leland et al. (2015) also studied the fate 438 439 of calcein marks in the endocuticle during the moult cycle: in five of nine specimens of C. 440 quadricarinatus used in their experiment a mark was present after one year and several moults; the exact positions of these marks was however highly variable. 441

442 The most likely explanation for the detection of calcein marks in specimens which have been 443 stained and allowed to moult afterwards is the resorption and remobilisation of calcium as suggested by Sheridan et al. (2016). Crustaceans are under high selective pressure to make the 444 445 moulting process as efficient as possible, because they are so vulnerable. In this circumstance, it makes sense that the material for a large solid calcified structure would be rapidly re-integrated 446 447 into the new skeleton. However, it clearly is shed into the stomach, and then reintegrated later. This transition, and concomitant degradation during digestion, means that the structure cannot 448 449 accumulate data about chronological age.

Kilada et al. (2012) addressed the phenomenon of potentially stored and remobilized calcium 450 451 carbonate as a possible cause for the detection of calcein marks in moulted specimens but argues 452 that the distinct nature of the observed mark does not support the reincorporation of stored calcium. However, images of the calcein mark presented in the supplementary material of their 453 454 publication do not show a well-defined mark, but rather a blurred area of variable thickness (Kilada et al., 2012: suppl. Figs. S10c, d, S11c). The authors furthermore argued that stained 455 456 remobilized calcium carbonate is unlikely to be incorporated solely into the most proximal 457 endocuticle but should be rather distributed throughout the whole layer. According to our point 458 of view, the understanding of the control and process of incorporating remobilized minerals from

different sources during the calcification of the cuticle is still too sparse to make predictions about 459 460 the fate and location of a calcein mark through such a complex process (see Greenaway, 1985). 461 Moreover, Sheridan et al. (2016) have demonstrated the persistence of calcein marks in gastric 462 mill ossicles through the moult in *N. norvegicus* despite that the trans-moult retention of the same 463 ossicles has been clearly disproved by the same authors. In conclusion, the trans-moult calcein 464 detection should not be regarded as a reliable tool to provide evidence for the retention of cuticle 465 structures before we have achieved a deeper understanding on the interaction of resorption and 466 remobilization of minerals during the moult. Previous evidence of retained calcified parts in the gastric mill ossicles apparently was an artefact of resorbed and re-used calcium. 467

468 **4.5 Potential mechanisms for cuticle bands**

The primary remaining argument that cuticle bands represent a record of chronological age, is the correlation between band counts and size-based age estimates. It is well established that such a correlation exists, and that the banding structures are visible. Positive linear correlation of band counts and age have been reported in several species (e.g. Hasyima Ismail et al., 2017), and there is also a positive correlation of chronological age with both ossicle size and cuticle thickness (Leland and Bucher, 2017): the crustacean cuticle is thicker in older specimens, and has more bands.

476 A general relationship between cuticle thickness and body size is obvious when comparing 477 differently sized individuals within a species: larger specimens have a thicker exoskeleton that is harder to crack open. However, this correlation has hardly been studied and the exact allometric 478 479 relationship is not clear. The thickness of the cuticle also varies during the different episodes of 480 the moult cycle; most obviously, the cuticle is thinner immediately after the moult before the 481 endocuticle deposition is completed. Unpublished data suggests the allometry of the thickness 482 deficit in the first week after moulting – the amount of thickening still required to fully harden the carapace – also increases with individual size, in in the snow crab *Chionecetes opilio* (pers. com. 483 Bernard Sainte-Marie, 2018). A possible explanation for this phenomenon is that the endocuticle 484 485 deposition and mineralisation takes longer in larger than in smaller specimens.

It seems likely that the number of any repetitive occurring cuticle layer increases with cuticle thickness. While we do not understand the cause and relevance of the cuticle bands interpreted as growth marks, the relationship between cuticle thickness and specimen size has the potential to explain why cuticle band counts are positively correlated to specimen size. A possible explanation for cuticle banding is available from comparison with studies on other arthropods.

Several studies have shown that the slow process of post-moult endocuticle deposition is subject 491 492 to a daily cycle. In insects, non-lamellate cuticle is laid down during the day and lamellate cuticle 493 deposited during the night, those layers are shown as dark and light cuticle bands (Neville, 1965; 494 Wiedenmann et al., 1986; Lukat et al., 1989; Weber, 1985; Ito et al., 2008). The images of insect 495 endocuticle bands in these studies strongly ressemble the cuticle bands in crustaceans. In some 496 insects, these cuticle bands record number of days that passed after the last moult during cuticle 497 hardening. Endocuticle formation can take 2-3 weeks in locusts (Neville, 1965), and once this process is completed, no further layers are added. 498

499 In crustaceans, the cuticle bands may also represent the length of time required to complete 500 cuticle deposition after the moult. Clearly the carapace is thicker in older specimens, and may form 501 more bands as it takes longer to harden. The observation that female snow crabs, *C. opilio*, have 502 significantly more cuticle bands in the eyestalks than males at the same size is consistent with the 503 knowledge that females grow more slowly than males in this species (Kilada et al. 2012); however, 504 it may be that cuticle deposition takes longer in females if their metabolism is generally slower, 505 or that cuticle thickness or density varies allometrically with age as well as size. Banding 506 apparently thus has a secondary correlation to chronological age, which could explain results 507 published in previous studies where banding and known age are correlated. But this somewhat 508 tenuous secondary correlation, and the clear evidence that bands are not a direct record of annual 509 increments, indicates that banding is not a reliable indicator of age.

510

511 **4.6 Conclusions and future directions**

512 Since the first proposal of direct age determination for crustaceans through presumed annual 513 growth bands in gastric mill ossicles, this method has been increasingly used (e.g. Clore, 2014; 514 Tang et al., 2015; Ibrahim and Kilada, 2016; see Table 1). The approach of analysing growth bands in enduring hard structures has been directly transferred from fisheries biology to crustaceans 515 516 (Leland et al., 2011), without considering the striking differences in growth mechanisms. In non-517 crustacean organisms that possess annual or otherwise periodic bands or rings, growth is 518 necessarily a continuous process, such that seasonal conditions that influence metabolism are 519 reflected in differentiated growth increments. Growth in crustaceans is profoundly different from 520 this model. As in all arthropods, growth in crustaceans is a discontinuous process. Being cast in a rigid, calcified exoskeleton, crustacean growth is a periodic event rather than a continuous 521 process, and body size increase is only possible through moulting. Studies using presumed growth 522 523 bands in crustaceans have been predominantly within the field of applied sciences, where these

data are sorely needed, but previous studies did not consider the fundamental mechanisms thatmight be held responsible for the formation of bands in the endocuticle.

526 Before demographic data collected through cuticle band counts for a certain species are implemented into stock assessment for fisheries and conservation management, the dynamics of 527 anatomical ageing structures during the moult should be studied. An explanation for the close 528 529 match of band counts with age estimates based on growth models (Kilada et al., 2015; 2017), 530 might arise with a deeper understanding of the underlying processes in the formation of bands in 531 the endocuticle. There are certainly bands that can be observed in the endocuticle, but we contend 532 they do not represent annual growth increments that indicate chronological age. However, the 533 mechanism that causes these patterns is not clear. There are other fine lamellation patterns that have been described in the procuticle of arthropods and are known to be caused by the twisted 534 plywood structure of deposited chitin layers (Fabritius et al., 2016), but these are finer-scale 535 structures than bands that have been attributed to ageing. Future studies on the mechanistic cause 536 for this broader layering may reveal what actually forms the observed bands. The control and 537 rhythmics of the process of postmoult endocuticle deposition might be responsible for the 538 539 formation of bands. In the current incomplete state of knowledge about the anatomy and physiology of moulting in fine structures, we cannot exclude that some calcified parts in other 540 crustacean species are actually retained through the moult. Future research should therefore 541 542 focus on understanding the process of postmoult endocuticle deposition and mineralisation in 543 additional species of crustaceans.

544

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693 Supporting information

- 694 Ethics approval
- **695** The conducted research adhered to legal requirements for animal care in the European Union.
- 696 **Consent for publication**
- 697 not applicable.

698 Availability

699 Specimens, samples and data used in this study are deposited at Queen's Marine Laboratory,

- 700 Portaferry, UK. The datasets used in this study are available from the corresponding author on
- 701 reasonable request.

702 Competing interests

None of the authors have any competing interests in the manuscript.

704 **Declaration of interest**

705 none

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709 Author Contributions

CB conducted lab work, collected and analysed the data, and prepared the manuscript. JDS, JTAD
and CB planned and coordinated the study. JDS and JTAD contributed to the manuscript. CS
studied samples using cryo-scanning electron microscopy, MEC prepared histological sections,
and JDS performed synchrotron x-ray scanning. All authors read and approved the final version
of the manuscript.

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716 **Figure captions**

Fig. 1 Petrological sections of gastric teeth in Norway Lobster *Nephrops norvegicus* (thickness of
sections 180-200 μm). (a) Cuticle bands in zygocardiac ossicle (male, carapace length 44 mm). (b)
Cuticle bands in zygocardiac ossicle (male, carapace length 40 mm). Red dots (white in print
version) indicate cuticle bands; Red questionmark (white in print version) indicates non-readable
bands. en – endocuticle, ex – exocuticle, ml – membraneous layer.

- Fig. 2 The zygocardiac ossicle of Norway Lobster Nephrops norvegicus studied with different
- 723 methods. (a) Synchrotron micro-computed tomography image shows bands. Red arrow (white in
- print version) indicates banded area. (b) The cryo-scanning electron micrograph only shows the

plywood-like structure of the cuticle. Red asterisks (white in print version) indicate cuticlelamellae which do not correspond to the cuticle bands.

727 Fig. 3 The gastric mill and the fate of ossicles during the moult in European Lobster Homarus gammarus; black asterisk on same structure for easier orientation. (a) Thorax of female specimen 728 729 with carapace removed to expose the gastric mill and other organs (carapace length 115 mm). (b) 730 Dorsal and (c) ventral aspect of intermoult gastric mill after ventro-longitudinal incision to expose 731 ossicles. The unpaired urocardiac ossicle lies centrally and carries the median tooth plate. 732 Adjoining anteriorly is the transversely elongated mesocardiac ossicle, flanked on each side by the 733 paired pterocardiac ossicles which are connected to the zygocardiac ossicles that carry the lateral 734 tooth plates. (d) The mesocardiac, zygocardiac and ptercoardiac ossicles were found loose in the stomach content of freshly moulted specimens. Two smaller, unassigned ossicles were also 735 present. The black asterisks in (a), (b) and (c) label the same structure to facilitate orientation; 736 Abbreviations: gm - gastric mill, he - heart, lt - lateral tooth plate, mc - mesocardiac ossicle, mt -737 738 median tooth plate, ov – ovary, pc - pterocardiac ossicle, uc - urocardiac ossicle, zc - zygocardiac 739 ossicle.

740 Fig. 4 The gastric mill during the moult in Norway Lobster Nephrops norvegicus. (a) Freshly 741 moulted, soft male specimen (CL: 47 mm) (b) Ventral aspect of dorsal ossicle complex (left) in 742 dissected intermoult specimen and extracted zygocardiac ossicle (right). (c) Unpaired urocardiac 743 ossicle with median tooth plate and paired zygocardiac ossicles with lateral tooth plates in freshly moulted specimen (ventral view). Note that the present ossicles are not fully calcified and the 744 745 mesocardiac ossicle is missing. (d) Subunits of gastrolith and (e) ossicles in the stomach of 746 stomach of freshly moulted specimens. Abbreviations: lt - lateral tooth plate, mc - mesocardiac 747 ossicle, mt - median tooth plate, pc - pterocardiac ossicle, uc - urocardiac ossicle, zc - zygocardiac 748 ossicle.

Fig. 5 Micro-computed tomography of anterior thorax of freshly moulted Norway Lobster *Nephrops norvegicus.* Volume rendering viewed with AMIRA software ("volren glow" colour
scheme), longitudinally cut using orthoslice tool. The lateral tooth plate, mandible and the eye of
the left body half are shown. The cardiac stomach is filled with the subunits of the disintegrated
gastroliths and gastric mill ossicles (black asterisk). Abbreviations: ey – eye, lt – lateral tooth plate,
md = mandible.

Fig. 6 The gastric mill during the moult in Velvet Crab *Necora puber*. (a) Exuvia of male with dorsal
carapace folded open along the moult line (carapace width 45 mm). (b) Ventral view on dissected
gastric mill in exuvia showing that teeth are moulted but calcified parts of ossicles are missing. (c)
Detail of zygocardiac ossicle. (d) Gastric mill of freshly moulted specimen with uncalcified

translucent ossicles (same aspect). (e) Ossicles found in stomach of freshly moulted specimen.
Abbreviations: lt - lateral tooth plate, mc - mesocardiac ossicle, mt - median tooth plate, pc pterocardiac ossicle, uc - urocardiac ossicle, zc - zygocardiac ossicle.

Fig. 7 The gastric mill during the moult in Brown Crab *Cancer pagurus.* (a) Exuvia (top, carapace width 124 mm) of and emerged specimen (bottom, carapace width 149 mm) of male. (b) Ventral view on dissected gastric mill showing calcified ossicles. (c) Gastric mill of freshly moulted specimen showing uncalcified transparent ossicles (ventral aspect). Abbreviations: lt - lateral tooth plate, mc - mesocardiac ossicle, mt - median tooth plate, pc - pterocardiac ossicle, uc - urocardiac ossicle, zc - zygocardiac ossicle.

Fig. 8 The zygocardiac ossicle in the Norway Lobster Nephrops norvegicus shortly before moulting 768 769 in histological sections. (a) Close to the moult, the old cuticle of the gastric tooth in the zygocardiac 770 ossicle (white label zc) is already delaminated from the newly formed tooth (black label zc). (b) 771 The shed cuticle consists of all three cuticle layers: endo-, exo- and epicuticle. (c) The newly 772 formed gastric tooth. (d) The cuticle in the newly formed tooth consists of a well-developed epicuticle that stains bright red and a thin, not yet fully developed exocuticle staining light 773 774 turquoise, which overlies the cuticle epithelium. The endocuticle is not formed until after the 775 moult. (e) The basal part of the zygocardiac ossicle showing the old cuticle (white label zc) 776 detached from the newly formed cuticle and its underlying hypodermis (black label zc). (f) The 777 cuticle of the newly formed ossicle shows a hypodermis, an exocuticle and a thin, red-staining epicuticle (black labels). The delaminated cuticle (white labels) shows all three cuticle layers: a 778 779 thin red-staining epicuticle, a turquoise and red staining exocuticle and a thick light turquoise 780 staining endocuticle. The endocuticle contains bands (indicated by white asterisks) and will be 781 completely shed at the upcoming moult. Abbreviations: en - endocuticle, ep - epicuticle, ex -782 exocuticle, gt – gastric teeth, hd – hypodermis, zc – zygocardiac ossicle.

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Freshly moulted Norway Lobster (Nephrops norvegicus)

zygocardiac ossicle

gastric mill ossicles are moulted and shed into the stomach content